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LECTIN RECEPTORS AND CYTOSKELETAL STRUCTURES IN SOYBEAN CELLS

By

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ABSTRACT

LECTIN RECEPTORS AND CYTOSKELETAL STRUCTURES IN SOYBEAN CELLS

By

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Receptor-cytoplasmic interactions in animal cells are believed to be modulated by a tripartite, metastable structure consisting of cell surface receptors and the cytoskeletal macromolecules - microfilaments and microtubules. Soybean cells were examined for the presence of the three components in order to extend this model of receptor-cytoplasmic interaction to the plant cells. First, receptors for the lectins, soybean agglutinin and concanavalin A, were shown to be present at the cell surface of soybean protoplasts by the binding of fluorescently labeled and radioactively labeled lectins. Second, an actin-like protein has been identified in soybean cells on the basis of three lines of evidence: (a) molecular weight of 45,000; (b) reactivity with rabbit antiserum directed against calf thymus actin; and (c) decoration of filamentous structures by heavy meromyosin yielding ATP sensitive arrowhead complexes. Finally, colchicine binding activity in soybean

protoplasts and cell extracts provided preliminary evidence for the presence of tubulin, the microtubule subunit. These results provide the basis for the next level of analysis, which is to show direct and dynamic interactions between the three components of the cell surface membrane complex.

DEDICATION

To Deb for her love and support

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I wish to thank John L. Wang for his support and guidance over the past years. I would also like to thank my co-workers for their help; Dr. Peter A. Steck, John Blenis, Calvin F. Roff, and Patricia G. Voss.

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ADP	adenosine-5'-diphosphate
ATP	adenosine-5'-triphosphate
ATPase	adenosine-5'-triphosphatase
BSA	bovine serum albumin
αCH_3-Man	α-methyl-D-mannoside
Con A	concanavalin A
CT	calf thymus actin
cDNA	complimentary deoxyribonucleic acid
DEAE	diethylaminoethyl
DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease I
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene-bis(β-aminoethyl ether)N,N'-tetraacetic acid
EPS	extracellular polysaccharide
F-actin	filamentous or polymerized form of actin
fl-G-anti-R-IgG	fluorescein conjugated goat antibodies directed against rabbit immunoglobulins
fl-SBA	fluorescently labeled soybean agglutinin
G-actin	globular or monomeric form of actin
GalNAc	N-acetyl-D-galactosamine
GaR	goat antibodies directed against rabbit immunoglobulins
GDP	guanine-5'-diphosphate

GTP	guanine-5'-triphosphate
[^{125}I]Con A	^{125}I-labeled concanavalin A
IgG	immunoglobulins
[^{125}I]SBA	^{125}I-labeled soybean agglutinin
LPS	lipopolysaccharide
MGB	modified Gamborg buffer
MGB	modified Gamborg buffer containing 0.01 M sodium azide and 0.2% (w/v) bovine serum albumin
MW	molecular weight
RaCon A	rabbit antibodies against concanavalin A
R-anti-Con A-IgG	rabbit antibodies against concanavalin A
RaCT	rabbit antibodies against calf thymus actin
Ra-anti-SBA-IgG	rabbit antibodies against soybean agglutinin
RaSBA	rabbit antibodies against soybean agglutinin
mRNA	messenger ribonucleic acid
PBS	phosphate buffered saline
PI	preimmune serum
SB	fraction C in Figure 18 from DEAE cellulose fractionation of soybean seedling extracts
SBA	soybean agglutinin
SDS	sodium dodecyl sulfate
SDS PAGE	polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate
Tris	tris(hydroxymethyl)aminomethane

INTRODUCTION

One of the fundamental problems in the field of cell surface biochemistry is to determine how interactions of the cell surface receptors with various ligands are linked to the metabolic machinery of the cytoplasm and nucleus. A description of this transduction mechanism is essential to an understanding of the differentiation process of various cells such as embryonic induction, hormone induced effects as well as a number of host-parasite interactions.

On the basis of work done in animal cells, it has been postulated that a tripartite, metastable structure, consisting of cell surface receptors and the cytoskeletal macromolecules - microfilaments and microtubules, forms a system by which control and regulation of cell growth, differentiation and movement is modulated. One stringent test of this model of receptor-cytoplasmic interaction is to show that both the components and the interactions exist in various eukaryotic cells in both animal and plant cells.

A paradigm plant system that is amenable for studies of receptor cytoplasmic interactions is the infection of the root hairs of leguminous plants by the bacterium Rhizobium, leading to a symbiosis capable of carrying out nitrogen fixation. There are several reasons for studying this system: (a) the two cell types of the symbiotic relationship, the root hair and the Rhizobium, are well defined in terms of their species and strain specificities; (b) the lectin molecules hypothesized

to mediate the specific recognition event between the two partners have been purified and characterized in several systems; (c) the overall sequence of events of the infection process as well as many detailed morphological alterations in plant and bacterial cells have been defined; (d) the successful establishment of a symbiotic system leading to nitrogen fixation can be easily assessed; and (e) the importance for an understanding of the molecular events in this process has been recognized as a prerequisite to expanding the range of plants involved in nitrogen fixation.

The work presented in this thesis details the search for the components of the lectin receptor-cytoskeletal tripartite structure. The results are presented in two sections describing evidence for: (a) the presence of lectin receptors at the cell surface of soybean cells; and (b) the presence of microfilaments and microtubules in soybean cells. Therefore, these studies provide the first step in analyzing the more general problem of receptor-cytoplasmic interactions during host-parasite infection.

LITERATURE REVIEW

Lectins

A lectin is a carbohydrate binding protein, which may have more than one carbohydrate binding site. Multivalent lectins can agglutinate cells or other components with the complementary carbohydrate structures on their surface. Some mixed function lectins have been found, but usually all carbohydrate binding sites are of identical specificity (1,2). Though initially localized as a cytoplasmic component on the basis of extraction by aqueous buffers, recent evidence has shown some lectins to be integral components of the cellular membrane (3-6).

The chemistry of lectins has been well studied especially with respect to those derived from plant extracts (for current reviews, see references 7-9). In the plant, the primary source of lectins is the seed, in which these proteins make up 2-10% of the total protein (9), but they have also been isolated in lesser amounts from other plant tissues (6). Recently, lectins have also been isolated from animal tissues, both invertebrate and vertebrate (10,11).

Though the initial interest in lectins was due to their ability to bind saccharides in a specific fashion, current research on plant lectins has centered on their physiological role in their homologous environment. Some of the hypotheses on the function of lectins in plants include: (a) serve as antibodies to counteract pathogenic bacteria; (b) serve as inhibitors to protect the plant against fungi (12-14). It has also been proposed that lectins play an important role

in the recognition and binding between the nitrogen fixing bacteria, Rhizobium, and the host legume root hair cell (11,14). This hypothesis is particularly attractive in light of the accumulating evidence that certain lectins bind to both the root hair and the bacteria in a saccharide specific fashion related to the capacity of the Rhizobium strain to infect the host cell (15-17). Thus, these proteins could account for the specific interaction of legumes with certain bacteria by binding to cross reacting, sugar specific antigens present on both cell types. It should be noted that a particular species of legume can be successfully infected and nodulated by only one strain of rhizobia, and that most rhizobia infect only one species of legume.

Role of Lectins in the Binding of Rhizobium to Root Hair Cells

The hypothesis that lectins mediate rhizobia-legume interaction was proposed by Hamblin and Kent (15). They showed that bean infecting bacteria were agglutinated following treatment with bean extract, which presumably contained a lectin (15). Furthermore, erythrocytes were bound to root hair cells following treatment with the bean lectin. Although the evidence was not conclusive, it prompted further work, primarily in two systems: Rhizobium japonicum-soybean (Glycine Max) and Rhizobium trifolii-clover (Trifolium).

In the soybean system, Bohlool and Schmidt showed that 22 out of 25 strains of R. japonicum bound fluorescently labelled soybean agglutinin (fl-SBA), whereas none of the rhizobia strains which do not infect soybeans bound the fl-SBA (16). Moreover, of the 22 reactive strains, the binding of soybean agglutinin (SBA) was inhibited by simple haptens in 15, and the remaining strains were shown not to be able to nodulate soybeans (18).

However, in two other studies it was shown that there was no SBA present (a) in the seeds, as detected by affinity chromatography (19); (b) in various tissues, using radioimmunoassay, at an age when nodulation occurs (20). Yet, in both studies there was normal nodulation of soybean roots. These results must be questioned in light of the results of Bowles and coworkers, who showed that detergent is required for efficient extraction of agglutination activity from soybean tissues (16). Furthermore, the detergent extracted agglutinin was efficiently inhibited by N-acetyl-galactosamine as well as by several glycoproteins including asiolofetuin. Therefore, it appears that there is a substantial level of SBA present in soybean roots when Rhizobium infection occurs.

