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Leslie John Szabo

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IDENTIFICATION AND PARTIAL CHARACTERIZATION OF ACTIN FROM *GLYCINE MAX* AND *TRIFOLIUM REPENS*

By

Leslie John Szabo

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ABSTRACT

IDENTIFICATION AND PARTIAL CHARACTERIZATION OF ACTIN FROM

GLYCINE MAX AND TRIFOLIUM REPENS

By

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The leguminous plants, Glycine max (soybean) and Trifolium repens (clover), were examined for the presence of actin. Fluorescence and electron microscopy were used to identify and determine the distribution of microfilaments in these species. Most tissues of seedlings and plants of both species contained filaments which could be decorated with heavy meromyosin. The decoration by heavy meromyosin was reversed by ATP. Biochemical evidence for the presence of actin in soybeans include: the ability of myosin to co-precipitate myosin-binding material from ^{125}I-labeled extracts of soybean, stimulation of the Mg-dependent ATPase activity of myosin by a fraction eluting from a Sepharose column to which myosin had been linked and the partial purification by chromatography on DEAE-cellulose of an actin-like protein that cross-reacts with rabbit anti-actin antibodies raised against calf thymus actin and has the same molecular weight as that of rabbit muscle actin.

DEDICATION

To my parents, and my wife, Cheryl,

for their abiding love.

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LIST OF ABBREVIATIONS

ADP	Adenosine 5'-diphosphate
ATP	Adenosine 5'-triphosphate
ATPase	Adenosine 5'-triphosphatase
BSA	Bovine serum albumin
C	Temperature in degree Celsius
DEAE	Diethylaminoethyl
DNA	Deoxyribonucleic acid
DNase I	Deoxyribonuclease I
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethyleneglycol-bis-(amino ethyl ether) tetraacetic acid
F-actin	Filamentous form of actin
g	Force of activity
G-actin	Monomeric form of actin
Mg	Magnesium (divalent form)
SDS	Sodium dodecyl sulfate
Tris	Tris (hydroxymethyl) aminomethane

INTRODUCTION

In the past few years, the muscle protein, actin, has been detected in a variety of non-muscle cells and it is now widely believed that actin is ubiquitous in all eukaryotic cells (47). Actin has been implicated in many diverse cellular activities such as nuclear migration, phagocytosis, cytoplasmic streaming, change in cell shape, and regulation of topographical distribution of membrane proteins (14,47). Most of these studies have been done exclusively in animal systems, but many of the same processes also occur in plants.

Microfilaments (F-actin) have been detected in a number of plant species and a variety of cell types, including vascular bundles, root hairs, and parenchyma cells (74). To date, most of the investigations concerning the possible function of actin in plants have dealt with studies of cytoplasmic streaming. This is only one of many processes in which plant actin may be involved. Another particularly intriguing system, and the genesis of this study, is the early infection process of the legume-Rhizobium symbiosis.

The first step of the infection process is the recognition and binding of the soil bacterium Rhizobium to the proximal end of the root hair of its leguminous host (for general review see reference 18). Once binding has occurred, the root hair will curl to form a "shepherd's crook." The first sign of infection is the swelling of the root hair wall and an increase in cytoplasmic streaming. The nucleus enlarges, migrates to the site of infection and preceeds the infection thread as it elongates, apparently directing the growth of the thread.

The infection thread is formed by an invagination of the plant membrane into the root hair cytoplasm, which usually grows towards the

center of the root. The thin tube formed by this process contains Rhizobium in a mucopolysaccharide matrix. In the cortex tissue of the root, the site of nodule development, the infection thread membrane fuses with the membrane of the cortex cell. The bacteria are encapsulated in a peribacteroid envelope by an endocytotic process.

Many of the steps involved in the infection process are analogous to actin mediated processes in non-plant cells, such as nuclear migration, phagocytosis (encapsulation of bacteria) and alteration of cell shape (root hair curling). The first phase of the investigation of actin and its possible role in the infection process of legumes, has been the identification, localization and characterization of plant actin. In this study, two leguminous plants, Glycine max (soybean) and Trifolium repens (white clover), were examined for the presence of actin.

LITERATURE REVIEW

Actin has been isolated and characterized from a variety of non--muscle animal cells (13,28,32,47,53,73,95) as well as other organisms of diverse evolutionary origin such as Mycoplasma (65,68), Acanthamoeba (30,75,96), Dictyostelium (39,91,102) and Saccharomyces cerevisiae (45,48). Actin can account for as much as 20-30% of the total cellular protein in Acanthamoeba (47,77,98) or as little as 1% in blood platelets (44,47). Actin from all sources examined are similar in composition and structure.

Properties of Monomeric Actin

The actin monomer (G-actin) is a single polypeptide chain with an approximate molecular weight of 42,000. The amino acid sequence is highly conserved (98). Only 6% of the amino acid residues Acanthamoeba in actin differ from those of rabbit muscle actin (44,47). All actins examined contain the unusual amino acid, N-3-methylhistidine (29,47) and bind 1 mole of ATP per mole of protein.

There are significant chemical differences, however, in the properties of actin isolated from different sources. Only actin from Acanthamoeba castellani, for example, contains the amino acid N-methyllysine, and all of the actins for which amino acid sequence data are available are different (47). At present, actin can be divided into three groups based upon differences in their respective isoelectric points. Alpha (α) actin which is obtained from muscle tissue is the most acidic form. Beta (β) and gamma ( ) are from non-muscle sources with gamma being the most basic. All non-muscle cells from vertebrates examined contain a mixture

of both β and γ actins (79,98). These isoforms of actin (β and γ) are not the result of post-translational modification, but are coded for by different genes (25).

Polymerization of Actin

Under the appropriate ionic conditions purified actin polymerizes to form filamentous actin (F-actin). During polymerization, the ATP bound to actin is hydrolyzed to form free inorganic phosphate and bound ADP. In the process of depolymerization, the ADP is replaced with ATP. Although the specific role of ATP is unclear, ATP presumably serves as an energy source for conformational changes which occur during polymerization (87). The actual process of polymerization involves two steps, nucleation and elongation. Nucleation, the formation of a short actin polymer 6-8 units long, is the rate limiting step and depends on the concentration of free monomeric actin present in solution. When the concentration exceeds a critical concentration, polymerization of G-actin to F-actin occurs, establishing an equilibrium between the two forms.

High concentrations of G-actin do occur in the cytoplasm of Phasarum (37), in bovine spleen cells and in the periacrosomal vesicle region of sperm (88,89). The presence of G-actin well above its critical concentration implies the presence of cellular control of polymerization. A 16,000 dalton protein (profilin) has been implicated as a regulatory molecular which controls polymerization of actin in spleen cells (10). Profilin forms a 1:1 crystalline complex with G-actin and inhibits polymerization. Another protein known to interact with G-actin is DNase I (48,64) which also forms a 1:1 crystalline complex with actin. Whether this interaction is a regulatory mechanism for the control of actin

polymerization, a regulator of DNase I enzymatic activity, or has no biological function, is unknown.

Properties of Filamentous Actin

The polymeric form of actin (F-actin) is comprised of a right handed, double stranded helix with a half pitch of 35 nm. The filaments upon electron microscopic examination exhibit a bead-like structure with a diameter of 5-7 nm and form an arrowhead pattern upon treatment with heavy meromyosin (19,33,36). Filament structure and decoration by heavy meromyosin are used as criteria for the identification of actin filaments. Heavy meromysin, the globular portion of the myosin molecule containing ATPase activity, is formed by tryptic digestion of myosin. Pyrophosphate or ATP inhibit or reverse the binding of heavy meromysin or myosin to F-actin. Moreover, F-actin activates the Mg-dependent ATPase activity of myosin, which has been widely used as a quantitative assay for actin.

Effects of Cytochalasin B and Phalloidin

Two families of drugs appear to modulate the polymerization state of actin. The first is the cytochalasins which disrupt the actin filament structure; although their precise mode of action is still disputed. The second group includes the cyclic peptide, phalloidin, from the mushroom Amanita, which has been shown to enhance polymerization of actin.

Cytochalasin B alters many processes in animal cells, including inhibition of phagocytosis (1), disruption of cell movement and severe alterations in cell shape (58). Cytochalasin B has been shown to inhibit the polymerization purified rabbit muscle actin, in vitro (54,59,63,84,85).

Cytochalasin B also inhibits transmembrane glucose transport (3,52) although cytochalasin D does not (3). Furthermore, cytochalasin D is more potent in altering cell shape of cultured fibroblasts than the B analog (3).

Cytochalasin B inhibits many plant processes which are believed to depend on microfilaments. These include cytoplasmic streaming (8,9,12,101), nuclear migration (32), and light-induced chloroplast movement in Mougeotia (94). In plants such as Zea mays and Avena sativa (9), treatment with cytochalasin B results in the cessation of cytoplasmic streaming and the aggregation of microfilaments into large bundles. In some cases, microfilaments can only be observed after the addition of cytochalasin B (9,99). Hepler et al. (32) suggested that in plants cytochalasins may affect actin structure by disruption of the membrane anchorage site for microfilaments.

Phalloidin has been shown to cause an increase in the amount of actin microfilaments in liver cells (78). In in vitro studies with rabbit muscle actin, phalloidin was shown to bind stoichiometrically, to enhance the rate of actin polymerization, and to stabilize the F-actin structure (17,52,60). The affect of phalloidin on plant cells has not been investigated.

Location and Function of Microfilaments in Plants

The occurrence of microfilaments in plant cells is now well documented. The most intensive work has been done with the characean algal cells, Nitella and Chara. These cells along with Physarum have been used as models to study cytoplasmic streaming. Palevitz demonstrated that Nitella contained actin filaments (72,73) and these were often organized

into large bundles. The polarity of the actin filaments within the bundles observed in these studies was parallel (44) and the orientation with respect to direction of movement was similar to that of muscle fibers. In addition, organelles such as chloroplasts are apparently attached to these microfilament bundles (44). These filaments are located in the stationary ectoplasm of the cell along the boundary of the streaming endoplasmic layer (40). This boundary is thought to be the site of generation of motive force for cytoplasmic streaming (41). The actomyosin complex is believed to be involved in the generation of this force (42,82,100).

Considerable cytological information is available about the distribution and localization of microfilaments in higher plants (see reference 74 for review). Parthasarathy et al. (74) observed that filament bundles are most often found in elongating or elongated nucleated root cells of vascular or cambium tissue and rarely seen in isodiameteric cells. However, Vahey et al. (92) reported that actin is present in the parenchyma cells of tomato fruit and O'Brien et al (69) observed microfilaments in the parenchyma cells of Avena sativa. Microfilaments are often orientated parallel to the long axis of the cell and are found either in the outer edge (69) or center of the cytoplasm (74). In addition Forer and Jackson (21) observed microfilaments in the mitotic spindles of Haemanthus katherinae. Organelles such as endoplasmic reticulum, plastids, vesicles, chloroplasts, and mitochondria are often found closely associated with these fibers (74).

Actin filaments have been characteristically identified in higher plants by reversible binding of heavy meromyosin. The decoration is done either in droplets of cytoplasm (6,15) or in sections of tissue perfused

Table 1

Occurrence of Microfilaments in Higher Plants*

Source	Reference
Amaryllis belladonna	Condeelis (1974)
Cuscuta sp	Bennett and Brown (1980)
Gossypium hirsutum	Bennett and Brown (1980)
Haemanthus katherinae	Forer and Jackson (1975)
Hibiscus esculentus	Bennett and Brown (1980)
Impatiens sultanii	Bennett and Brown (1980)
Lemna minor	Bennett and Brown (1980)
Liriodendron tulipfera	Bennett and Brown (1980)
Mimosa sp	Arraes-Hermans *et al.* (1976)
Phaseolus aureus	Bennett and Brown (1980)
Phaseolus vulgaris	Jackson and Doyle (1972)
Pinus taeda	Bennett and Brown (1980)
Pueraria lobata	Bennett and Brown (1980)
Raphanus sativus	Bennett and Brown (1980)
Setraeasea purpurca	Bennett and Brown (1980)
Vica faba	Ilker *et al.* (1974)
Xylosma congestum	Ilker *et al.* (1974)
Zea mays	Bennett and Brown (1980)

*Filaments were identified by electron microscopic examination of samples treated with heavy meromyosin.

with heavy meromyosin in glycerol (35). A list of higher plants in which actin filaments have been identified by heavy meromyosin decoration is presented in Table I. In addition, Bennett and Brown (6) examined representative species from each taxonomic class of the plant kingdom and found microfilaments were present in all but five.

Although actin has been shown to be a component in numerous higher plant species, very few biochemical studies have been conducted (Table II). The first biochemical report of an actomyosin-like complex was from vascular bundles of Nicotiana and Cucurbita (104). This material exhibited an ATP-dependent reduction in viscosity and ATPase activity. Little progress has been made in obtaining purified actin from higher plants although partial purification of actin from Phaselous vulgaris (38), Lycopesican esculentum (92), and Triticum aestivum (34) has been reported. Actin from higher plants still needs to be purified and characterized biochemically in terms of the kinetics of polymerization and the stimulation of Mg-dependent ATPase of myosin as well as amino acid composition and sequence. This would be an important initial step in understanding the role of actin in plants. Very little is known about the function of actin in plants, but progress is being made in studies of cytoplasmic streaming. There are many plant processes that involve movement, such as cytokinesis, opening of stomata, thigmotrophism, root hair curling and migration of chloroplasts and nuclei. With a clearer understanding of the biochemical properties of plant actin, the mechanism and regulation of these processes may be elucidated.