The molecular identity of the lectin receptor in the bacterial cell surface has not been elucidated to date. Wolpert and Albersheim have suggested the lipopolysaccharide (LPS) fraction of the cell membrane contains the lectin receptor on the basis of binding of LPS to affinity columns of homologous lectins covalently coupled to agarose (21). Yet, it has been shown by Tsien and Schmidt that the extracellular polysacchiarde (EPS) from R. japonicum forms a precipitin line with SBA in agarose gels (22). In a second experiment, Rhizobium were incubated with ferritin labeled lectin, and subsequently fixed so as to preserve the EPS and LPS matrices. Electron micrographs demonstrated the association of the labeled lectin with the EPS, rather than at the cell surface with the LPS (23). Furthermore, Schmidt has shown, using strain specific antibodies which react with the outer membrane LPS, that (a) the LPS of bacteroids is immunologically similar to that of the free rhizobia, and (b) the homologous lectin will not bind to the bacteroid

(14). Thus, it appears that receptor for SBA on the R. japonicum cell surface is contained within the EPS.

Research done with Rhizobium trifolii and clover has generated further support for the hypothesis of the role of lectins as mediators in the specific root-bacterial symbiosis (for a recent review see reference 24). Dazzo and coworkers have isolated a lectin, trifoliin, from clover seeds and from seedling roots (25). Trifoliin specifically agglutinates R. trifolii, and this interaction can be inhibited completely by 2-D-deoxyglucose. The clover lectin was shown to be present on the surface of root hair cells by immunofluorescence (25). Furthermore, trifoliin could be eluted by incubation with 2-deoxyglucose, suggesting that it is anchored through its carbohydrate binding sites.

Dazzo had previously demonstrated that the interactions between R. trifolii and clover root hairs was sensitive to inhibition by 2-deoxyglucose, and that noninfective strains of rhizobia were adsorbed at 20-25% of the levels of infective R. trifolii (26). Furthermore, it was shown by fluorescence microscopy that the bacterial capsular material was preferentially bound by the root hair cells of clover (27). It has also been shown that as the availability of biologically usuable nitrogen (as NO_3^- or NH_4^+) increases, the binding R. trifolii to root hair cells is decreased, although nitrogen concentrations does not affect agglutination of R. trifolii by trifoliin (28). Finally, Dazzo and Brill have demonstrated that the lectin trifoliin and antibodies to clover root antigenic determinants bind competitively to 2 acid polysaccharides isolated from the capsular material of R. trifolii (29). Moreover, 2-deoxyglucose inhibited the binding of both proteins. Furthermore, the purified capsular antigen from infective strains of

R. trifolii induced root hair deformation to a greater extent than capsular material isolated from non-infective strains. Although these studies strongly implicate a complex polysaccharide as being the lectin receptor on the bacteria, little information is available on the corresponding lectin receptor on the root hair.

Other Possible Roles of Lectins in Nodulation Events

Although the prevailing idea is that lectins mediate the initial recognition event, there are a number of other properties of these proteins which suggest that they may play other key roles during the nodulation process. It has been documented in a number of mammalian cell systems for example, that lectins can mediate a variety of processes including cell-cell recognition, morphogenetic changes in cell shape, surface-to-nucleus signaling, membrane fusion, and stimulation of cell division. Each of these processes is analogous to events which occur during the infection and nodulation of leguminous plants by Rhizobium. The analogous events include: (a) plant cell-Rhizobium recognition by lectin-mediated ligation of the two organisms; (b) root hair curling; (c) redirection of root hair growth with subsequent formation and growth of the infection thread; (d) infection of the host cortex cells and encapsulation of Rhizobium; and (e) differentiation and growth of nodule tissue cells.

Two specific examples are particularly illuminating. First, recent studies have shown that the binding of lectins to cell surface receptors results in "receptor-mediated endocytosis" (30), a process that involves microfilaments and clustered ligand receptor complexes (31). In addition, it has also been documented that the presence of lectin-receptor complexes within endocytosed vesicles function to stabilize these

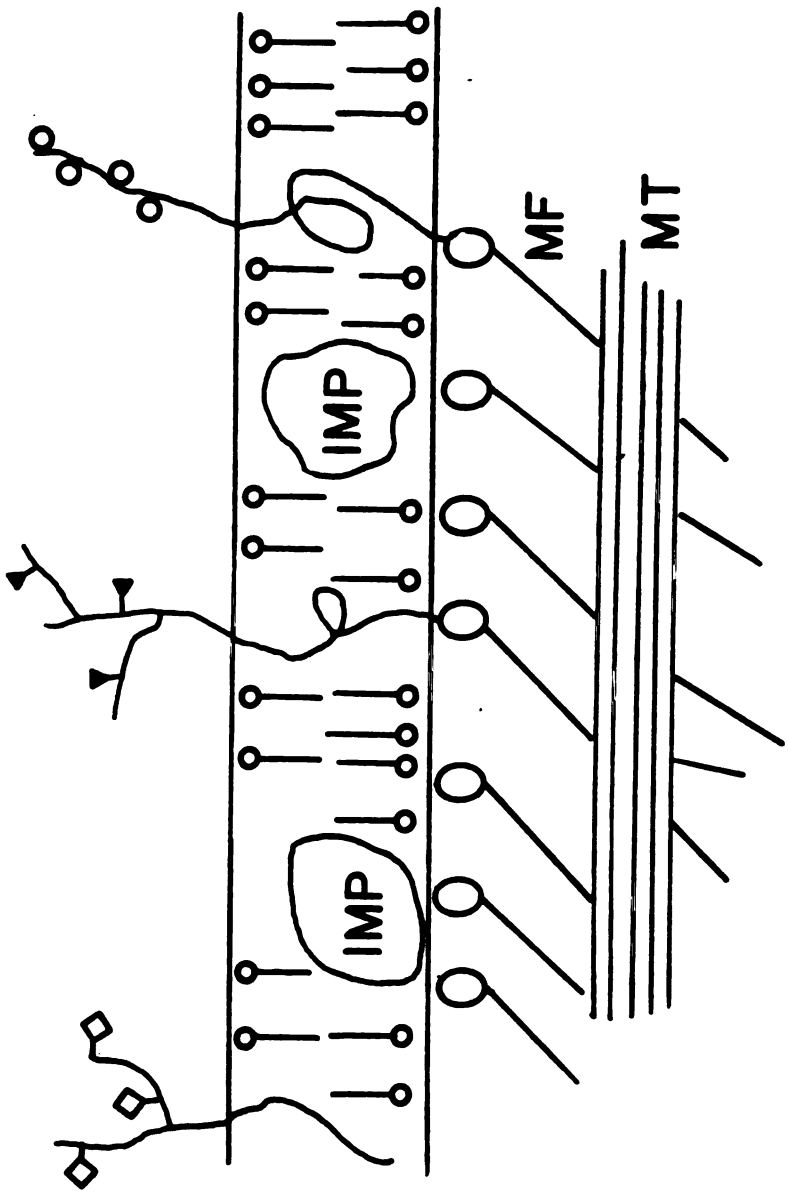
vesicles within certain animal cells (32,33). Assuming that these processes have analogous counterparts in plant systems, it is possible to hypothesize that the fusion of the host cell membrane with the infection thread membrane, the formation of the encapsulated bacteria, and the maintenance of the vesicle within the host may all be mediated by lectins and their interactions with surface receptor-microfilament complexes. This hypothesis may explain the endocytotic uptake of Rhizobium leguminosarum by pea leaf protoplasts, resulting in stable vesicles within the host cell cytoplasm (34). In light of the various different lines of evidence implicating the involvement of microfilaments and microtubules in each of the lectin-mediated effects in animal cell systems (35,36), the intriguing possibility is raised that lectin receptor-cytoskeletal structure interactions may also play a role in one or more of the early events during infection.

Receptor-Cytoskeletal Structure Interactions In Eukaryotic Cells

As a result of several independent lines of experimentation from various laboratories, a new view of the cell surface has been put forth in which surface receptors are hypothesized to interact with cytoplasmic assemblies of macromolecular structures (35-37). This assembly is thought to consist of a metastable, tripartite structure: (a) certain surface receptors that bind to external ligands; (b) microfilaments and associated proteins that are responsible for systematic movement of receptors; and (c) microtubules that are responsible for reversible anchorage of receptors (Figure 1).

Most of the early evidence suggesting the existence of this macromolecular assembly was derived from perturbation experiments using

Figure 1. A model for the organization of various components in the cell surface membrane complex. The model assumes that microfilaments (MF) interact with receptors, possibly via vinculin, and that microfilaments in turn interact with microtubules (MT). The interactions among the various components are assumed to involve reversible association-dissociation reactions. It is assumed that the intramembraneous particles (IMP) are not involved in these equiliberia.


MF
MT
IMP
IMP

external ligands to reveal certain cellular states (for a review, see reference 35).

These experiments can be arbitrarily classified into the following categories: (a) the induction of receptor redistribution (patch and cap formation) on lymphocytes and other cell types and the inhibition of receptor movement by cytochalasin B which disrupts microfilaments (38,39); (b) the modulation of receptor mobility at the level of individual receptors by the binding of the lectin concanavilin A (Con A) (40) and the reversal of this effect of Con A by colchicine which dissociates the microtubules (41); (c) the selective and directed movement of certain surface transport sites on polymorphonuclear leukocytes during the process of phagocytosis and the loss of the selective effect after treatment with colchicine (42,43); and (d) the co-redistribution of cell surface receptors and the microfilament and microtubule components as revealed by fluorescence and electron microscopy (44-46).

More recently, however, there has been an intensive accumulation of structural evidence in support of the receptor-cytoplasmic assembly interaction hypothesis. The key experiments that have to bear on this hypothesis are summarized below. First, it has been shown that certain cell surface receptors penetrate the lipid bilayer and are exposed to both the cytoplasmic and external environment simultaneously (47,48). This is particularly important as it immediately suggests a means by which receptor-cytoplasmic interactions may take place. Second, the existence of microfilaments and microtubules has been documented in a survey of many different animal cell types including non-muscle cells (49-51). Moreover, there is now strong evidence to indicate that these

microfilament and microtubule proteins interact with the cell surface membrane (52-54). Third, there is also recent evidence for direct molecular interactions between cell surface receptors and the actin subunit of microfilaments (55,56) as well as between the microtubule and microfilament proteins themselves within the assembly (57). The dynamic evidence, which served as the basis of the receptor-cytoplasmic interaction hypothesis (Figure 1) is supported, therefore, by the presently available structural information.

Cytoskeleton Proteins

The cytoskeleton of eukaryotic cells consists of 4 major components: (a) microtubules (25 nm), (b) microfilaments (6 nm), (c) intermediate filaments (10 nm), and (d) a group of smaller filaments (2-3 nm) (58,59). Present evidence suggests that the role of the latter two components are structural and relatively static, thus not participating in movement of cellular components, and therefore they will not be discussed (for recent reviews, see references 60, 61).

Microtubules

The development of glutaraldehyde fixation permitted the first visualization of microtubules (62), which are described as long, hollow filaments, 25 nm in diameter with 5 nm walls (for a review, see reference 63). Staining of cells with fluorescently-labeled antibodies directed against microtubules shows that a fine network of microtubules is associated with the plasma membrane and extends throughout the cell (49,64). The growth of unidirectional microtubules has been shown to originate at identifiable cellular structures called microtubule organizing centers.

The principal protein component is tubulin, which is composed of two related protein subunits, α and β tubulin. The subunits are present in a one to one ratio, and it is thought that tubulin is an αβ heterodimer. Isolation of tubulin from various sources has shown that there is conservation of amino acid sequence (65), subunit molecular weights, as well as immunological reactivity between species (63). Each tubulin dimer binds two molecules of guanine nucleotide, GTP and/or GDP. Some of the major chemical properties documented for tubulin are summarized in Table 1.

It is generally accepted that microtubules exist in an equilibrium relationship with a pool of depolymerized microtubular components. This equilibrium between tubulin and microtubules is sensitive to the presence of various agents including colchicine (66), Vinca alkaloids (67) low temperature as well as high concentrations of divalent cations (68). Colchicine binds to tubulin dimers and can displace the equilibrium in favor of the depolymerized form of the microtubular protein. The addition of the drug to cells causes, therefore, the dissociation of the microtubule assembly.

The elucidation of the conditions required for <u>in vitro</u> polymerization of tubulin (69) has enabled researchers to examine the roles of non-tubulin components of microtubules in the polymerization reaction. Such research has led to the hypothesis that the polymerization of tubulin dimers is controlled by nucleation elements present at very low levels (70-72). This hypothesis is particularly attractive inasmuch as the state of microtubules in the cell could be modulated by the physiological state of the cell.

Table 1

Properties of Tubulin

Properties		References
1. Molecular Weight		
a. native	110-120 K	(73-75)
b. sodium dodecyl sulfate		
α subunit	53 K	
β subunit	56 K	
2. Sedimentation coefficient	6 S	(76)
3. Isoelectric point	5.2 - 5.4	(77)
4. Binding sites		
a. colchicine	one/110 K dimer	(66,74)
b. podophyllotoxin	one/110 K dimer (competitive with colchicine)	(78)
c. vinblastine	one/110 K dimer (at one of the GTP sites)	(67)
d. GTP	two/110 K dimer	(73)

Microfilaments

The belief that all eukaryotic cells contain the same general apparatus for a variety of movements such as cytoplasmic streaming, amoeboid movement and phagocytosis has prompted an extensive search for muscle-like structures. Observations using electron microscopy have shown that filamentous structures (5-7 nm in diameter) similar to muscle thin filaments exist in many types of eukaryotic cells, including a variety of non-muscle cells of animal origin, vascular plants, slime molds, algae, amoeba and fungi (for reviews, see references 79-81). This distribution of microfilaments in animal cells has been dramatically depicted by immunofluorescence staining (49,50).

Actin, the principal protein component of microfilaments, has been isolated from various animal cells (79) and also from Acanthamoeba (82-84), Amoeba (85), Dictyostelium (86,87), Physarum (88-90), Saccharomyces (91), Mycoplasma pneumonia (92), and Chlamydomonas (93). Three classes of actin have been established on the basis of isoelectric points: α, β, and γ. α (most acidic form) is found in striated muscle, while β and γ are cytoplasmic species, with γ being the most basic (91-98). Despite these small differences, the basic molecular structure of actin has been highly conserved in a wide variety of cell types. Some key features of the actin molecule are summarized in Table 2.

The product of the conversion of monomeric G actin into fibrillar F actin can be visualized by electron microscopy, particularly using heavy meromyosin decoration to form the characteristic arrowheads (99). It is known that a number of regulatory factors may be involved. First, it has been shown that cytochalasin B inhibits or disrupts microfilament assembly states, possibly by inhibiting the attachment of the actin

Table 2

Properties of Actin from Various Sources

Properties		References
1. Subunit molecular weight	42-46 K	(79-81)
2. Sedimentation coefficient	3-3.7 S	(86)
3. Characteristics of the amino acid composition	contains methylhistidine	(79)
4. ATP (ADP) binding	one ATP/42 K	(80)
5. G actin $\rightleftharpoons$ F actin	ATP and salt dependent	
6. Myosin binding		
a. stimulates Mg^{2+}-dependent ATPase		(99)
b. arrowhead decoration of heavy meromyosin		(99)
7. DNase I binding	irreversible	(102)

filaments to membrane anchorage sites (100). In addition, it has been documented that cell extracts containing actin can undergo gelation in the presence of activating factors (101). Finally, there is a recent report that assembly of microtubules and microfilaments may be coordinately modulated by factors such as Tau, or other microtubule associated proteins (57).

Cytoskeletal Proteins in Plant Cells

The majority of the work on microtubules and microfilaments in plant cells has centered on ultrastructural (for reviews, see references 103-105) and immunofluorescence (106,107) demonstrations of fibrillar structures that show sensitivity to the drugs colchicine and cytochalasin B. Very little is known concerning the chemical components of these structures and their assembly properties. Cytoplasmic tubulin and microtubule organizing center proteins have been purified from the algae Polytomella; the molecular weights of these isolated proteins are similar to those obtained from animal sources (108). In addition, there is recent evidence for the purification of tubulin from tobacco cells (109). The identification of the actin subunit of microfilaments have been reported in abstracts for Phaseolus vulgaris (110), Lycopesium esculentum (111) and in partially fractionated form for Nitella flexilis (112). The data all indicate that plant actin, like actin isolated from muscle and from other animal cells, is a protein with a molecular weight of about 45,000 (see Table 2). Much remains to be learned, however, concerning the detailed chemical structures, binding properties, and assembly characteristics of both tubulin and actin from plant cells.

Microtubules in plant cells, as in animal cells, appear to constitute a major component of spindle fibers which are involved in mitotic

processes such as chromosomal separation and cytokinesis. There is accumulating evidence to suggest that microtubules may mediate cell wall deposition in terms of microfibril alignment and secondary cell wall pattern formation (113-116). The most striking example of the latter is seen in the disappearance of organized secondary cell wall in vessel elements treated with colchicine (117). These observations are consistent with the larger hypothesis on the general role of microtubules in the intracellular movements of organelles and vesicles (109). Finally, there are at present conflicting reports concerning the effect of colchicine on the infection process of Rhizobium on legume root hairs. In one experiment, colchicine increase the number of infection threads and nodules at low concentrations while those nodules formed at high drug concentrations were ineffective (118). In contrast, it has also been reported that colchicine will inhibit the growth of infection threads in Trifolium (119).

In recent years, a number of laboratories have documented the ultrastructural observation of microfilaments in plant cells such as Avena sativa, Nicotiana tabacum, Nitella flexilis, Amaryllis belladonna, Haemanthus katherina, Mougeotia, and Chara (120-126, see Table 3). These structures have also been implicated in cytoplasmic streaming and organelle movement. In the algae Mougeotia, light induced chloroplast movement is modulated by cytochalasin B and Ca^{2+} ions (129,130). In addition, a number of plant cells such as Nitella (131), Zea, Avena, and Pisum (132) also show reversible inhibition of cytoplasmic streaming by cytochalasin B. In studies with Nitella, the effect of cytochalasin B appears to disrupt microfilament structures, not by dissociation into subunits, but more likely by detachment from anchorage sites (103,105).