Table 2

Purification and Characterization of Actin from Plants

Source	Procedure	Characteristics
Cucurbita and Nicotiana (Yen et al., 1965)	Enrichment by actomyosin ppt	ATPase
Nitella (Palevitz, 1976)	Enrichment by polymerization	dec. mf
Phaseolus vulgaris (Jackson et al., 1976)	Enrichment by gel filtration, polymerization	dec. mf MW
Lycopesicon esculentum (Vahey et al., 1978)	Partial purification - ion exchange chromatography, polymerization	dec. mf MW
Triticum aestivum (Ilker et al., 1979)	Partial purification - actomyosin ppt, gel filtration	MW ab

Abbreviations used:

ppt - Precipitation

ATPase - Stimulation of myosin ATPase activity

dec. mf - Microfilaments decorated with heavy meromyosin

MW - Same molecular weight as actin from other sources

ab - Cross reacts with anti-actin antibodies

Actin Immunology

Anti-actin antibodies are difficult to obtain, because actin is a ubiquitous protein and its structure is highly conserved. For these reasons a number of immunization methods are used. These include the injection of F-actin treated with glutaraldehyde (66), or SDS denatured actin (51). In addition, the amount of antigen used and the interval between injections vary widely (34,51).

The properties of antisera produced also vary. Not all anti-actin antibodies form antigen-antibody complexes which produce precepetin lines in Ouchterlony double-diffusion tests. However, the presence of anti-actin antibodies is clearly demonstrable by double-antibody co-precipitation tests and radioimmunoassay (66). Moreover, not all antibodies react with all actins. Antiserum raised against chicken embryo brain actin cross-reacts with actins from bovine cardiac muscle and brain, rabbit skeletal muscle, and chicken embryo brain. On the other hand cardiac actin antiserum bound cardiac and skeletal muscle actin but did not bind actin (66) from brain tissue of either source.

MATERIALS AND METHODS

Soybean Cell Culture

SB-1 cell line of soybean (Glycine max) cells was kindly provided by Dr. O.L. Gambory (Prairie Regional Laboratory, Saskatoon, Saskatchewan, Canada). Cultures were grown in 125 ml Erlenmeyer flasks containing 40 ml of solution at 25 to 30°C on a gyratory shaker. Liquid cultures were subdivided (25) every 3 to 4 days by transferring 10 ml of culture to 30 ml of fresh 1-B5 medium (26). Cultures of SB-1 cell lines were also maintained as callus which was grown on agar plates containing 0.5% (w/v) Bacto agar in 1-B5 medium. Callus cultures were transferred to new agar plates every month, sealed with parafilm and stored at room temperature in the dark. When necessary new liquid cultures were started by placing callus into flasks containing 20 mls of 1-B5 medium and incubated on a gyratory shaker. Initially spent medium was removed and replaced with fresh medium every week until a uniform cell suspension was formed, at which time the culture was subdivided by mixing 20 ml of suspension with 20 ml of fresh medium until the culture was growing rapidly.

Growth Conditions for Soybean and Clover

Soybean (Glycine max (L.) Merr. var. Ansoy 71) and white clover (Trifolium repens L. var. Ladino) seeds were surface sterilized by immersing seeds first in 75% (v/v) ethanol and then in an acidified solution of mecuric chloride (2 g $HgCl_2$ and 5 ml concentrated HCl per liter) for 45 seconds each. The seeds were washed 8 to 10 times by soaking in sterile water for 5 minutes. Soybean seedlings were germinated in sterilized trays which were lined with paper towels moistened with water

and covered with aluminum foil. Soybean plants used in this study were grown in Perlite under greenhouse conditions. Clover seeds were germinated on 0.5% (w/v) water agar plates and seedlings were transferred and grown on Fahraeus slides (20), to which KNO_3 was added to the Fahraeus medium to a final concentration of 2 mM.

Purification of Muscle Proteins

Acetone powder was prepared from rabbit back muscle by grinding 100 gm of tissue in a commercial meat grinder and washed with 10 mM sodium EDTA, 0.4% sodium bicarbonate, pH 7.0 for 15 minutes and rinsed with distilled water (5). The solution was decanted and the muscle tissue was added to 1 liter of acetone at -10°C. After stirring for 30 minutes, the suspension was filtered through a Buchner funnel and reextracted twice for 10 minutes with 500 ml of cold acetone. The residue was dried in a vacuum jar overnight under aspiration, ground and stored with dessicant at 4°C.

Actin was isolated from 10 g of acetone powder by extraction with 200 mls of extraction buffer (2 mM Tris, 0.2 mM ATP, 0.5 mM β-mercaptoethanol, 0.2 mM $CaCl_2$, pH 8.0) for exactly 10 minutes at 0°C (85). The suspension was filtered through 8 layers of cheesecloth and 1 layer of Miracloth and the filtrate was clarified by centrifugation at 10,000 x*g* for 1 hour. Actin was polymerized in the pooled supernatant fluids by adding 2 M KCl and 1 M $MgCl_2$ to a final concentration of 50 mM and 2 mM, respectively, and was incubated for 2 hours. The concentration of KCl was adjusted to 0.8 M by adding solid KCl and the solution was gently stirred for 1.5 hours to dissociate actin from actin-binding proteins. The polymerized actin was pelleted by centrifugation at 80,000 x*g* for 3

hours. The pellet was resuspended in 30 mls of extraction buffer and dialyzed against 500 mls of the same buffer for 2 days with 2 changes of the buffer. After dialysis, the solution was centrifuged for 1.5 hours at 80,000 xg to remove any insoluble material and the supernatant fluid containing monomeric actin was stored at 4°C. To prevent bacterial growth, sodium azide was added to a final concentration of 0.05%.

Myosin was isolated from fresh rabbit back muscle (45,89). The tissue was ground in a meat grinder and myosin was extracted with 3 volumes of buffer A (0.5 M KCl, 0.1 M K_2HPO_4, 1 mM DTT, 15 mM sodium EDTA, pH 6.8). After 20 minutes, the suspension was centrifuged for 30 minutes at 13,200 xg, and the supernatant fluid was filtered through glass wool. Myosin was made insoluble and collected by dilution of the filtrate with 10 volumes of water, incubated for 30-45 minutes and centrifuged at 13,200 xg for 15 minutes.

The pellet containing myosin was resuspended in 100 mls of buffer B (0.5 M KCl, 50 mM K_2HPO_4, 1 mM DTT, pH 6.8), and gently stirred for 20 minutes. Insoluble material was removed by centrifugation at 20,200 xg for 30 minutes. Actin was removed by precipitation of actomyosin by dilution of the supernatant fluid with 80 ml of water, incubation for 20 minutes and centrifugation for 90 minutes at 55,000 xg. The clear liquid was decanted, diluted with 7 volumes of water, incubated for 30 minutes, and centrifuged for 15 minutes at 13,200 xg to collect the myosin. The process was repeated two more times as described above. The final myosin pellet was resuspended in 100 mls of buffer B and stored at 4°C. Sodium azide was added to a final concentration of 0.05% to prevent bacterial contamination.

Heavy meromyosin was prepared by tryptic digestion of myosin (103). Myosin (4 mg/ml) was digested with 0.01 volume of TPCK-Trypsin (0.15 mg/ml in 1 mM HCl) for exactly 7.5 minutes at 25°C with constant stirring. The reaction was stopped by adding 0.1 volume of soybean trypsin inhibitor (1 mg/ml). Undigested myosin and light meromysin were precipitated by dialysis overnight of the digestion solution against 800 mls of 20 mM imidazole buffer, pH 6.6, with 2 changes of buffer. The insoluble material was removed by centrifugation at 80,000 xg for 1.5 hours. The supernatant fluid was fractionated by precipitation of heavy meromyosin with the addition of solid ammonium sulfate to a final concentration of 2.4 M and centrifugation at 25,000 xg for 30 minutes (72). The pellet was resuspended in 10 mls of 5 mM Tris 0.5 mM EGTA, 1 mM DTT, pH 8.0, and dialyzed against 400 mls of the same buffer for 2 days with 3 changes of buffer. The final dialyzed solution was diluted with 1 volume of glycerol and stored at -20°C. All steps were carried out at 4°C unless noted otherwise.

Staining of Soybean Protoplasts with Fluorescent-DNase I

Protoplasts were isolated by a modified procedure of Constabel (16). Actively growing SB-1 cells (24-48 hours after transfer) were digested with an equal volume of enzyme solution containing 400 mg cellulysin, 200 mg pectinase and 2 g of D-sorbitol, pH 5.5 in 20 mls. After 2 hours, the protoplast suspension was filtered through a 48 µM nylon filter and pelleted by centrifugation in a clinical centrifuge for 2 minutes. The pelleted protoplasts were gently resuspended in 10 mls of protoplast medium (16) which was modified by substituting 20 g of D-sorbitol for sucrose. After 3 washes, protoplasts were resuspended in 1 ml of

protoplast medium, counted in a Thomas C-10 counting chamber and diluted to 5 x 10^5 cells per milliliter.

The effects of the drugs cytochalasin B and colcochine on protoplasts were examined. To 5 mls of protoplast suspension, 50 µg of cytochalasin B (1 mg/ml), 20 µg of cholchicine (0.5 mg/ml) or 50 µl of modified protoplast medium were added and incubated at room temperature. After 1 hour incubation, the protoplasts were pelleted and resuspended in formaldehyde and fixed.

Soybean protoplasts were fixed by a modified procedure of Fowke (23). Protoplasts were pelleted and resuspended in modified protoplast medium containing 1% formaldehyde for 1 hour followed by 3% formaldehyde solution for 2 hours at room temperature and washed 3 times with protoplast medium. The fixed protoplasts were incubated in 0.1% BSA solution in PBS (16 g NaCl, 0.4 g KCl, 2.3 g Na_2HPO_4, 0.4 g KH_2PO_4 per liter) to remove any remaining aldehyde groups and spread on microscope slides to air dry. Protoplasts bound to glass slides were dehydrated by incubation for 15 minutes each in an ethanol series of 10% to absolute ethanol, in increments of 10%. All solutions were at 0-4°C. After 2 treatments of absolute ethanol, the slides were air dried.

Fluorescein labeled DNase I (fl-DNase) was used as a stain (11). To a DNase I solution (20 mg in 2 mls of 0.1 M $NaCO_3$, 0.1 mM $CaCl_2$, pH 8.2) 0.4 mg of fluorescein isothiocyanate was added, and stirred overnight at 4°C in the dark. The solution was dialyzed against 250 mls of PBS for 48 hours with 5 changes of buffer. The absorbance ratio (500 nm to 280 nm) of the final dialyzed solution was 1.5.

The dehydrated protoplasts were stained by covering the slides with fl-DNase (diluted 1:10 with PBS) for 1 hour at room temperature in the

dark and excess stain was removed by washing the slides 3 times for 5 minutes with PBS. The slides were wet mounted in 50% glycerol and examined with a Leitz fluorescence microscope. Micrographs were taken with Kodak Pan X-100 film.

Myosin-Binding Assay

Calf thymus extract was prepared by homogenizing 50 g of tissue with 150 mls of 10 mM Tris, 10 mM EDTA, 10 mM NaCl, 0.5% triton X-100, pH 7.5, in a Waring blender for 2 minutes (56). The homogenate was centrifuged for 10 minutes at 10,000 xg and the supernatant fluid recentrifuged for 2 hours at 100,000 xg. Extract of soybean cultured cells was prepared in a similar fashion except 200 g (fresh weight) of cells were ground with 200 mls buffer, 15 g of polyvinylpyrrolidone, and alumina.

Bovine serum albumin (3 mg/ml) and extracts of calf thymus (13 mg/ml) and soybean (2 mg/ml) were iodinated (24) with ^{125}I and dialyzed against 1 liter 10 mM Tris, 5 mM $CaCl_2$, pH 7.5. Chicken muscle myosin which was a generous gift from Dr. C. Suelter and Ms. D. Thompson was radiolabeled with ^{131}I and dialyzed against 1 liter of precipitation buffer containing 10 mM Tris, 1 mM sodium EDTA, 0.1 mM DTT, 0.1 M KCl, pH 7.5 (13). All samples were dialyzed for 2 days with 4 changes of buffer.

Extracts of [^{125}I]-labeled calf thymus or soybeans (0.4 mls) were incubated with myosin (0.1 ml) for 30 minutes at room temperature, and centrifuged for 4 minutes in an Eppendorf microfuge. Pellets were resuspended in 0.5 ml of precipitation buffer and transferred to fresh tubes. Control experiments were performed with [^{125}I]-labeled bovine serum albumin. Radioactivity was determined by gamma counting

(Beckman Biogamma), with channel settings of 0-50 for ^{125}I and 50-1000 for ^{131}I. Radioactivity was converted to micrograms of precipitated protein by the specific activity of each solution and was reported as such.

Chromatography on DNase I-Sepharose

Affinity columns were prepared by covalently coupling BSA or DNase I to Sepharose 4B (43,56). Cyanogen bromide (20 mls of 30 mg/ml solution) was added to Sepharose 4B (20 mls) and stirred for 6 minutes at room temperature while the pH was maintained between 11.0-11.3 by the addition of 2 N NaOH (4,77). The reaction was stopped by immediately washing the packing in 500 mls of ice cold water and 0.1 M $NaHCO_3$ and resuspended in 25 mls of 0.1 N $NaHCO_3$. A solution of DNase I or BSA (20 mg in 2 mls of 0.1 N $NaHCO_3$) was added to the activated Sepharose 4B, gently mixed overnight, and washed with a liter of water and 0.1 N $NaHCO_3$ each. Columns (0.75 cm x 6.5 cm) were packed with BSA or DNase I-Sepharose and washed with 50 mls of 4 M guanidine HCl, 0.5 M sodium acetate, 30% glycerol followed by 500 mls of DNase column buffer (10 mM Tris, 5 mM $CaCl_2$, pH 7.5).

Calf thymus extract was prepared by homogenizing 39 g of tissue with 100 mls of 10 mM Tris, 10 mM EDTA, 10 mM NaCl, 0.5% triton X-100, pH 7.5, in a Waring blender for 2 minutes (56). The homogenate was centrifuged at 12,000 xg for 10 minutes to remove cell debris and was further clarified by centrifugation at 100,000 xg for 2 hours. An extract of soybean cells was prepared in a similar fashion except 200 g (fresh weight) of cells were ground with alumina, 15 g of polyvinylpyrrolidone and 200 mls of buffer. After centrifugation, the supernatant fluids were subjected to affinity chromatography.