Table 3

Occurrence of Microfilaments in Higher Plants*

Source	Reference
Amaryllis belladonna	124
Cuscuta sp	127
Gossypium hirsutum	127
Haemanthus katherinae	125
Hibiscus esculentus	127
Impatiens sultanii	127
Lemna minor	127
Liriodendron tulipfera	127
Phaseolus aureus	127
Phaseolus vulgaris	110
Pinus taeda	127
Pueraria lobata	127
Raphanus sativus	127
Setraesea purpurca	127
Vica faba	128
Xylosma congestum	128
Zea mays	127

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

Finally, it is interesting to note that cytochalasin B treatment resulted in cessation of cytoplasmic streaming and stoppage of tip growth in root hairs of Hepidium stuivum, Raphanus sativus, as well as in pollen tubes of Lillum longiflorum (133). A summary of the present status on the purification and characterization of actin from higher plants is presented in Table 4.

Table 4

Purification and Characterization of Actin From Plants

Source	Procedure	Characteristics
Cucurbiat and Nicotiana (134)	Enrichment by actomyosin ppt	ATPase
Nitella (135)	Enrichment by polymerization	dec. mf
Phaseolus vulgaris (110)	Enrichment by gel filtration, polymerization	dec. mf MW
Lycopesicon esculentum (111)	Partial purification - ion exchange chromatography polymerization	dec. mf MW
Triticum aestivum (128)	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 - Reacts with anti-actin antibodies

MATERIALS AND METHODS

Soybean Cell Culture

SB-1 cell line of soybean (Glycine max) cells was kindly provided by Dr. O.L. Gamborg (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 every 3 to 4 days by transferring 10 ml of culture to 30 ml of fresh 1-B5 medium (136). 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 2-B5 medium (136). 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. Amsoy 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 mercuric chloride (2 gm $HgCl_2$ 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 (137), to which KNO_3 was added to the Fahraeus medium to a final concentration of 2 mM.

Preparation of Affinity Absorbent for Soybean Agglutinin (138)

1.5 gm of CH-Sepharose 4B (Pharmacia, Uppsala, Sweden) was swollen in 0.5 M NaCl and washed three times with the same solvent by centrifugation, followed by three washes with water. The gel was suspended in water to make a slurry of 25 ml and stirred at room temperature. 100 mg of galactosamine HCl (Sigma, St. Louis, MO) was added, and the pH was adjusted to 5.0 with 0.1 M NaOH. 50 mg of solid 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl (Sigma) was added over a period of 5 min. After 10 minutes, the pH had risen to a constant value of 5.2. The reaction was stirred at room temperature for 1 hour, then left at room temperature without stirring for 20 hours. The gel was washed successively with 250 ml of 1 M NaCl, 1 M NaCl in 0.1 M Tris (pH 8.6), 1 M NaCl in 0.05 M sodium formate (pH 3.0), 1 M NaCl in 0.1 M Tris (pH 8.6), water and phosphate buffered saline (PBS). The gel was then ready for use. If not used immediately, the gel was stored in 2 M NaCl in PBS.

Isolation and Characterization of Lectins

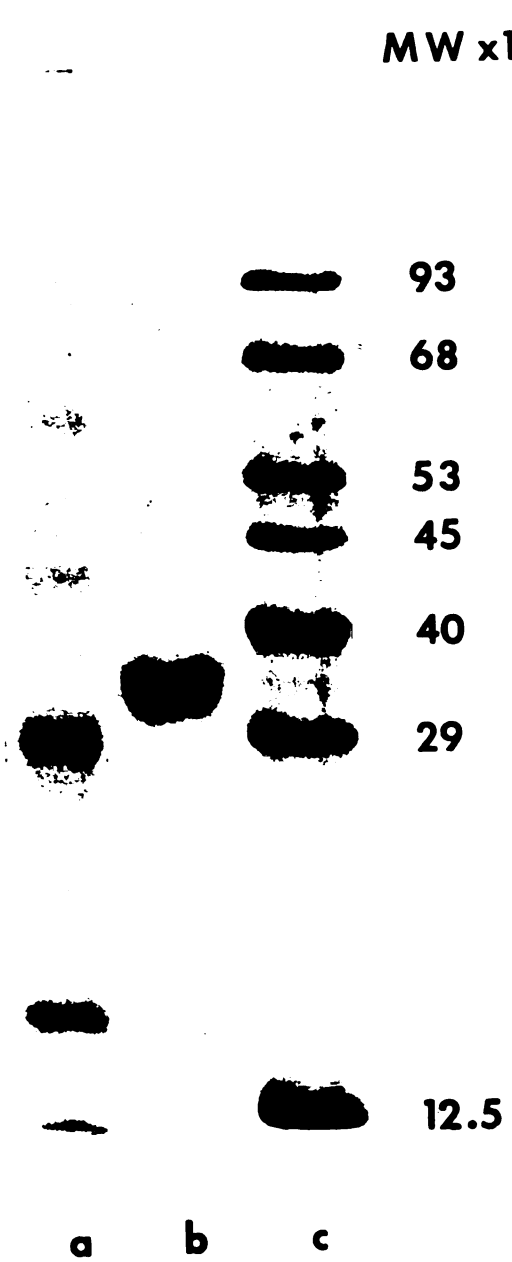
Soybean meal was prepared by grinding seeds in a commercial grinder. The 12.5 gm meal was defatted by stirring with 300 ml hexane

for 10 min. The suspension was filtered on a Buchner funnel, until the meal was dry. The defatted meal was stored at 4°C.

All further steps are done at 4°C. 10 gm of defatted soybean meal was extracted with 200 ml phosphate buffered saline (PBS) overnight with stirring. The suspension was centrifuged at 9,000 xg for 15 min, and the supernatant was further classified by filtration using a Buchner filter. The clarified extract was incubated with the Sepharose-N--caproylgalactosamine packing for 60 min. The suspension was washed with 1 l PBS by filtration on a coarse fritted glass filter. It was then transferred to a column and washed with PBS until absorbance at 280 nm was below 0.005. Elution of the column with 0.2 M D-galactose yielded pure SBA as determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS PAGE; see lane b, Figure 2, 138). The lectin was extensively dialyzed against water and lyophilized.

1,000 gm of jack bean meal (Sigma) was suspended in 300 ml of 0.01 M Tris (pH 7.4) and stirred for 2 hours at room temperature. The suspension was filtered through cheesecloth and the filtrate was centrifuged at 1020 xg for 40 minutes. The supernatant fraction was subjected to affinity chromatography on a column (5 x 30 cm) of Sephadex G-75 (Pharmacia) in the same buffer. After washing the column with 1 liter of buffer, the protein was eluted with 0.01 M Tris (pH 7.4) which was 0.1 M in D-glucose (Sigma). The protein eluted from the column was dialyzed against 0.01 M Tris (pH 7.4) followed by extensive dialysis against water and lyophilization (139). The purified concanavalin A was analyzed by SDS PAGE, and shown to be >95% pure (see lane a, Figure 2).

Figure 2. SDS polyacrylamide gel electrophoresis of soybean agglutinin and concanavalin A. The concentration of acrylamide in the running gel was 10% (w/v). (a) Concanavalin A (15 μg); (b) soybean agglutinin (15 μg); (c) molecular weight standards: phosphorylase a (93,000), bovine serum albumin (68,000), glutamic dehydrogenase (53,000), calf thymus actin (45,000), aldolase (40,000), carbonic anhydrase (29,000) and cytochrome c (12,500).


MW x10-3
93
68
53
45
40
29
12.5
a
b
c

Purification and Characterization of Animal Actin Proteins

Acetone powder was prepared from rabbit back muscle by grinding 100 gm of tissue in a commercial meat grinder and washing with 10 mM sodium EDTA, 0.4% (w/v) sodium bicarbonate (pH 7.0) for 15 minutes and finally rinsing with distilled water (140). 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. Unless noted otherwise, all further steps were carried out at 4°C.

Actin was isolated from 10 gm of acetone powder by extraction with 200 ml of extraction buffer (2 mM Tris, 0.2 mM ATP (Boehringer-Mannheim Biochemicals, Indianapolis, IN), 0.5 mM β-mercaptoethanol, 0.2 mM $CaCl_2$, pH 8.0) for exactly 10 minutes at 0°C (141). The 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. Actin was polymerized in the pooled supernatant fluids by adding solid KCl and 2.5 M $MgCl_2$ to a final concentration of 50 mM and 2 mM, respectively, and was incubated for 2 hours at 37°C. 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 xg for 3 hours. The pellet was resuspended in 30 ml of extraction buffer and dialyzed against 500 ml of the same buffer for 2 days with 4 changes of the buffer. After dialysis, the solution was centrifuged for 1.5 hours at 80,000 xg to remove 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% (w/v).

Analysis of the isolated rabbit muscle actin by SDS PAGE (Figure 3, lane a) showed that the major Coomassie blue stained component had an electrophoretic mobility which corresponded to a molecular weight of 45,000. The second band (molecular weight 39,000) is a known degradative fragment of actin (142). The rabbit muscle actin was also subjected to two dimensional gel electrophoresis with isoelectric focusing in the first dimension, followed by SDS PAGE in the second dimension. As shown in Figure 4, there is a single major spot which has an isoelectric point of 5.7, and an electrophoretic mobility in the second dimension which corresponds to a molecular weight of 45,000. These results are in agreement with previously published reports (94-98).

The cytoplasmic form of actin was purified from calf thymus by a modification of the method of Gordon et al. (82). All steps were done at 4°C unless noted. Briefly, 320 gm of tissue was homogenized in 700 ml of buffer A (3 mM imidazole HCl (pH 7.5), 0.1 mM $CaCl_2$, 0.5 mM ATP, 0.75 mM β-mercaptoethanol) with 0.02 units per ml of the protease inhibitor Trayslol (Sigma) in a Waring blender. The homogenate was clarified by centrifugation at 16,318 xg for 30 minutes followed by a second centrifugation step at 100,000 xg for 2 hours. The supernatant fraction was applied to a DEAE cellulose column (DE52, Whatman, Springfield Mill, England; 4.1 x 25 cm) which was equilibrated in buffer A. The column was washed with 100 ml buffer A, followed by 1 l of buffer B (10 mM imidazole HCl (pH 7.5), 0.1 mM $CaCl_2$, 0.5 mM ATP, 0.75 mM β-mercaptoethanol). A linear gradient from 0 to 0.3 M KCl in a total volume of 3 l of buffer B was used to elute the column. The fractions

Figure 3. SDS polyacrylamide gel electrophoresis of rabbit muscle actin and calf thymus actin. The concentration of acrylamide in the running gel was 7.5% (w/v). (a) Rabbit muscle actin (15 μg); (b) calf thymus actin (12 μg). Molecular weight standards: phosphorylase a (93,000), bovine serum albumin (68,000), glutamic dehydrogenase (53,000), aldolase (40,000) and chymotrypsinogen (25,000), and the dye bromophenol blue.

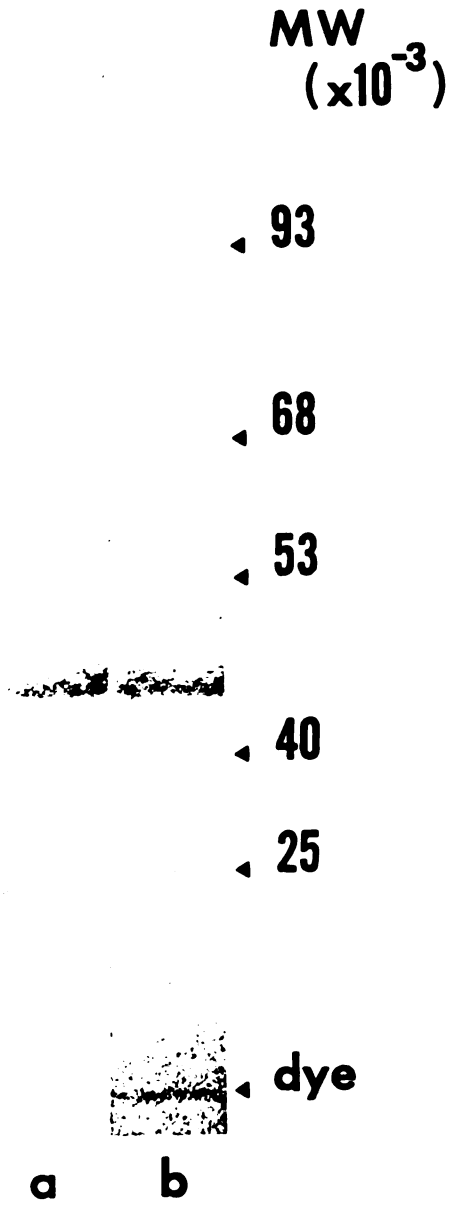
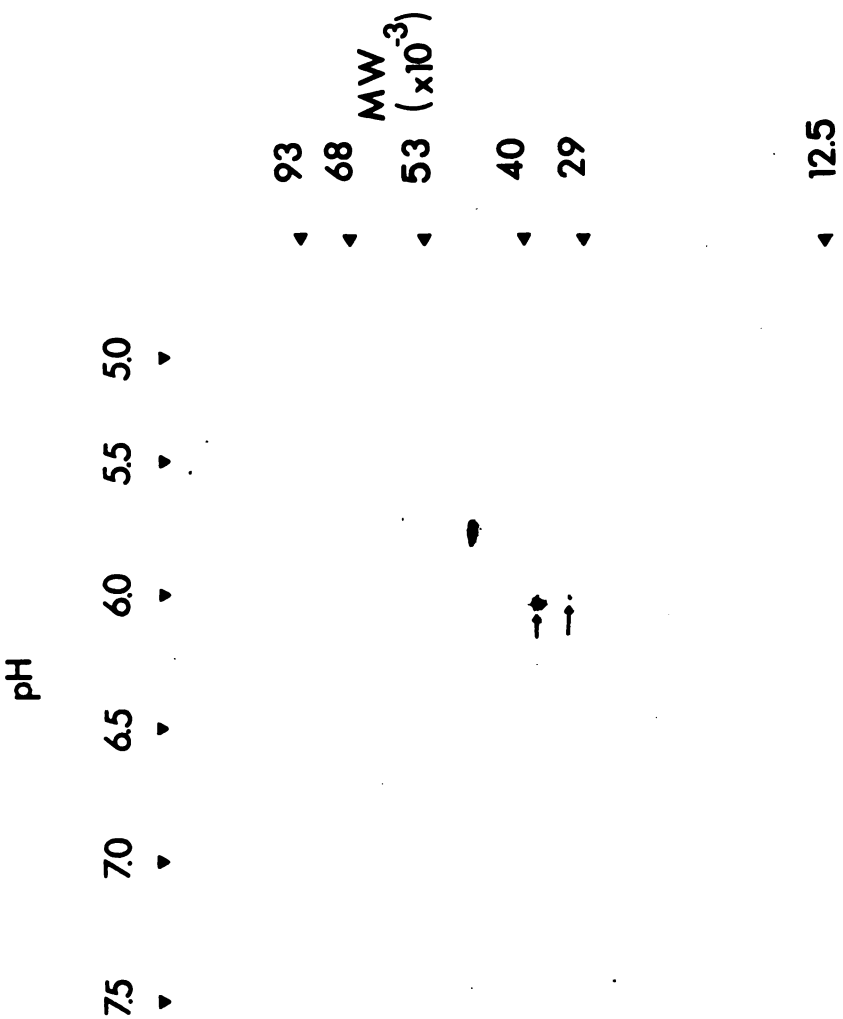

MW
(x10-3)
93
68
53
40
25
dye
a
b

Figure 4. Two dimensional gel electrophoresis of rabbit muscle actin. Lyophilized rabbit muscle actin was dissolved in sample buffer containing urea and, a sample containing 15 μg protein was subjected to analysis as described in Materials and Methods. Molecular weight standards are the same as in Figure 2. The arrows indicate degraded forms of actin.


MW (x10^-3)
93
68
53
40
29
12.5
pH
5.0
5.5
6.0
6.5
7.0
7.5

which contained actin were pooled and solid KCl and 2.5 M $MgCl_2$ were added to a final concentration of 0.2 M and 2 mM, respectively. The solution was incubated at 37°C for at least 4 hours. The solution was then centrifuged at 100,000 xg for 3.5 hours at 20°C. The polymerized actin pellet was resuspended in buffer A which contained 0.05% (w/v) NaN_3, and depolymerized by dialysis against the same buffer at 4°C for 3 days with 6 changes of buffer. Following centrifugation to pellet insoluble material (100,000 xg for 90 minutes), the resulting supernatant was applied to a Sephadex G-150 column (Pharmacia; 2.5 x 95 cm) equilibrated in buffer A containing 0.05% (w/v) NaN_3. The purified, globular form of actin (G-actin) eluted as the second fraction from the column. Analysis of the G-actin by SDS PAGE (see lane b, Figure 3) demonstrated that there was only one Coomassie blue stained component present, and it had a molecular weight of 45,000.

The purified protein was demonstrated to be the cytoplasmic form of actin by two dimensional gel electrophoresis (see Figures 5 and 7). This analysis showed that the calf thymus actin consisted of 2 spots which had different isoelectric points (5.8 and 5.9) but identical electrophoretic mobilities in the presence of sodium dodecyl sulfate corresponding to a molecular weight of 45,000. These values are in agreement with previous published reports concerning the characterization of the non-muscle, or cytoplasmic, form of actin (94-98). As demonstrated in Figure 6, and the last panel of Figure 7, when rabbit muscle actin and calf thymus actin were analyzed simultaneously, one could distinguish muscle actin (most acidic form actin) from the cytoplasmic form (β actin pI 5.8, γ actin pI 5.9). Therefore, the actin purified from calf thymus was shown to be the non-muscle, or cytoplasmic, forms of actin (94-98).

Figure 5. Two dimensional gel electrophoresis of the calf thymus actin. Lyophilized calf thymus actin was dissolved in sample buffer containing urea, and a sample containing 15 μg protein was subjected to analysis as described in Material and Methods. Molecular weight standards are the same as in Figure 2.