Extracts of calf thymus (50 mls) or soybean (200 mls) were applied to 15 ml columns, washed with DNase column buffer and 0.75 M guanidine-HCl, 0.5 M sodium acetate, 30% glycerol, pH 6.5. Actin was eluted by washing the column with 3.0 M guanidine-HCl, 1.0 M sodium acetate, 30% glycerol, pH 6.5. Fractions from the column were pooled, dialyzed, lyophilized and analyzed by SDS gel electrophoresis.

Chromatography on DEAE-Cellulose

Ten-day-old soybean seedlings (350 g fresh weight) were homogenized for 2 minutes in a Waring blender in 700 mls of 3 mM imizadole buffer (3 mM imidazole, 0.5 mM ATP, 0.1 mM $CaCl_2$, 0.75 mM β-mercaptoethanol, pH 7.5 with 70 g of polyvinylpyrrolidone and 0.02 units·ml^{-1} of the protease inhibitor, Trasylol. The homogenate was filtered through 8 layers of cheesecloth, 1 layer of Miracloth, centrifuged for 20 minutes at 16,300 x*g*, and further clarified by centrifugation at 100,000 x*g* for 90 minutes. The resulting supernatant fluid was chromatographed on a DEAE-cellulose column (4 cm x 25 cm) and eluted with a linear gradient from 0 to 0.25 M KCl, according to the procedure of Gordon *et al.* (30).

Immunochemistry

Rabbit anti-actin antibodies raised against calf thymus actin were a generous gift from Mr. T.N. Metcalf. The actin used as an antigen was purified according to the procedure of Gordon *et al.* (30), subjected to SDS slab gel electrophoresis and extracted from mascerated gel sections. Rabbits were given primary injections of 0.8 mg denatured actin in complete Freund's adjuvant, followed by booster injections of 0.4 mg (in complete Freund's adjuvant) at 6 week intervals. Antisera was

collected bi-weekly. Ouchterlony immunodiffusion tests were performed as described by Munoz (67) and precipitin lines were usually observed after 1-2 weeks. Soybean material in fraction C and calf thymus extract were labeled with ^{125}I (31) and dialyzed against 3 mM imidazole buffer (see Chromatography on DEAE-cellulose section). Immunoprecipitation was performed by mixing [^{125}I]-labeled calf thymus extract, calf thymus actin or fraction C with antisera and incubated for 30 minutes at 37°C, followed by overnight at 4°C. Goat antibodies directed against rabbit immunoglobulin were added, incubated for 30 minutes at 37°C and overnight at 4°C, and centrifuged for 5 minutes at 1,000 xg. The pellets were washed in 3 mM imidazole buffer, and analyzed by SDS gel electrophoresis.

Myosin and Heavy Meromysin Affinity Chromatography

Affinity columns were prepared by covalently coupling myosin and heavy meromysin to Sepharose 4B (7). Activation of Sepharose 4B was performed as previously described (see Chromatography on DNase I-Sepharose section). Activated packing (15 g) was washed and suspended in a total volume of 40 ml of coupling buffer (0.5 M KCl, 0.1 M K_2HPO_4, pH 8.0). Solutions of myosin (40 mg) or heavy meromyosin (36 mg) in coupling buffer were added to the packing and gently mixed on a shaker overnight. The packing was washed in 500 mls of coupling buffer, 100 mls of 1 M Tris, pH 8.0, and incubated for 2 hours in 100 mls of 1 M Tris, pH 8.0, to remove any remaining active sites. Heavy meromyosin and myosin-Sepharose were equilibrated in phosphate buffer (20 mM K_2HPO_4, 50 mM KCl, 5 mM $MgCl_2$, 5 mM EGTA, 2 mM DTT, pH 7.0) and triethanolamine buffer (10 mM triethanolamine, 50 mM KCl, 2.5 mM $MgCl_2$, pH 7.5), respectively.

Extracts of soybean roots were prepared by homogenizing roots (132 g fresh weight) with 130 mls of equilibration buffer in a Waring blender for 2 minutes, and the homogenate was filtered through 8 layers of cheesecloth and 1 layer of Miracloth. The filtrate was incubated for 1 hour at room temperature and centrifuged for 30 minutes at 22,000 xg.

Extracts of rabbit muscle were prepared by stirring 2 g of acetone powder with 40 mls of extraction buffer (see section on Purification of Muscle Proteins) for exactly 10 minutes at 0°C. The tissue suspension was filtered through 8 layers of cheesecloth and 1 layer of Miracloth and, the filtrate was clarified by centrifugation at 10,000 xg for 1 hour. The supernatant fluid was dialyzed against 250 ml of equilibration buffer overnight with 2 changes of buffer.

Extracts of soybean or rabbit muscle were mixed with 15 mls of column packing, gently agitated for 1 hour at room temperature, and poured into a 20 ml column (1.8 cm x 8.0 cm). The column was washed with equilibration buffer, followed by equilibration buffer containing 2 mM ATP. The elution of proteins was monitored by absorbance at 280 nm, and by stimulation of the Mg-dependent ATPase activity of myosin. Fractions were dialyzed against water, lyophilized, and analyzed by SDS gel electrophoresis.

DNase I Inhibition Assay for Monomeric Actin

Monomeric actin was measured by inhibition of DNase I activity as described by Lindberg (55). Various amounts of inhibitor (0-100 µl) were added to 20 µl of DNase I solution (0.05 mg DNase I per ml of 50 mM Tris, pH 7.5), and incubated for 30 seconds. Three milliliters of DNA solution (24 mg of double-stranded calf thymus DNA in 600 mls of 0.1 M Tris, 2.4

mM $MgSO_4$, 1.7 mM $CaCl_2$, pH 7.5) was added, mixed, and the change in absorbance at 260 nm was monitored. One unit of inhibition activity was expressed as the amount of inhibitor needed to cause a decrease in DNase I activity of 0.01 absorbance units per minute.

Measurement of ATPase Activity of Myosin

Filamentous actin was detected by its ability to stimulate the Mg-dependent ATPase activity of myosin (14). Each assay mixture contained: 350 µl of buffer (15 mM KCl, 5 mM imidazole, 1 mM $MgCl_2$, 1 mM ATP, pH 7.0), 100 µl of sample and 100,000-200,000 cpm of γ-[^{32}P]-ATP. The reaction was initiated with the addition of 50 µl of myosin (1 mg/ml), incubated for 30 minutes at 37°C, and stopped with the addition of 0.5 ml of cold 5% perchloric acid. The assay was a modification of the [^{32}P]-transfer assay used by Schubert et al. (81). The amount of activity was measured by determining the quantity of non-charcoal absorbable radioactivity (80).

Activities of myosin and heavy meromyosin were determined by the same method, except that the reaction mixture contained 450 µl of buffer (0.6 M KCl, 10 mM imidazole, 2 mM EDTA, 1 mM ATP, pH 7.0), 100,000-200,000 cpm of γ-[^{32}P]-ATP and 50 µl of sample (49).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Discontinous polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was carried out as described by O'Farrell (70). Gels were stained and fixed in 25% isopropanol, 10% acetic acid (v/v) and 0.125% Coomassie Blue (80). Gels were destained in 25% isopropanol and 10% acetic acid (v/v).

Electron Microscopic Examination of Microfilaments

Squashes of plant material were prepared as described by Bennett and Brown (6). Small sections of plant material were minced in 2 to 5 drops of buffer (20 mM K_2HPO_4, 50 mM $MgCl_2$, 5 mM EGTA, 2 mM DTT, pH 7.0, containing 0.2-0.4 mg/ml heavy meromyosin) on a glass slide with a razor blade. A coverslip was placed over the minced tissue and pressure was applied until the cells ruptured. The liquid was removed with a Pasteur pipet and used immediately.

Copper grids (200 mesh) were coated with formvar/carbon. Films of Formvar were prepared glass slides dipped in a solution of 0.5% Formvar in chloroform and air dried. The film was removed by scraping the edges of the slide with a razor blade and by floating the film off on the surface of a water reservoir by slowly immersing the slide. Acetone washed grids were placed on the floating film, removed by inversion on an index card, and air dried. The Formvar coated grids were carbon coated with a Ladd carbon evaporator.

Samples (1 drop) were applied to coated grids for 10 to 60 seconds, washed with 1 to 3 drops of buffer (20 mM K_2HPO_4, 50 mM $MgCl_2$, 5 mM EGTA, 2 mM DTT, pH 7.0) and stained with 2 to 3 drops of uranyl acetate (2% in water and stored in the dark). After 30 to 60 seconds, the stain was removed with filter paper and air dried. Grids were examined with a Philips 201 C electron microscope at 60 KV. Electron micrographs were taken using Kodak electron image film 4463.

Protein Determination

The concentration of protein in a sample was measured either by the method of Lowry et al. (62) or by absorbance at 280 nm. The following

extinction coefficients were used: myosin 0.543 $ml \cdot cm^{-1} \cdot mg^{-1}$ (61) and actin 1.15 $ml \cdot cm^{-1} \cdot mg^{-1}$ (19).

Materials

Cholchicine, cytochalasin B, fluorescein isothiocyanate, pectinase, soybean trypsin inhibitor, and Trasylol were purchased from Sigma. Bovine pancreatic DNase I and TPCK [L-(tosylamido-2-phenyl) ethyl chloromethyl ketone] trypsin were obtained from Worthington. Uranyl acetate and 200 mesh copper grids were supplied by Ted Pella Co. (P.O.B. 510; Tustin, Ca. 92680). Cellulysin was purchased from Calbiochem. Polyvinylpyrrolidone, a gift of the G.A.F. Corporation, was acid washed as described by Loomis (57). Sepharose 4B and Sephadex G-150 were purchased from Pharmacia. Formvar was obtained from E.F. Fullman, Inc. DEAE-cellulose was suppled by Whatman. Highly polymerized, double-stranded calf thymus DNA was purchased from P-L Biochemicals, Inc. Iodogen (1,3,4,6-tetrachloro-3a-6a-diphenylglycoluril) was donated by Drs. P. Fraker and J. Speck. γ-[^{32}]-ATP was prepared by K.R. Schubert using a modified procedure of Glynn et al. (27). Goat anti-bodies directed against rabbit immunoglobulin were purchased from Miles Laboratories.

Figure 1. Identification of microfilaments by electron microscopy. Samples were treated with rabbit muscle heavy meromyosin, and stained with 2% uranyl acetate. a, rabbit muscle actin; b,c, microfilaments from roots of clover seedlings; d,e, microfilaments from stems of soybean plants. The bar (——) represents 100 nm.

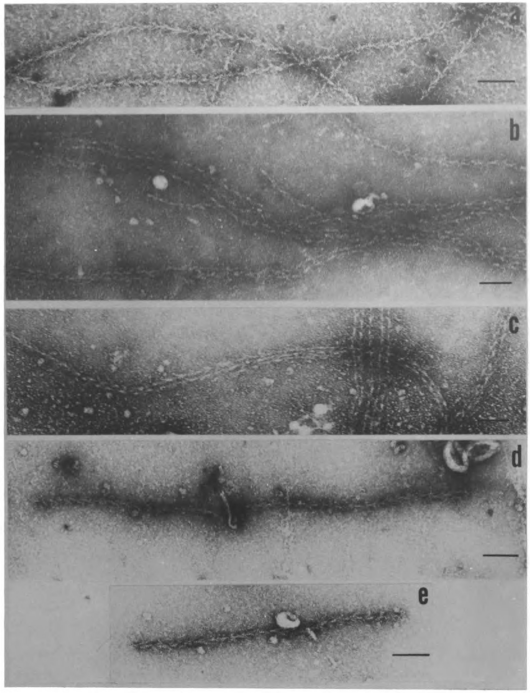

FIGURE 1

RESULTS

Identification and Localization of Microfilaments by Electron Microscopy

Microfilaments were observed in samples from soybean and clover plants that were examined by electron microscopy using the squash technique of Bennett and Brown (6). These filaments exhibited the classical arrowhead decoration with heavy meromyosin (Figure 1). When the grids were treated with 1 mM ATP prior to staining, decorated filaments were not observed. The heavy meromyosin decorated filaments showed a repeat of 30 to 34 nm for rabbit actin and 28 to 33 nm for soybean and clover microfilaments. The majority of microfilaments were quite short in length, ranging from 0.7 to 1.2 μM. Occassionally, longer filaments were observed (4 μM or larger), these often being in bundles with parallel polarity. The longer microfilaments tended to be associated with or attached to organelles. The heavy meromyosin decoration of these filaments always pointed toward the site of attachment. As a control, grids were prepared with only heavy meromyosin and no decorated microfilaments were observed.

The various anatomical structures of seedlings (Figure 2) and plants (Figure 3,4) were examined in both clover and soybean for the presence of filamentous actin. Microfilaments were observed in all regions except cotyledons, soybean petiols and clover leaves (Table 3). The frequency of microfilaments was found to be the highest in the root tip region, where one grid square may contain 15 to 30 filaments. Samples prepared from stems and leaves contained on the average 1 to 10 filaments per grid square. In some cases, several preparations were made and examined before decorated microfilaments were observed.

Figure 2. Schematic drawing of a typical seedling. Regions that were examined for the presence of microfilaments are indicated.