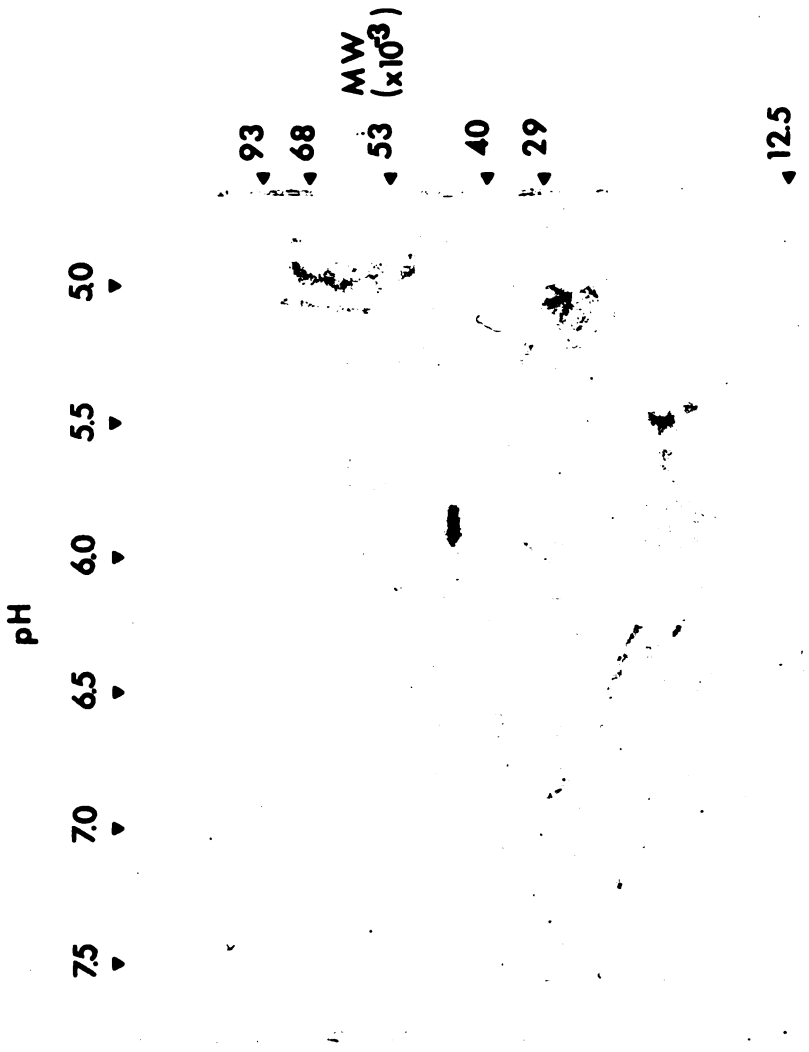

MW
(x10^3)
93
68
53
40
29
12.5
pH
5.0
5.5
6.0
6.5
7.0
7.5

Figure 6. Two dimensional gel electrophoresis of calf thymus actin and rabbit muscle actin. Lyophilized samples of each protein were dissolved in sample buffer containing urea. A sample consisting of 15 µg protein of calf thymus actin and 15 µg protein rabbit muscle actin was analyzed as described in Material and Methods. Molecular weight standards are the same as in Figure 2. The origin of the Coomassie blue spot indicated by the arrowhead is not known.

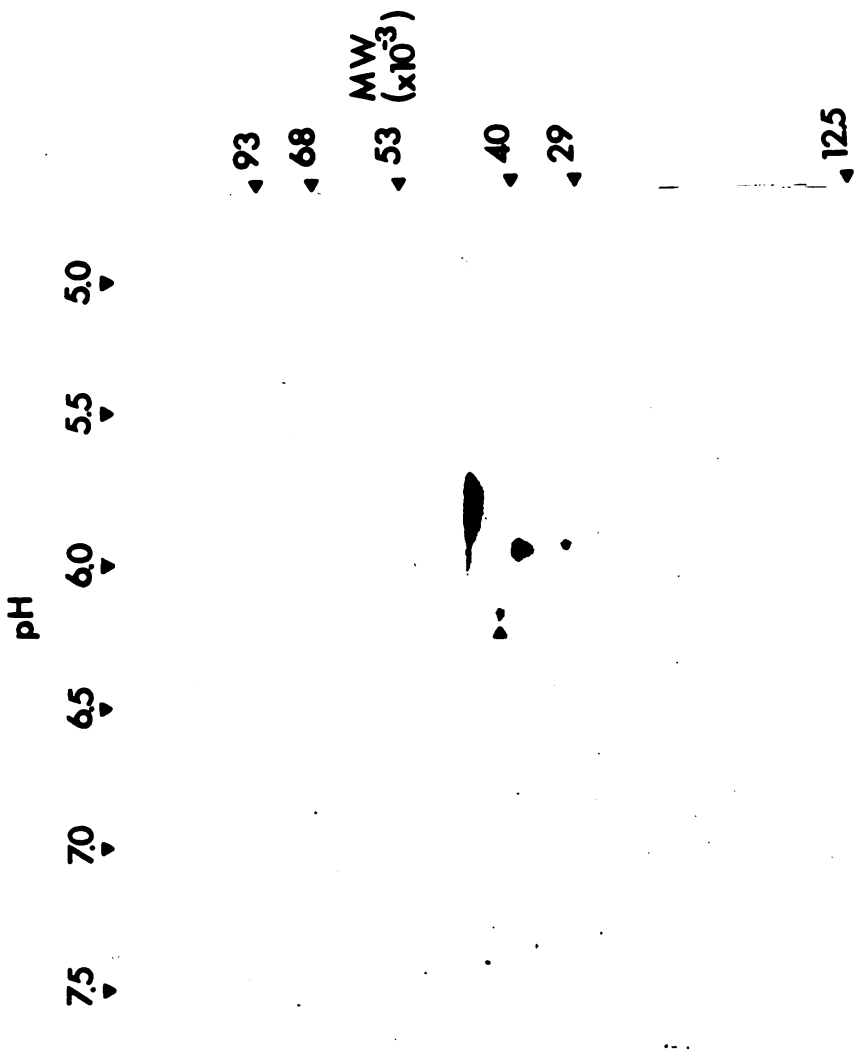
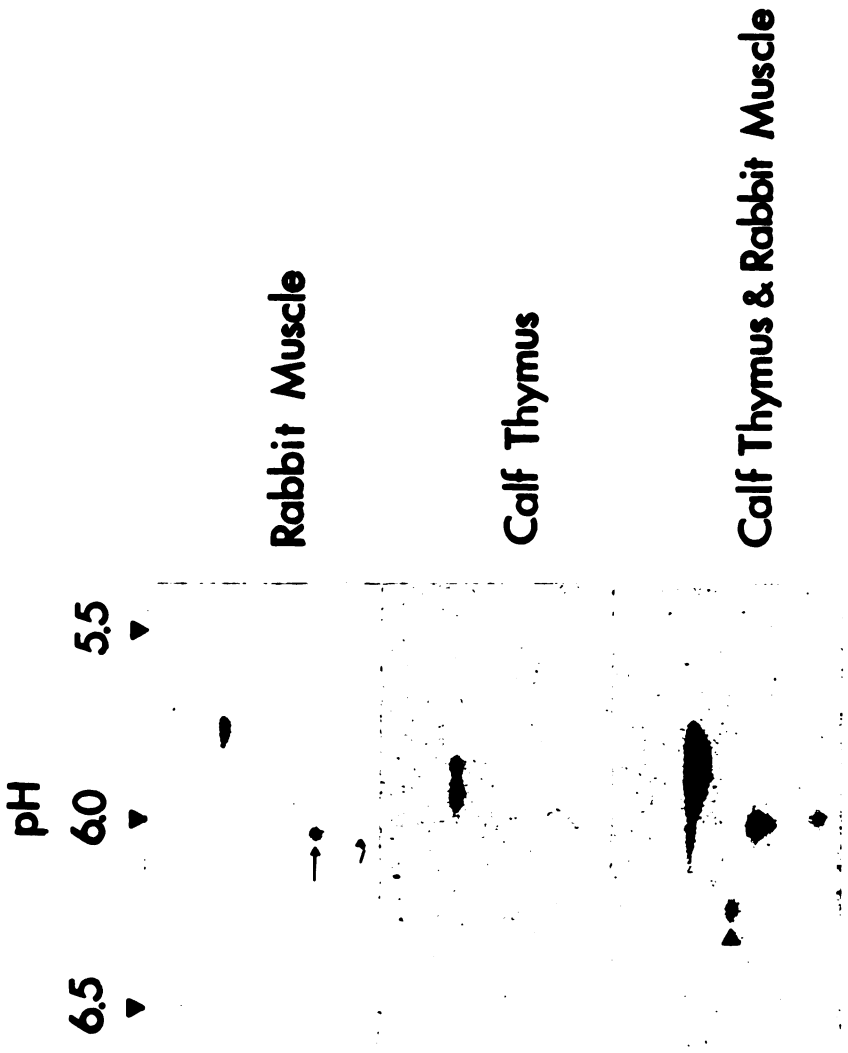

MW (x10^-3)
93
68
53
40
29
12.5
pH
5.0
5.5
6.0
6.5
7.0
7.5

Figure 7. Enlargement of the actin area in two dimensional gel electrophoretic analyses of calf thymus actin and rabbit muscle actin - separately and together. 15 μg of each protein was subjected to analysis as described in Materials and Methods. The arrows indicate degraded forms of actin. The origin of the Coomassie blue spot (arrowhead), when calf thymus actin and rabbit muscle actin were run together, is not known.