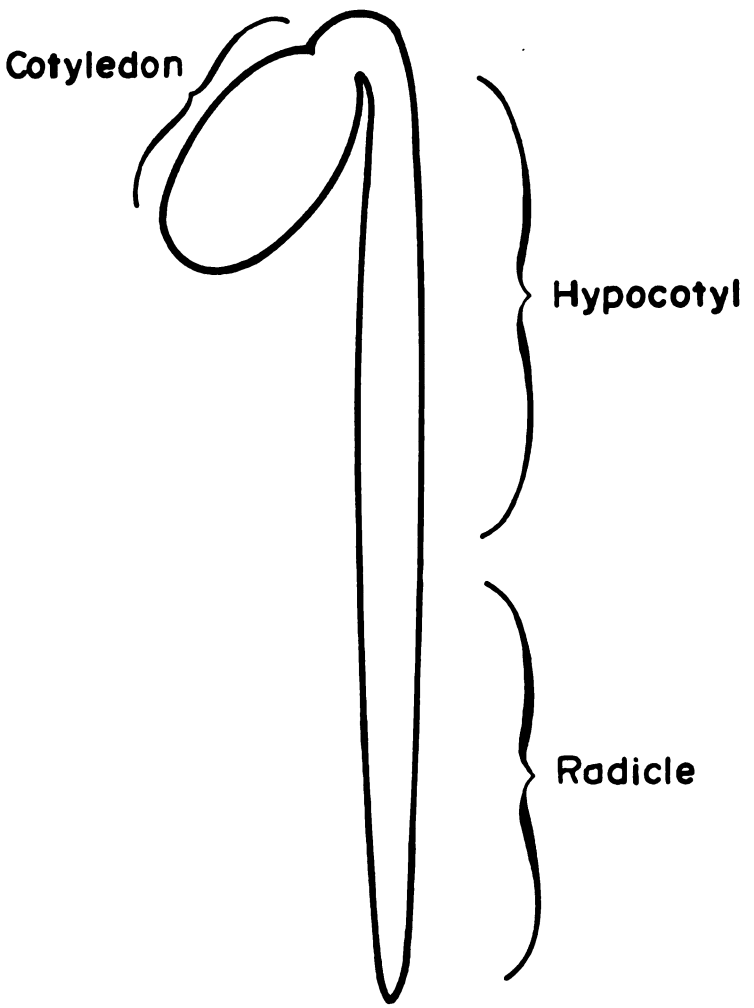

FIGURE 2

Figure 3. Schematic drawing of a soybean plant. Regions that were examined for the presence of microfilaments are indicated.

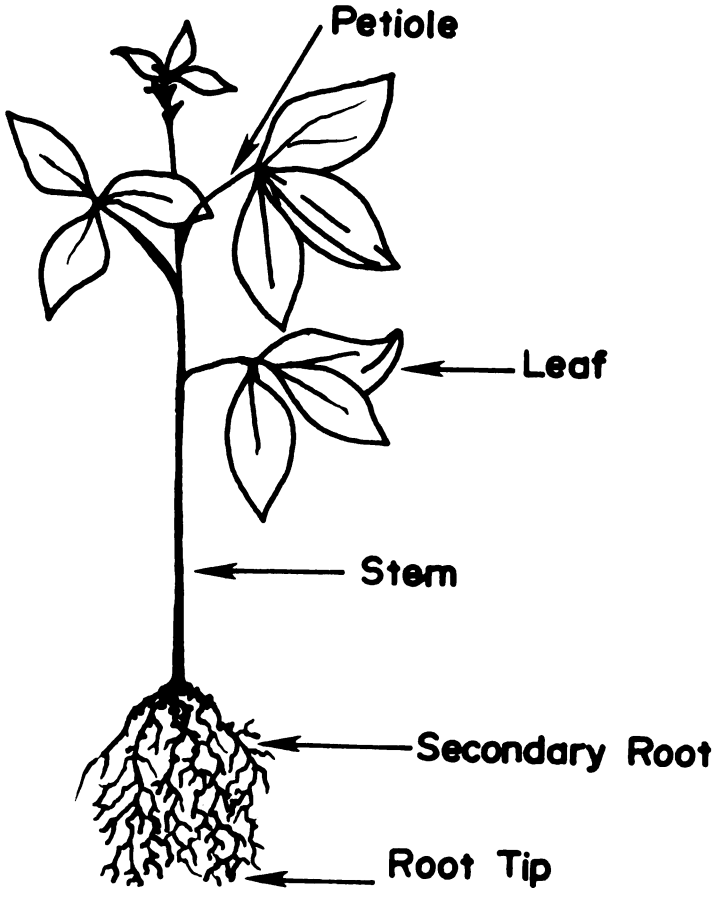

FIGURE 3

Figure 4. Schematic drawing of a clover plant. Regions that were examined for the presence of microfilaments are indicated.

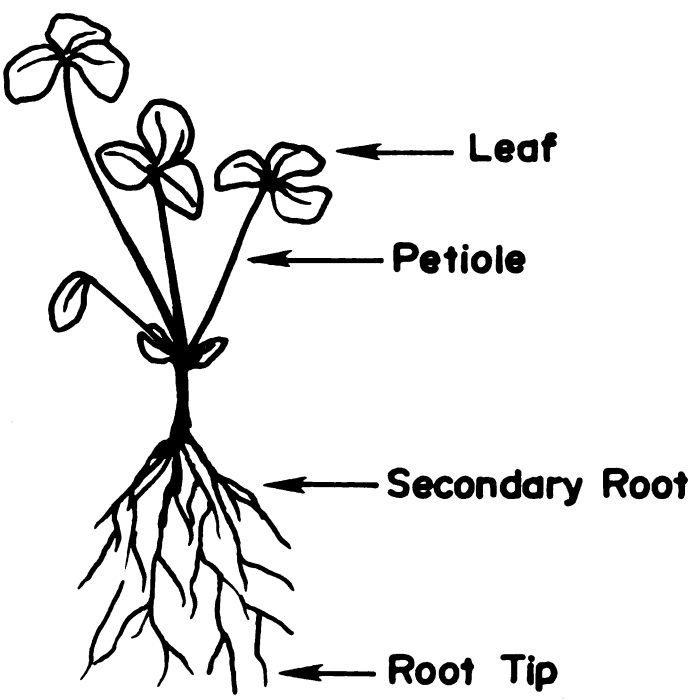

FIGURE 4

Table 3

Distribution of Microfilaments

Source	*Glycine max* (soybean)	*Trifolium repens* (white clover)
A. Seedlings		
radical	++	++
hypocotyl	+	+
cotyledons	ND	ND
B. Plants		
root tips	++	++
mid section of secondary root	++	++
stems	+	--
petioles	ND	+
leaves	+	ND

Symbols and Abbreviations

ND - None detected

++ - 10 to 30 microfilaments were observed per grid square

+ - 1 to 10 microfilaments were observed per grid square

-- - No true stem structure in this plant (see Figure 4)

Both clover and soybeans contained microfilaments which decorated with heavy meromyosin. In order to confirm that these filaments were composed of actin, soybeans were examined biochemically for the presence of the subunit protein, actin. Soybeans were selected because of the availability of a cell culture line and the ease in which large quantities of material could be obtained.

Myosin-Binding Protein in Soybeans

Myosin-Binding Activity

The selective activity of myosin to bind actin and to be separated out as an actomyosin complex was used as another indication for the presence of an actin-like protein in soybean cells. BSA and extracts of calf thymus and soybean cells were labeled with ^{125}I, while chicken myosin was labeled with ^{131}I. Mixing [^{125}I]-labeled calf thymus extract with [^{131}I]-labeled myosin resulted in the co-precipitation of myosin as well as [^{125}I]-labeled material (Table 4) presumed to be actin. Similarly, incubation of [^{131}I]-myosin with [^{125}I]-labeled extract of soybean cells caused co-precipitation of both [^{125}I] and [^{131}I]-labeled material. In contrast, when [^{125}I]-labeled BSA was used, there was no precipitation of either [^{125}I]-labeled BSA or [^{131}I]-labeled myosin.

Affinity Chromatography on Myosin-Sepharose and Heavy Meromyosin--Sepharose

The selective ability of myosin to bind filamentous actin was also used as a purification technique. Affinity columns were initially

Table 4

The Binding of Myosin to Proteins from Calf Thymus and Soybean Cell Extracts

Conditions*	^{125}I Protein Precipitated	^{131}I Protein Precipitated
^{125}I CT + ^{131}I Myosin	10.5 ± 2.5	0.9 ± 0.1
^{125}I SB + ^{131}I Myosin	2.4 ± 2.1	1.6 ± 1.6
^{125}I BSA + ^{131}I Myosin	0	0

*Abbreviations: CT, calf thymus extract; SB, soybean cell extract; BSA, bovine serum albumin; SEM, standard error of the mean. The specific activities of ^{125}I CT, ^{125}I SB, ^{125}I BSA, and ^{131}I Myosin were: 60 mg/μg, 1100 cpm/μg, 1620 cpm/μg and 100 cpm/μg, respectively.

Figure 5. Affinity chromatography of soybean root extract on myosin-Sepharose column. The column (1 cm x 5 cm) was washed with 10 mM triethanolamine, 50 mM KCl, 2.5 mM $MgCl_2$, pH 7.5 and eluted with 10 mM triethanolamine, 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM ATP, pH 7.5 (o—o), absorbance 280 nm; (Δ—Δ), ATPase activity.

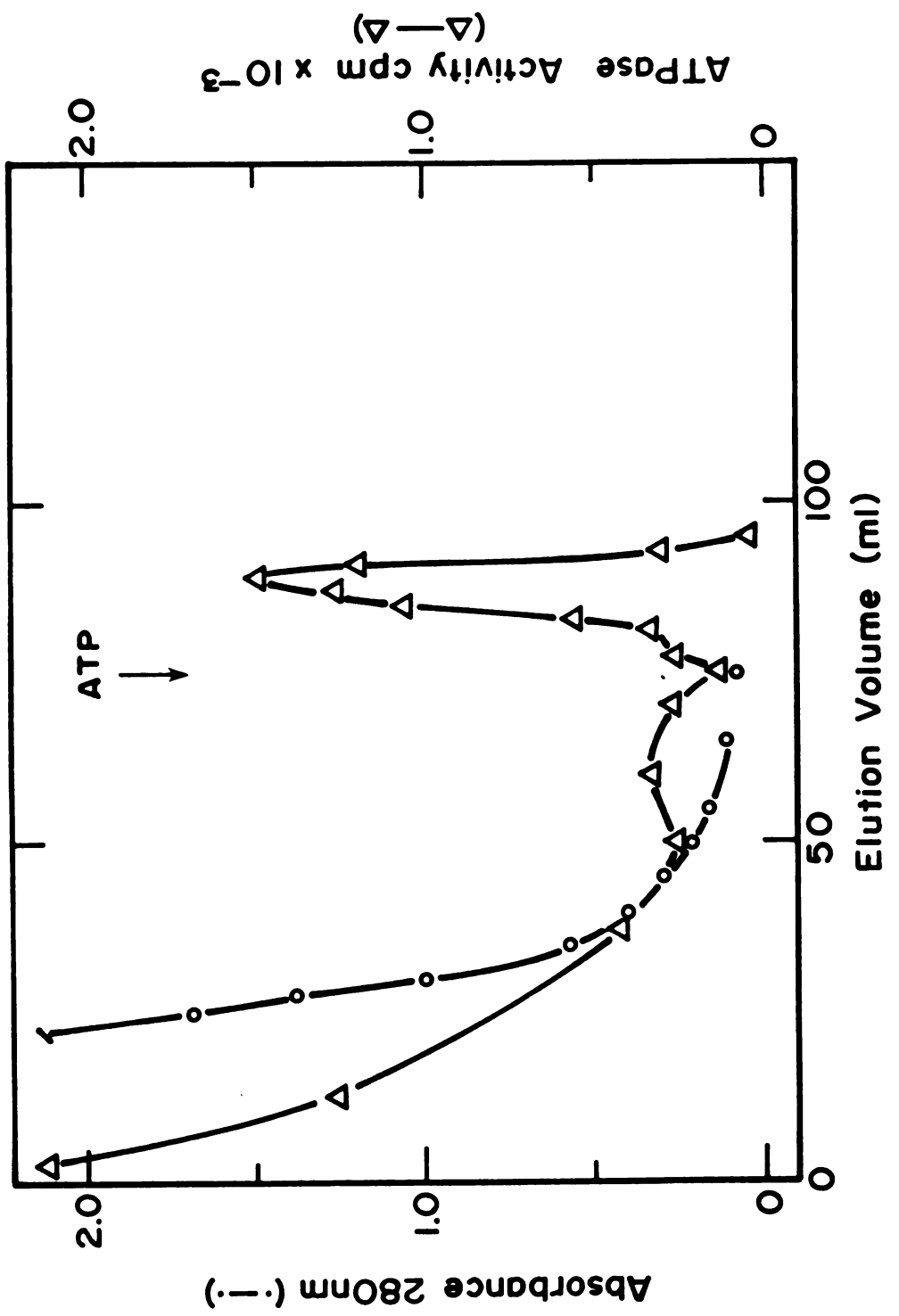
ATPase Activity cpm x 10⁻³
(Δ—Δ)
2.0
1.0
0
ATP
100
50
0
Elution Volume (ml)
Absorbance 280nm (·—·)
2.0
1.0
0

FIGURE 5

Figure 6. SDS polyacrylamide gel electrophoresis of material eluted with ATP from the myosin-Sepharose column. SDS gels (7.5%) were prepared according to O'Farrell (1975). Gel was stained with Coomassie Blue. The arrow indicates the position of migration of rabbit muscle actin.