Rabbit Muscle
Calf Thymus
Calf Thymus & Rabbit Muscle
pH
5.5
6.0
6.5

Preparation of Rabbit Muscle Myosin and Heavy Meromyosin

Myosin was isolated from fresh rabbit back muscle (143,144). The tissue was ground in a meat grinder and myosin was extracted with 3 volumes of buffer E (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 ml of buffer F (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 ml of buffer F and stored at 4°C. Sodium azide was added to a final concentration of 0.05% (w/v) to prevent bacterial contamination.

Heavy meromyosin was prepared by tryptic digestion of myosin (145). Myosin (4 mg/ml) was digested with 0.01 volume of TPCK-Trypsin (0.15 mg/ml in 1 mM HCl; Sigma) 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; Sigma). Undigested myosin and light meromysin were precipitated by dialysis overnight of the digestion solution

against 800 ml 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 (122). The pellet was resuspended in 10 ml of 5 mM Tris, 0.5 mM EGTA, 1 mM DTT (pH 8.0) and dialyzed against 400 ml 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.

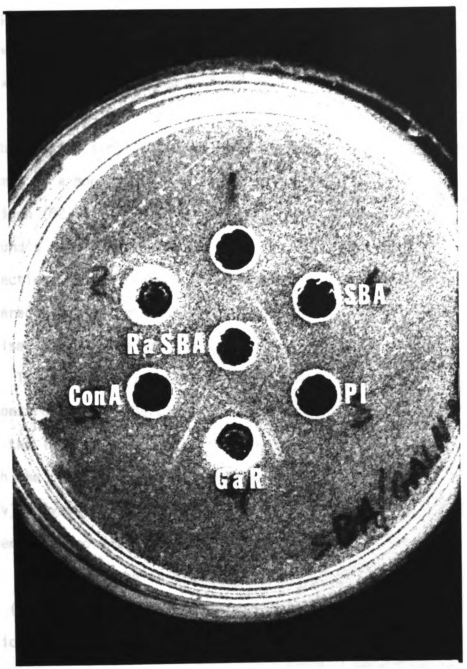
Antibody Reagents

Antibodies against soybean agglutinin were raised in rabbit, using the previously purified protein as antigen (138). The primary injection consisted of 800 µg of protein in Freund's complete adjuvant (Difco, Detroit, Mich.) Booster injections of 200 µg protein in Freund's incomplete adjuvant (Difco) were administered at bi-weekly intervals. Antiserum was collected one week after boosting.

The presence of immunological activity was detected by Ouchterlony double immunodiffusion (146). The immunodiffusion plates (1% (w/v) agar (Difco) with 0.2 M N-acetyl-D-galactosamine (Sigma), 0.05% (w/v) NaN_3) were incubated at 37°C for 24 hours, then kept at 4°C until the precipitin lines developed. Figure 8 shows a picture of an Ouchterlony plate for rabbit anti-SBA, run in the presence of N-acetyl-galactosamine, which showed a single precipitin line between the antigen and the immune serum. No precipitin line was seen between the antigen and the preimmune serum or between Con A and the immune serum.

Rabbit anti-Con A antiserum was obtained using the purified protein (127) as antigen, following the protocol outlined above. The

Figure 8. Ouchterlony double immunodiffusion plate demonstrating reactivity of rabbit anti-soybean agglutinin serum against soybean agglutinin. The immunodiffusion plate was run as described in Materials and Methods. SBA - soybean agglutinin (10 mg/ml in 0.2 M GalNAc in PBS). PI - preimmune serum. GaR - goat antibodies directed against rabbit immunoglobulin. Con A - concanavalin A (10 mg/ml in 0.2 GalNAc in PBS). RaSBA - rabbit anti-soybean agglutinin serum.


SBA
RaSBA
ConA
PI
GaR

Ouchterlony plate shown in Figure 9 depicts the reaction between Con A and the immune serum. The immunodiffusion was run in the presence of 0.2 M α-methyl-D-mannoside (Sigma) in order to eliminate the binding properties of the lectin. There is a single broad precipitin band between the immune serum and the antigen, with no precipitin line seen between Con A and preimmune serum or between immune serum and SBA.

Antibodies directed against calf thymus actin were raised in rabbits using a modified protocol. Pure actin, isolated from calf thymus by a modification of the method of Gordon et al., was used as antigen (82). The initial injection consisted of 800 μg protein in Freund's complete adjuvant. Three months later, the first booster injection (400 μg protein in Freund's incomplete adjuvant) was administered. Subsequent boosts were given at six week intervals. Antiserum was collected one week after boosting.

The Ouchterlony double immunodiffusion plate shown in Figure 10 demonstrates that there was a single precipitin line between calf thymus and the immune serum, while there was no precipitin line seen associated with the preimmune control. The Ouchterlony plates were run with 0.5% (w/v) polyethylene glycol 6800 (Aldrich Chemical Co., Milwaukee, WI) in order to improve the formation of the precipitin lines.

The immunoglobulin fraction of the various antisera was obtained by 37% $(NH_4)_2SO_4$ precipitation at room temperature. After centrifugation (16,318 xg for 15 minutes), the pelleted material was resuspended in 0.0175 M phosphate buffer (pH 7.0), and dialyzed against the same buffer for 24 hours with 2 changes of buffer. The dialyzed material was then applied to a DEAE cellulose (1 x 10 ml) equilibrated in 0.0175 M phosphate buffer (pH 7.0) (147).

Figure 9. Ouchterlony double immunodiffusion plate demonstrating reactivity of rabbit anti-concanavalin A serum against concanavalin A. Immunodiffusion plate was run as described in Materials and Methods. Con A - concanavalin A (10 mg/ml in 0.2 M α-methylmannoside in PBS). PI - preimmune serum. GaR - goat antibodies directed against rabbit immunoglobulins. SBA - soybean agglutinin (10 mg/ml in 0.2 M α methylmannoside in PBS). RaCon A - rabbit anti-concanavalin A serum.

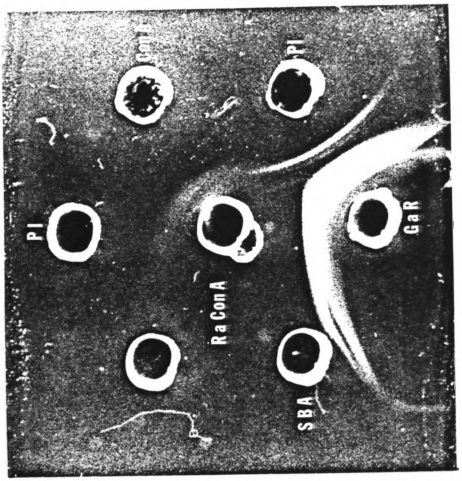
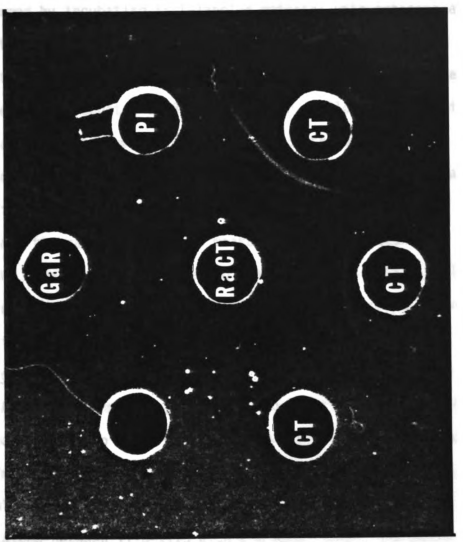

PI
RaConA
PI
SBA
GaR

Figure 10. Ouchterlony double immunodiffusion plate demonstrating reactivity of rabbit anti-calf thymus actin against calf thymus actin. Immunodiffusion plate was developed as described in Material and Methods. GaR - goat antibodies directed against rabbit immunoglobulin. PI - preimmune serum. CT - calf thymus actin (from right to left, 1, 0.5, and 0.25 mg/ml calf thymus actin). RaCT - rabbit anti-calf thymus actin serum.


PI
CT
GaR
RaCT
CT
CT

Immunoprecipitation

Fractions derived from soybean cell extracts, calf thymus extract and calf thymus actin were labeled with ^{125}I (Amersham, Arlington Heights, IL, 148) and dialyzed versus buffer A (see Purification and Characterization of Animal Actin Proteins). Immunoprecipitation was performed by incubating ^{125}I-labeled material with antisera at 37°C for 1 hour, followed by 6 hour incubation at 4°C. Goat antibodies directed against rabbit immunoglobulins (Difco) were added, and the samples were incubated at 37°C for 1 hour, followed by overnight incubation at 4°C. The immunoprecipitate was centrifuged at 1,500 x g for 5 minutes, and then washed twice with 0.5 ml PBS. After a final resuspension in 0.5 ml PBS, the immunoprecipitate was transferred to a fresh tube and the radioactivity was measured by a gamma counter. Subsequently, the samples were subjected to analysis by SDS PAGE. Following electrophoresis, the gels were cut into 2 mm slices and the radioactivity was measured in a gamma counter.