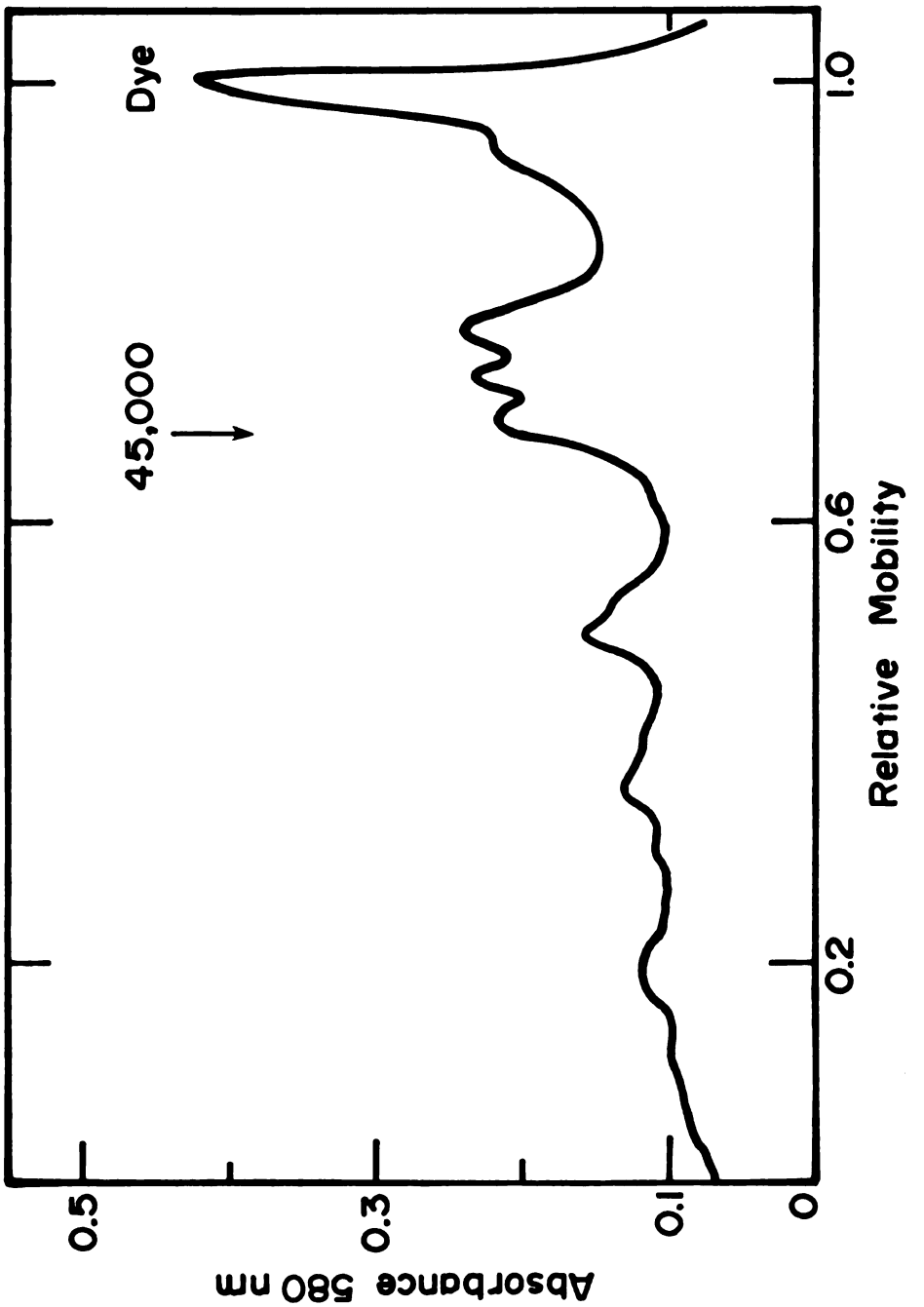
Dye
45,000
0.5
0.3
0.1
0
0.2
0.6
1.0
Absorbance 580 nm
Relative Mobility

FIGURE 6

prepared using heavy meromyosin, because actin filaments in extracts of soybean plants decorated with heavy meromyosin. However, coupling of heavy meromyosin to Sepharose resulted in a substantial loss of ATPase activity and the ability to bind rabbit muscle actin. Therefore, affinity columns were prepared with myosin in the hope that the majority of coupling sites would be along the tail region of the molecule and thus leave an active head region. Although the amount of ATPase activity bound was not greatly enhanced, the capacity of the column to bind rabbit muscle actin increased approximately 60 fold upon replacing heavy meromyosin with myosin as the ligand.

An extract of 34-day-old soybean roots were prepared and chromatographed on a myosin-Sepharose column (Figure 5). The column was monitored for protein by absorbance at 280 nm and for actin by the ability to stimulate the Mg-dependent ATPase activity of myosin. Stimulatory activity was observed both in the fractions containing material not bound to the column and those eluted with ATP. The fractions eluted by ATP were pooled, dialyzed, lyophilized and analyzed by SDS gel electrophoresis. This fraction contained 3 major bands (Figure 6) with the largest polypeptide having the same mobility as rabbit actin. In successive experiments, "actin activity" was consistently detected in the pass through volume but there was no longer a peak of "actin activity" which was specifically eluted with ATP. Alteration of chromatographic conditions, variation in the age of the plant material used, and newly prepared affinity columns were tried but again, no peak of "actin activity" was detected. No significant loss of actin binding ability was observed when myosin-Sepharose columns were tested for the ability to bind rabbit muscle actin before and after chromatography with soybean extracts.

Figure 7. Staining of soybean protoplasts with fl-DNase I. a,b, control; c,d, protoplasts incubated for 1 hour with 10 µg/ml of cytochalasin B; e,f, protoplasts incubated for 1 hour with 4 µg/ml of cholchicine. Frames a,c,e, and b,d,f, present results of fluorescence and phase-contrast microscopy, respectively.

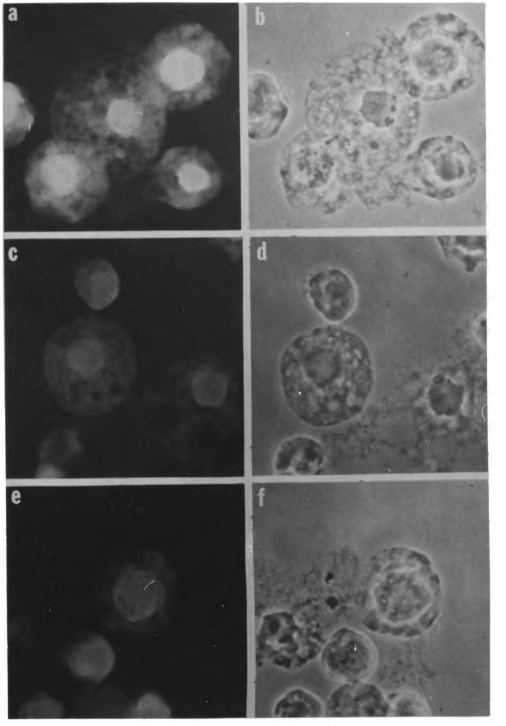

FIGURE 7

DNase I-Binding Proteins in Soybeans

Staining of Protoplasts with fl-DNase I

Since the discovery of Lazarides and Lindberg (50) that actin was a specific inhibitor of DNase I, this enzyme has become a useful tool for the identification and localization of actin within cells. In this study, fl-DNase was used to detect the presence and distribution of an actin-like protein in soybean protoplasts. The following staining pattern was observed: the nuclear region was stained most intensely, sharply delineating the nucleus from the rest of the cell; the cytoplasm showed a more diffuse general staining pattern (Figure 7). No distinct bundles or "stress fibers" were observed. Control experiments showed that the protoplasts did not autofluoresce. In addition, pretreatment of the protoplasts with cytochalasin B (10 μg/ml) or cholchicine (4 μg/ml) for 60 minutes before fixation did not significantly alter the observed staining pattern (Figure 7). These results indicate that the enzyme DNase I does label soybeans, possibly by binding to actin-like protein. In order to characterize the DNase I binding material, this enzyme was used as a ligand for affinity chromatography.

Affinity Chromatography on DNase I-Sepharose

The specific ability of DNase I to bind actin served as the basis for affinity chromatography. Columns were prepared by covalently coupling DNase I to Sepharose 4B. This technique was used successfully to purify actin from calf thymus. The elution profile after chromatography of calf thymus extract on a DNase I-Sepharose column is presented in Figure 8.

Figure 8. Affinity chromatography of calf thymus extract on DNase I-Sepharose column. The column (0.75 cm x 6.5 cm) was washed with 10 mM Tris, 5 mM $CaCl_2$, pH 7.5, and eluted with 0.75 M guanidine·HCl, 0.5 M sodium acetate, 30% glycerol, pH 6.5, followed by 3 M guanidine·HCl, 1.0 M sodium acetate, 30% glycerol, pH 6.5. The arrows indicate the points at which the buffer containing 0.75 M or 3M, guanidine·HCl was added to the column.

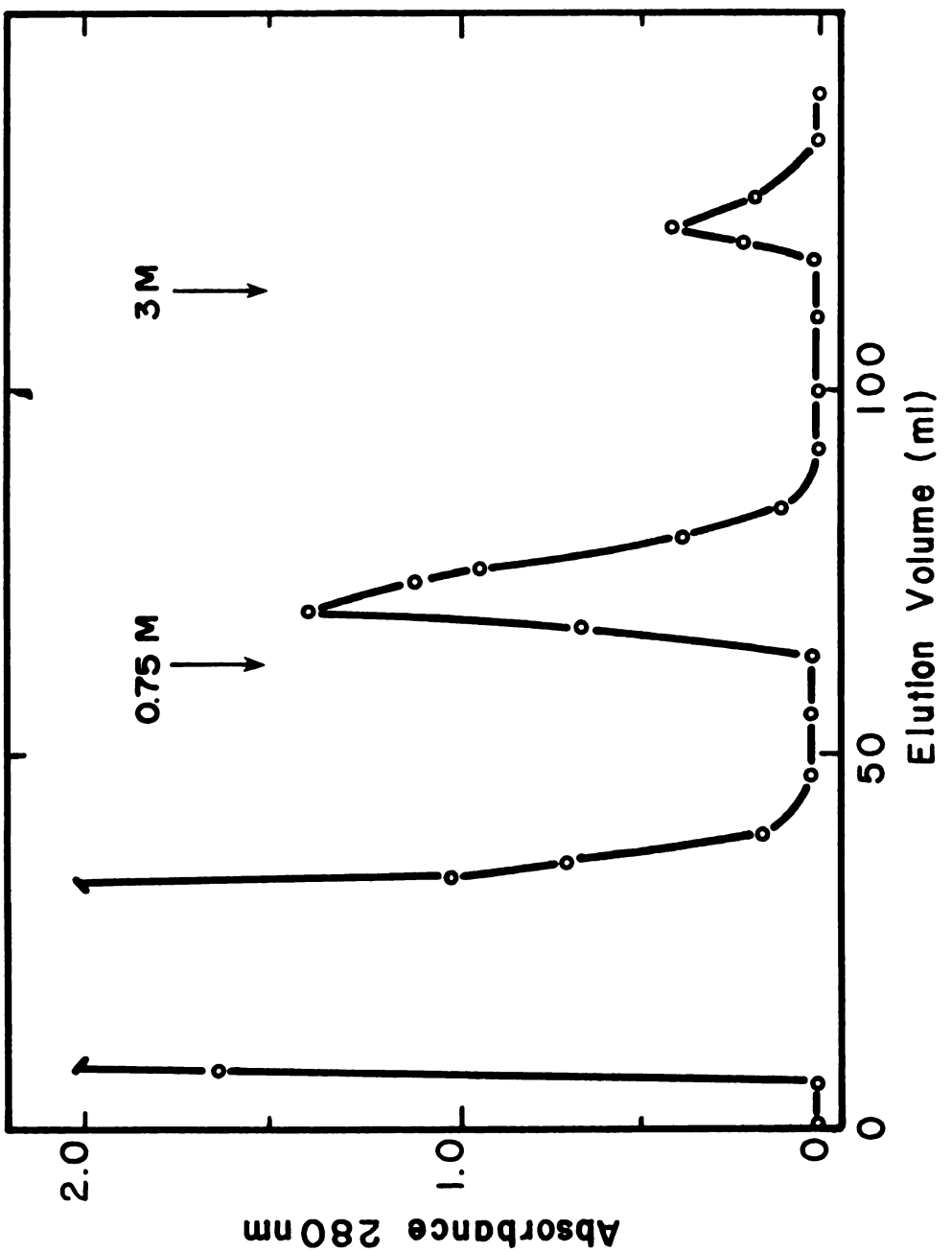

FIGURE 8

Figure 9. SDS polyacrylamide gel electrophoresis of calf thymus actin which was eluted from a DNase I-Sepharose column. The acrylamide composition of the gel was 7.5%. Approximately 75 μg of protein was loaded on this gel. The gel was stained with Coomassie Blue.

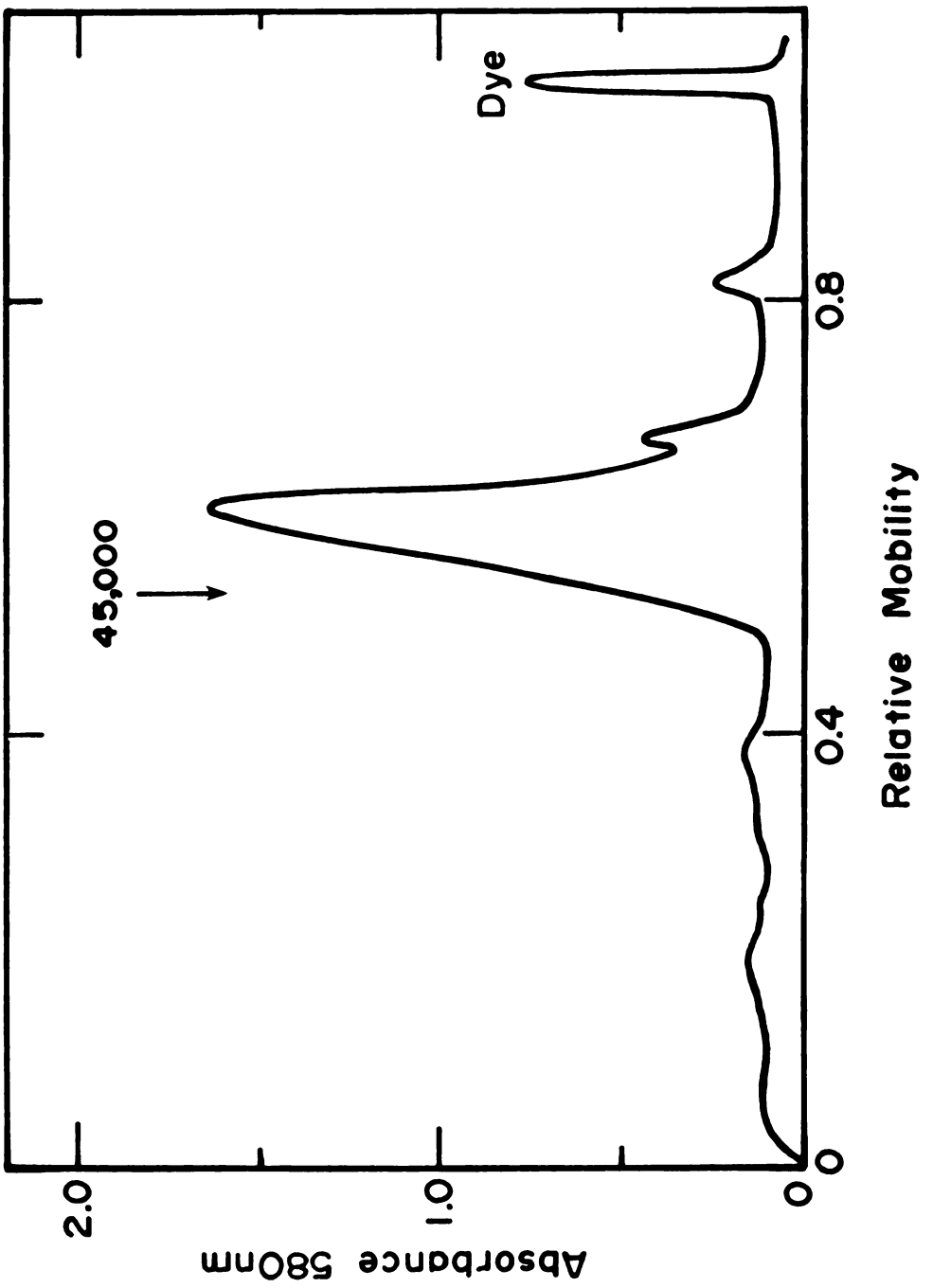

FIGURE 9

Figure 10. Affinity chromatography of soybean cell extract on DNase I-Sepharose column. The column (0.75 cm x 6.5 cm) was washed with 10 mM Tris, 5 mM $CaCl_2$, pH 7.5 DNase I binding material was eluted by stepwise addition of 0.75 M guanidine·HCl, 0.5 M sodium acetate, 30% glycerol, pH 6.5 and 3 M guanidine·HCl, 1.0 M sodium acetate, 30% glycerol, pH 6.5. The arrows indicate the points at which the buffer containing 0.75 M or 3 M guanidine·HCl was added to the column.

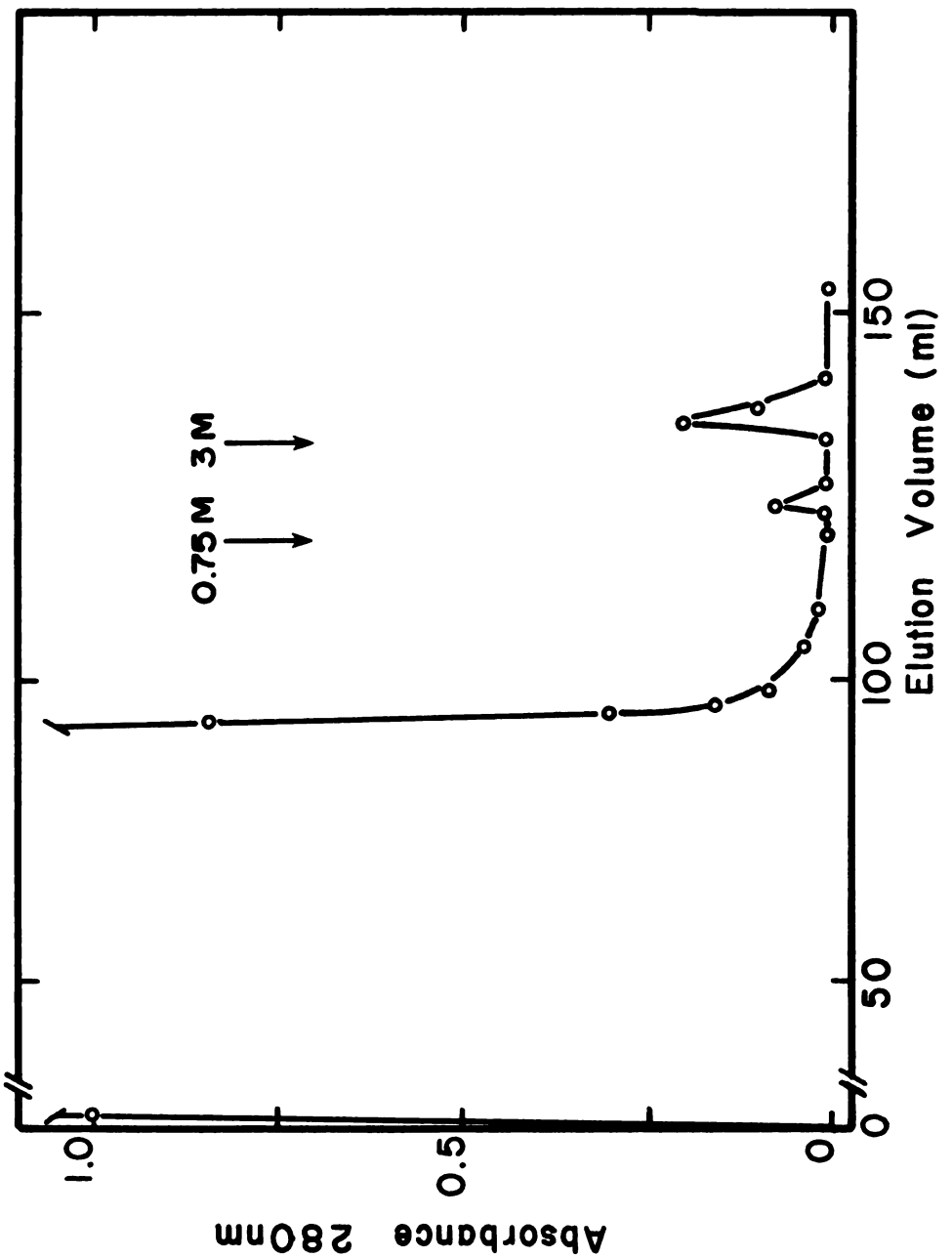
Absorbance 280nm
1.0
0.5
0
0
50
100
150
Elution Volume (ml)
0.75M
3M

FIGURE 10

Figure 11. SDS polyacrylamide gel electrophoresis of the material which was eluted with 3 M guanidine·HCl from a DNase I-Sepharose column chromatographed with soybean cell extract. The acrylamide composition of the gel was 7.5%. The arrow indicates the position of migration of calf thymus actin. Bromophenol blue was used as a tracking dye.

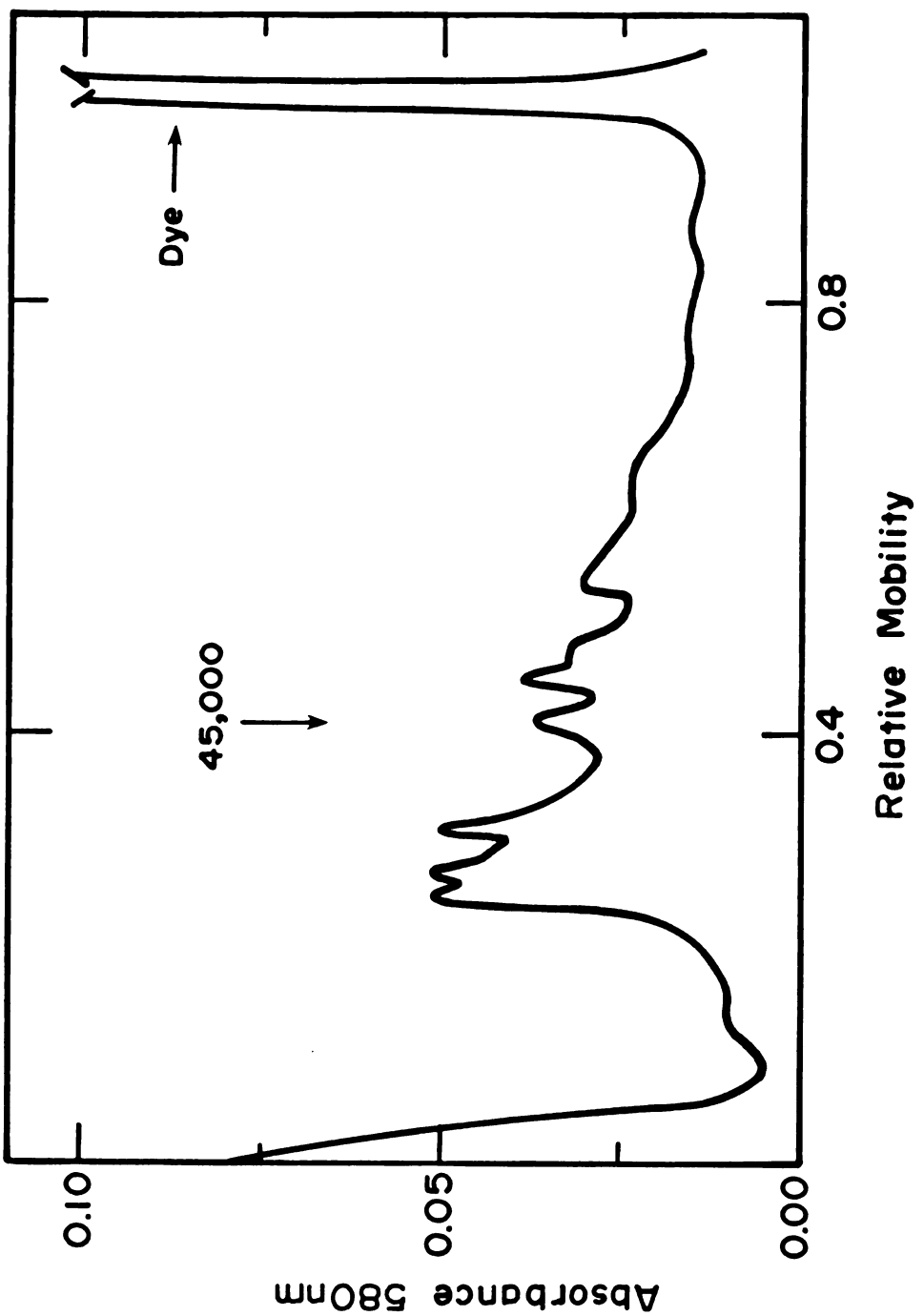
Absorbance 580nm
0.10
0.05
0.00
0.4
0.8
Relative Mobility
Dye
45,000

FIGURE 11

Figure 12. Chromatography of an extract of soybean cells on a Sepharose 4B column. The column (0.75 cm x 6.5 cm) was washed with 10 mM Tris, 5 mM $CaCl_2$, pH 7.5. At the point indicated by the arrows, stepwise elution by 0.75 M guanidine·HCl, 0.5 M sodium acetate, 30% glycerol, pH 6.5, or 3 M guanidine·HCl, 1.0 M sodium acetate, 30% glycerol, pH 6.5, was initiated.

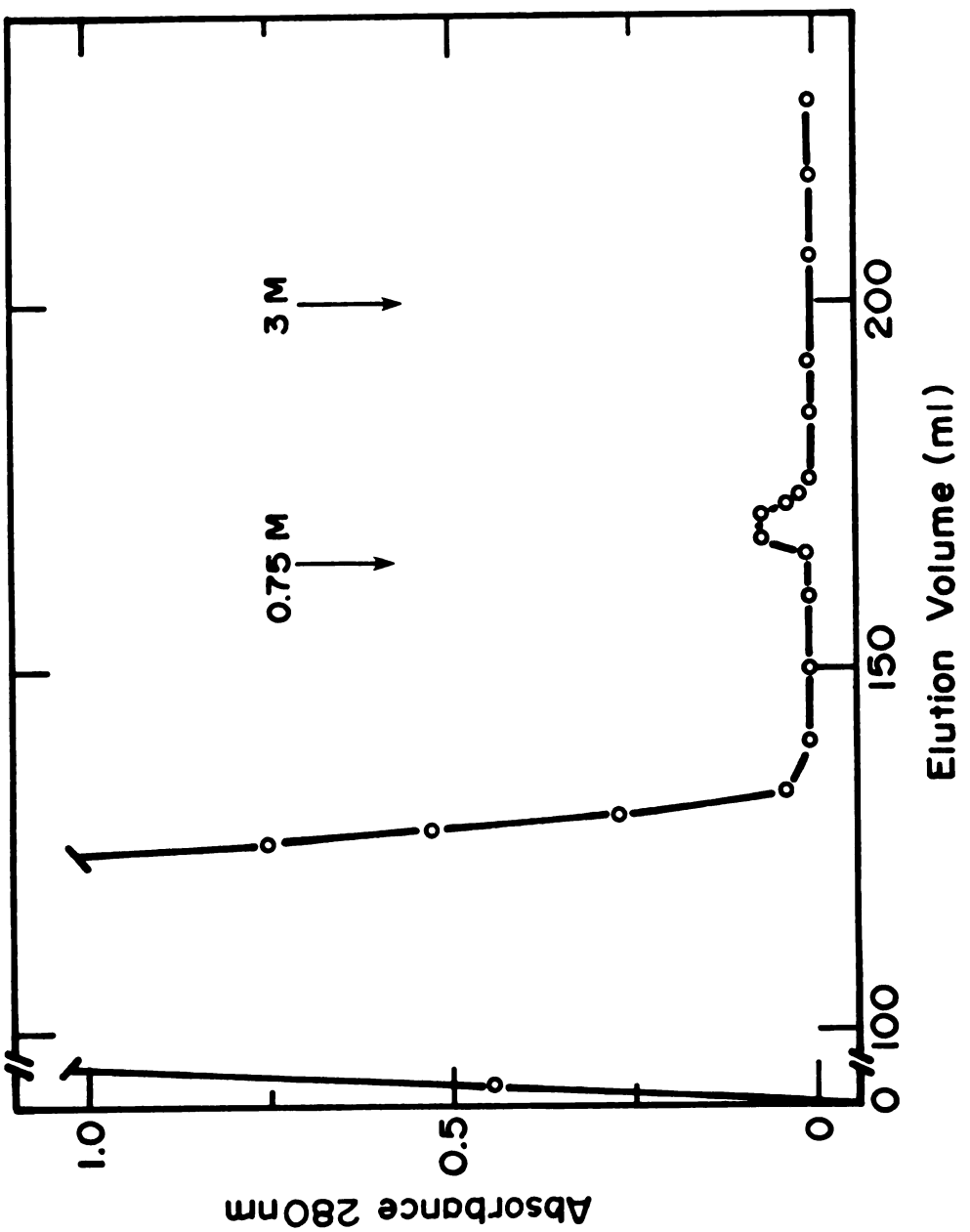
Absorbance 280nm
1.0
0.5
0
0
100
150
200
Elution Volume (ml)
0.75 M
3 M

FIGURE 12

Figure 13. Chromatography of an extract of soybean cells on a BSA-Sepharose column. The column (0.75 cm x 6.5 cm) was washed with 10 mM Tris, 5 mM $CaCl_2$, pH 7.5 and eluted by stepwise addition of 0.75 M guanidine·HCl, 0.5 M sodium acetate, 30% glycerol, pH 6.5, and 3 M guanidine·HCl, 1.0 M sodium acetate, 30% glycerol, pH 6.5. The arrows indicate the points at which the buffer containing 0.75 M or 3 M guanidine·HCl was added to the column.

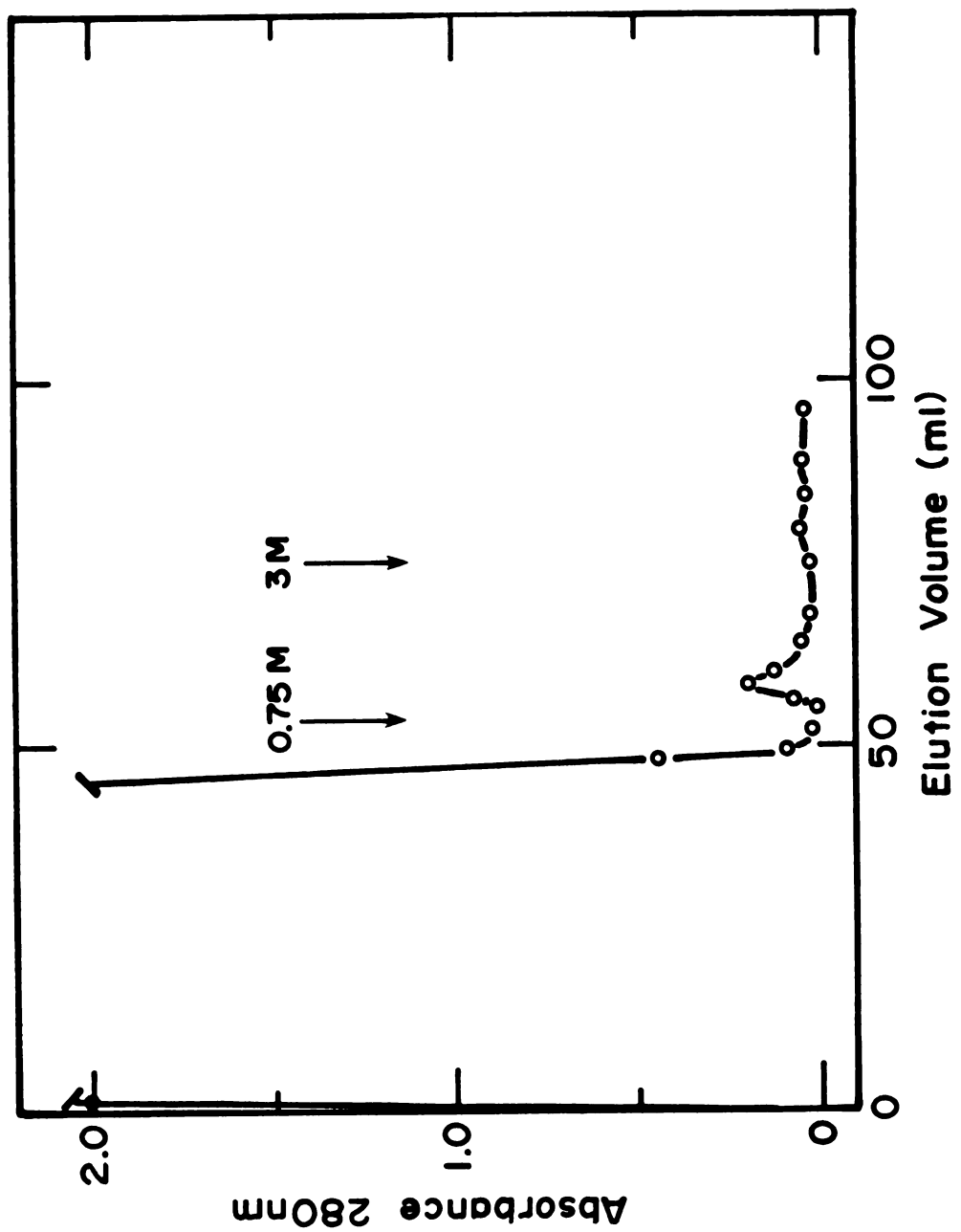

FIGURE 13

The material eluted with 3 M guanidine·HCl was analyzed by SDS gel electrophoresis. This fraction contained one predominate protein with the apparent molecular weight of 45,000 (Figure 9).

An extract of soybean cells chromatographed on a DNase I-Sepharose column resulted in an elution profile similar to the profile obtained with calf thymus (Figure 10). The fraction which was eluted with 3 M guanidine·HCl was pooled, lyophilized and analyzed by SDS gel electrophoresis. Based on staining with Coomassie Blue this fraction contained several polypeptides, one of which had the same apparent molecular weight (45,000) as purified calf thymus actin (Figure 11).

The heterogeneity of proteins in the soybean fraction which was eluted with 3 M guanidine·HCl could be due to a number of possibilities including the binding of lectin-like proteins to the polysaccharide matrix of the column or non-specific interactions between DNase I and other proteins. To test these possibilities, a series of control experiments were run. Soybean cell extracts were chromatographed on columns of Sepharose 4B (Figure 12) and Sepharose 4B linked with BSA (Figure 13). In both cases, a small amount of material was eluted with 0.75 M guanidine·HCl, but there was no significant amount of protein eluted with 3 M guanidine·HCl.

Partial Purification and Immunochemical Identification of Soybean Actin

Since the microgram quantities of material that was eluted from DNase I-Sepharose column was denatured and non-homogenous, a modification of the method reported by Gordon *et al.* (30) was used. Actin was purified from extracts of calf thymus by this method, which involved DEAE-cellulose chromatography, polymerization-depolymerization

Figure 14. Chromatography of an extract of soybean seedlings on a DEAE-cellulose column. The column (4 cm x 25 cm) was equilibrated with 10 mM imidazole, 0.5 mM ATP, 0.1 mM $CaCl_2$, 0.75 mM β-mercaptoethanol, pH 7.5. The solid line denotes the absorbance of the effluent fractions at 290 nm. At the point indicated by the arrow, a linear gradient (---) from 0 to 0.25 M KCl was initiated. The total gradient volume was 3.1.

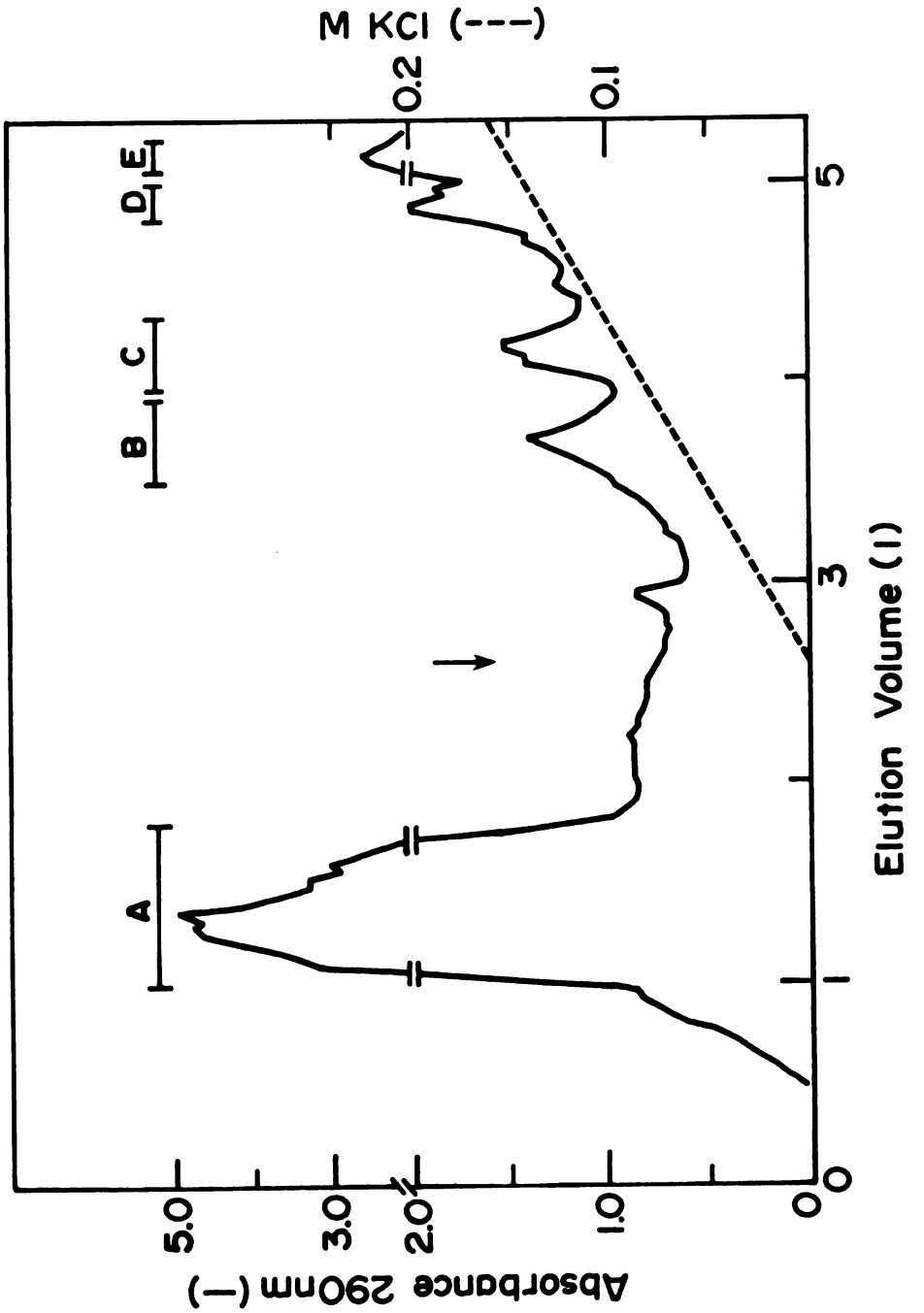
M KCl (---)
0.2
0.1
D
E
B
C
A
5
3
1
0
Elution Volume (l)
5.0
3.0
2.0
1.0
0
Absorbance 290nm (—)

FIGURE 14

Figure 15. SDS polyacrylamide gel electrophoresis of protein obtained from soybean cell extracts after fractionation by DEAE-cellulose chromatography and by affinity chromatography using rabbit anti-actin antibodies. a-e, fractions A-E from Figure 14; f, material from fraction C (Figure 14 purified by affinity chromatography on a column coupled with rabbit antibodies directed against calf thymus actin; g, calf thymus actin. The acrylamide composition of the gel was 7.5%. The arrows on the right indicate the positions of migration of molecular weight markers: bovine serum albumin (68,000); glutamic dehydrogenase (55,000); ovalbumin (43,000) and pancreatic deoxyribonuclease I (33,000). Approximately 10-30 µg of protein was loaded in each lane of the gel.

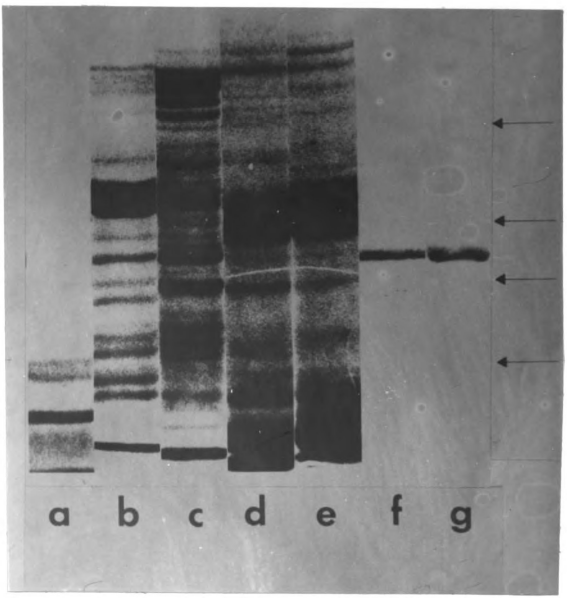

FIGURE 15

Figure 16. SDS polyacrylamide gel electrophoresis of ^{125}I-labeled proteins after immunoprecipitation. a, [^{125}I]-labeled calf thymus actin (1.7 x 10^5 cpm μg^{-1}); b, [^{125}I]-labeled fraction C (Figure 14) from soybean seedlings (8.3 x 10^3 cpm μg^{-1}). o, Radioactivity profile of immune precipitates obtained using rabbit anti-actin antiserum; ●, radioactivity profile of immune precipitates obtained using preimmune serum. The arrows indicate the position of migration of molecular weight markers. bovine serum albumin (68,000); glutamic dehydrogenase (55,000); ovalbumin (43,000); and pancreatic deoxyribonuclease I (33,000). The acrylamide composition of the gel was 7.5%.

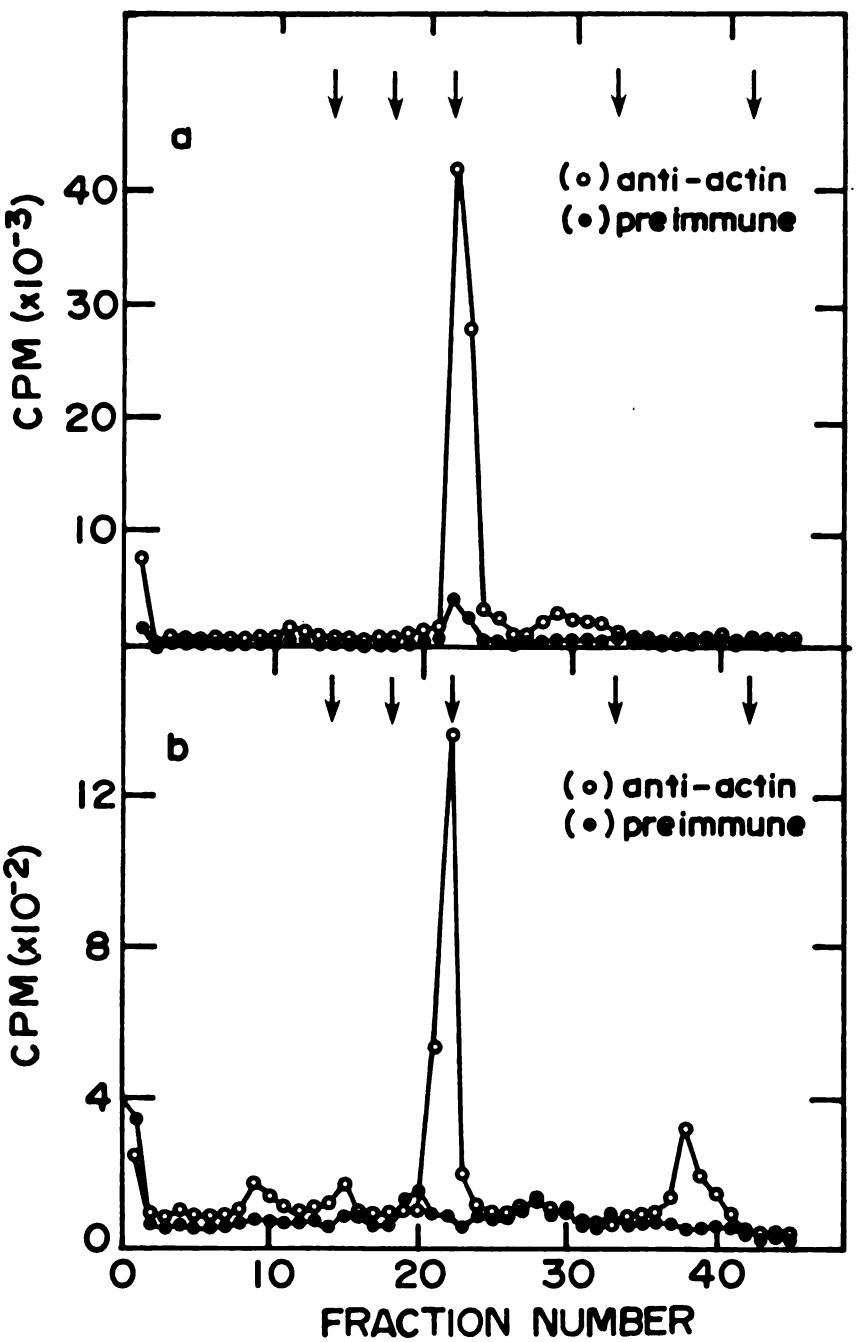
a
(o) anti-actin
(•) preimmune
CPM (x10⁻³)
40
30
20
10
b
(o) anti-actin
(•) preimmune
CPM (x10⁻²)
12
8
4
0
0
10
20
30
40
FRACTION NUMBER

FIGURE 16

and gel filtration on Sephadex G-150.

This approach was then applied to the purification of actin from soybean seedlings. The elution profile after chromatography of soybean seedling extract on a DEAE-cellulose column is presented in Figure 14. The 5 major fractions that were eluted from the DEAE-cellulose column were analyzed by SDS polyacrylamide gel electrophohresis (Figure 15) which showed that each fraction consisted of highly heterogenous populations of polypeptides.

Fraction C, eluted from the DEAE-cellulose, was found to react with rabbit antibodies raised against purified calf thymus actin in an Ouchterlony immunodiffusion test. None of the other fractions cross-reacted with the rabbit anti-actin antibodies. In addition, no precipitin line was observed when fraction C was tested against preimmune serum.

The material in fraction C was labeled with ^{125}I and immunoprecipitated with rabbit anti-actin antibodies plus goat antibodies directed against rabbit immunoglobulin. Analysis of the precipitated radioactive protein by SDS gel electrophoresis showed a single major polypeptide band, with an electrophoretic mobility identical to that of calf thymus actin (Figure 16). The molecular weight of this protein was estimated to be 45,000. No [^{125}I]-labeled material was observed when the preimmune serum was substituted for the rabbit anti-actin antibody. In addition, when unlabeled calf thymus actin was added to the reaction mixture, the amount of [^{125}I]-labeled soybean protein precipitated was reduced. Moreover, the precipitation of radioactivity was directly proportional to the amount of unlabeled actin added.

DISCUSSION

In this report, microfilaments, the polymerized form of actin, were identified in two leguminous plants, Glycine max and Trifolium repens. These microfilaments were reversibly decorated with heavy meromyosin. The characteristics of these filaments in terms of size, pattern of decoration, and polarity within bundles are identical to that of microfilaments from other sources. However, the polarity of the decorated microfilaments with respect to organelle attachment is opposite of that observed in muscle z-bands and microvilli. In this case, the arrowhead decoration pointed toward the site of attachment, not away.

The distribution of microfilaments in soybean and clover plants were examined. Microfilaments were present in all parts of the plant except cotyledons, soybean petioles, and clover leaves. The failure to observe microfilaments in some parts of the plant, such as cotyledons, probably represents a limitation of the assay procedure rather than an actual lack of microfilaments. There are several limitations of the squash technique that may account for the absence of detectable filaments. First, the assay depends on the relative ability of the squash technique to expell microfilaments from the ruptured cells. If the microfilaments of one cell type tended to be more highly bound to organelles or plasmalemma such as those associated with chloroplasts observed in Nitella (71), the ability to extract microfilaments would be diminished. In addition, the cell shape may affect the extraction in that elongated root cells are more easily disrupted by the mincing procedure than the round parenchyma cells of the leaves.

A second limitation of this assay involves the dependency on protein concentration of samples examined by electron microscopy. In the case of cotyledons, the majority of the cells are specialized for the storage of protein and starch. This will result in a higher concentration of extractable material per cell for cotyledons as compared to root cells. Even though the quantity of actin in root and cotyledon cells may be equal, the percentage of total extractable protein is quite different. Therefore, the extracted solution from cotyledons was diluted before examination and this dilution may result in lowering the actin concentration below the detection limit of the assay.

In addition to the general distribution of microfilaments, the frequency of their occurrence was also determined. Microfilaments were observed most often in the root region, followed by stem tissue, and least frequently, in the leaves. This general pattern is consistent with the work of Parthasarathy and Muhlethaler (7) in which actin was found most often in elongating or elongated nucleated cells of the roots and stems and seldom observed in the isodiameteric cells of leaf tissue. Bennett and Brown (6) have observed microfilaments in roots, stems and leaves, but gave no indication of relative frequency.

The intracellular distribution of actin in cultured soybean cells was also examined. Protoplasts stained with fl-DNase I exhibited a general diffuse staining pattern throughout the cytoplasmic region, with intensive staining in the nucleus.

The specificity of the fl-DNase I probe is questionable because of due to the multiplicity of proteins that were bound to the DNase I-Sepharose column. Although protoplasts stained with anti-actin antibodies directed against actin isolated from calf thymus, gave the same

general diffuse staining pattern (83). Whether actin is also found in the nucleus of soybean cells can not be determined with the probe used in this study because fl-DNase I could bind to DNA as well as to actin. However, Forer and Jackson (22) have reported the presence of microfilaments in the mitotic spindles of Haemanthus katherinae.

The presence of the subunit protein of microfilaments, actin, in soybeans was substantiated. In a preliminary experiment, the presence of a myosin-binding protein was detected in extracts of soybean cells. [^{131}I]-labeled myosin was added to [^{125}I]-labeled extracts of soybean cells, which resulted in the co-precipitation of both labels. Similar results were obtained when [^{125}I]-labeled calf thymus extracts were substituted for soybean extracts. The mixing of BSA with myosin did not result in the precipitation of either protein, and therefore, the co-precipitation observed with soybean extract and myosin was due to a specific interaction between myosin and a myosin-binding protein. These results only indicate the presence of actin in soybeans because of the large experimental variation obtained. This variation may have resulted from the low specific activity of the labeled proteins. Another indication that soybeans contain actin was the ability of fractions obtained after chromatography of an extract of soybean roots on a myosin-Sepharose column to stimulate the Mg-dependent ATPase activity of myosin.

The most conclusive evidence for the presence of soybean actin was that the material in fraction C, which was eluted from a DEAE-cellulose column chromatographed with soybean seedling extract, cross-reacted with rabbit anti-actin antibodies. Immunoprecipitation of this material resulted in the isolation of one major protein with the apparent molecular weight of 45,000. This is identical to the molecular weight

of calf thymus and rabbit muscle actin. Moreover, when fraction C was chromatographed on a Sepharose column containing covalently linked rabbit anti-actin antibodies, the material which was eluted with 1 M acetic acid contained only one polypeptide with the same molecular weight as calf thymus actin.

The integrity of these results depends on the specificity of the antisera used. Rabbits were immunized with highly purified calf thymus actin, which contained a single polypeptide, when analyzed by SDS gel electrophoresis. Second, one protein with the molecular weight of 45,000 was precipitated from [^{125}I]-labeled extracts of calf thymus by rabbit anti-actin antiserum and goat antibodies directed against rabbit IgG. The electrophoretic mobility of this material was identical to the purified calf thymus actin used for immunization. Parallel experiments using preimmune serum showed no precipitation of [^{125}I]-labeled material. Finally, in Ouchterlony immunodiffusion tests, the rabbit anti-actin antiserum produced a single precipitin line when tested against both the crude calf thymus extract and purified calf thymus actin. Moreover, no reaction was observed with a variety of other proteins such as chicken muscle myosin, BSA, fetuin and DNase I.

Another goal of this project, besides the identification of actin, was to explore purification schemes for soybean actin. Affinity columns were prepared with heavy meromyosin or myosin. This chromatography technique selects for F-actin, the more stable and biologically active form of actin. Affinity columns prepared with heavy meromyosin as the ligand did not bind significant quantities of rabbit actin. This problem was alleviated by using myosin as the ligand instead of heavy meromyosin. The results obtained from chromatography of soybean root extracts were

inconsistent. This inconsistency was not the result of the inactivation of myosin by soybean extracts. In all cases, "actin activity" was detected in the column wash; therefore, alterations of chromatographic conditions or extraction procedures may result in better binding of the actin protein.

Affinity chromatography on DNase I-Sepharose columns was another purification approach used. The protein which bound to the column, and was eluted with 3 M guanidine·HCl, was heterogenous with respect to molecular weight. One of the polypeptides had the same apparent molecular weight as rabbit actin. Two control experiments were performed to examine the specificity of binding between soybean proteins and DNase I-Sepharose. The first checked for the presence of a lectin-like protein that would bind to the polysaccharide matrix of the column. No detectable amount of protein was eluted with 3 M guanidine·HCl from a Sepharose column upon which a soybean extract was chromatographed. The second control examined the possibility of non-specific protein-protein interaction by chromatography of soybean extract on a BSA-Sepharose column. Again, no detectable protein was eluted with 3 M guanidine·HCl. Therefore, the multiplicity of proteins bound to the affinity column must be due to interaction directly or indirectly with DNase I.

The final approach used to purify actin was based on the procedure reported by Gordon et al. (30). Extracts of soybean seedlings were chromatographed on a DEAE-cellulose column and eluted with a linear gradient of KCl. Fraction C contained a protein that cross-reacted with rabbit anti-actin antibodies and has a molecular weight identical to that of rabbit actin. A similar approach was reported in the partial purification of actin from tomato fruit (90).

Attempts to polymerize the actin-like material which was eluted from the DEAE-cellulose column were unsuccessful. Whether this fraction contained some factor which inhibited polymerization, the concentration of actin was below the critical point for polymerization, the protein had become deactivated, or some factor was removed that was required for polymerization, is unclear. In addition, actin activity was not detected using the DNase I inhibition or the Mg-dependent ATPase activity of myosin assays. Therefore, the actin in this fraction may no longer be biologically active in that the only assay that detected actin did not depend on biologically active protein.

Evidence for the two forms of actin in soybeans is presented in this report. Microfilaments decorated with heavy meromyosin were observed in both clover and soybeans. The subunit protein of microfilaments, actin, is identified by cross-reactivity with rabbit anti-actin antibodies. However, soybean actin still needs to be purified in a form that shares the biological characteristics of other actins: polymerization into a bead-like filamentous structure, 5 to 7 nm in diameter, stimulation of the Mg-dependent ATPase activity of myosin and stoichiometric binding of ATP.

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