Immunoaffinity Absorbent

Affinity column was prepared by covalently coupling of isolated immunoglobulins to Sepharose 4B (Pharmacia). Cyanogen bromide (30 mg CNBr/ml packing in 5 ml acetonitrile; Sigma) was added to Sepharose 4B (20 mls) and stirred for 8 minutes at room temperature while the pH was maintained between 11.0-11.5 with 2 N NaOH (149). The packing was then washed on a frittered glass filter with 500 ml ice cold water, then 500 ml of 0.1 M $NaHCO_3$, and resuspended in 40 ml 0.1 M $NaHCO_3$. Immunoglobulins (20 mg in 2 ml of 0.1 M $NaHCO_3$) was added to the activated Sepharose 4B and gently mixed overnight. Then packing was then washed

with 500 ml of 0.1 M $NaHCO_3$, followed by 500 ml PBS. A column (1 x 10 ml) was poured using the prepared packing and washed with buffer A (see Preparation and Characterization of Animal Actin Proteins) before being used.

Binding of Fluorescent-Lectin to Protoplasts

Protoplasts were isolated by a modified procedure of Constabel (150). Actively growing SB-1 cells (24-48 hours after transfer) were digested with an equal volume of enzyme solution containing 400 mg cellulysin (Calbiochem, LaJolla, CA), 200 mg pectinase (Sigma) and 2 g D-sorbitol (Sigma), (pH 5.5) in 20 ml. After 2 hours, the protoplast suspension was filtered through 48 μm nylon filter and pelleted by centrifugation in a clinical centrifuge for 4 minutes at 460 xg. The pelleted protoplasts were washed by gentle resuspension and centrifugation using 5 ml of protoplast medium (150) which was modified by substituting 20 g D-sorbitol for sucrose (MGB). After three washes, the protoplasts were resuspended in 0.5 ml of MGB.

The binding of various lectins to protoplasts was assayed by fluorescence microscopy following the representative protocol detailed below: (a) incubation of protoplasts (5 x 10^5 per ml) with lectin solution (50 μg/ml) SBA, Con A or fluorescein-conjugated wheat germ agglutinin (Miles, Elkhart, IN); (b) the protoplasts were washed by centrifugation and resuspension in modified protoplast media containing 0.01 M NaN_3 and 0.2% (w/v) bovine serum albumin (BSA; MGB-N_3-BSA); (c) in cases where SBA or Con A were used, the protoplasts were further incubated for 15 minutes with 100 μg/ml rabbit antibodies directed against SBA or Con A, followed by fluorescein-conjugated (Difco, Detroit, MI) or rhodamine-conjugated goat antibodies directed against

rabbit immunoglobulin (Cappel, Downington, PA); (d) after washing the protoplasts were suspended in a final volume of 50 µl of MGB-N_3-BSA and observed under a Leitz fluorescence microscope. Micrographs were taken with Kodak Tri-X pan film. In experiments where the effect of drugs on receptor distribution was examined the protoplasts were preincubated with colchicine (10^{-5} M; Sigma) or with cytochalasin B (2.1×10^{-5} M; Sigma) for 15 minutes at 25°; during the subsequent incubations with lectins and antibody reagents, the concentration of each drug was maintained at the same level. In experiments done at 4° or 37°, the protoplasts were preincubated at the appropriate temperature for 15 minutes prior to the addition of the lectin solution.

The demonstration of sugar specificity with respect to lectin binding was accomplished by incubating the lectin and protoplast together in the presence of 0.2 M sugar for 60 minutes at room temperature (N-acetyl-D-galactosamine for SBA, α-methyl-D-mannoside for Con A, N-acetyl-D-glucosamine (Sigma) for wheat germ agglutinin).

Binding of ^{125}I-Labeled Lectin to Protoplasts

Concanavalin A was labeled with radioactive iodine (Amersham) using the iodogen, 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril (a gift from Drs. Speck and Fraker (151)). SBA was iodinated according to the method of Greenwood and Hunter (148).

Protoplasts (5×10^5 per ml) were incubated with [^{125}I]-lectin for 60 minutes, in duplicate, at room temperature. Nonspecific binding of [^{125}I]lectin was determined by incubation of the [^{125}I]lectin in the presence of the competing sugar. After three washes by centrifugation and resuspension of the cells in MGB-N_3-BSA,

the protoplasts were transferred to gamma counting vials, and the radioactivity was determined. In testing the effect of drugs or temperature on the binding of [^{125}I]lectin, the protoplasts were preincubated as described above (see Binding of Fluorescent Lectin to Protoplasts).

Electron Microscopic Examination of Microfilaments

Squashes of plant material were prepared as described by Bennett and Brown (127). Small sections of plant material were minced in 2 to 5 drops of buffers (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; Ted Pella, Tustin, CA) were coated with formvar/carbon. Films of formvar were prepared glass slides dipped in solution of 0.5% (w/v) formvar (C.F. Fullman, Inc., Schenectady, NY) 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 and stained with 2 to 3 drops of uranyl acetate (2% (w/v) in water and stored in the dark; Ted Pella). 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.

Chromatography of Soybean Seedling Extracts on DEAE Cellulose

All steps were done at 4°. Ten day old soybean seedlings (350 gm fresh weight) were suspended in 700 ml of buffer A (see Preparation of Animal Actin Proteins) containing 70 gm of acid washed polyvinylpyrrolidone (G.A.F. Corporation, New York, NY; 152) and 0.02 units per ml of Trayslol. This suspension was homogenized in a Waring blender for 2 minutes, filtered through cheesecloth and centrifuged at 16,300 xg for 30 minutes. The supernatant was further clarified by centrifugation at 100,000 xg for 2 hours. The resulting supernatant fraction was applied to a DEAE cellulose column (4.1 x 25 cm) which was equilibrated in buffer B (see Preparation and Characterization of Animal Actin Proteins). The column was washed with 100 ml buffer A, then with 1 l buffer B. A linear gradient of 0-0.25 M KCl in total volume of 3 l of buffer B was used to elute the column (82).

Binding of [^{3}H] Colchicine to Soybean Protoplasts and Cell Extracts

Colchicine binding activity in soybean protoplasts was determined by incubating protoplasts (5 x 10^5 per ml) with [^{3}H]colchicine (10 Ci/μmole, New England Nuclear, Boston, MA) for 3 hours at various temperatures. The protoplasts were then deposited on GF/C filters (Whatman), washed five times with 5 ml cold MGB and subjected to scintillation counting.

Extracts of soybean cells were prepared from cells which had been preincubated at 4° for at least 30 minutes. All further steps were done at 4° unless noted otherwise. 100 gm of cells, 16 gm polyvinylpyrrolidone and 40 ml of 0.25 M sucrose, 0.5 mM $MgCl_2$, 1 mM GTP (Sigma),

10 mM sodium phosphate (pH 6.95; 153) were ground in a motar and pestel in the presence of alumina. After centrifugation at 12,000 xg for 10 minutes to remove cell debris, the extract was further clarified by centrifugation at 100,000 xg for 1 hour. Aliquots (100 µl) of this supernatant material were preincubated at various temperatures for 15 minutes. [^{3}H]Colchicine was then added, and the samples were incubated for 3 hours at the appropriate temperature. The unbound [^{3}H]colchicine was removed by adding 0.1 ml of 2 mg/ml BSA and 0.5 ml of 5 mg/ml activated charcoal to all tubes followed by a 10 minute incubation period. The tubes were centrifuged (1620 xg for 10 minutes), and aliquots of the supernatant were analyzed for radioactivity by scintillation counting or for protein content (153).

Lumicolchicine was produced by irradiating 0.3 µM colchicine in ethanol solution with long wavelength UV source until there was no change in absorbance at 350 nm (154). The lumicolchicine was air dried, and resuspended in [^{3}H] colchicine (final lumicolchicine concentration = 0.5 mM). Experiments examining the binding of [^{3}H]colchicine in the presence of lumicolchicine were done as described above using 100,000 xg supernatant fraction.

Analytical Methods - Protein Determination

The concentration of protein in a sample was either by the method of Lowry et al. (155) or by absorbance at 280 nm. The following extinction coefficients were used:

immunoglobulins	1.5 ml/cm mg (156)
Con A	1.39 ml/cm mg (157)
SBA	1.28 ml/cm mg (158)
Myosin	0.543 ml/cm mg (159)

Gel Electrophoresis

Discontinuous polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was done according to the method of Laemmli (160). The acrylamide composition of the stacking gel was 4% (w/v), while that of the running gel was either 7.5% (w/v) or 10% (w/v). The gels were fixed in 10% (w/v) trichloroacetic acid for 30 minutes, then stained for at least 3 hours in 0.125% (w/v) Coomassie blue in 25% (v/v) isopropanol, 10% (v/v) acetic acid. The gels were destained in 25% (v/v) methanol, 10% (v/v) acetic acid and dried down.

The immunoprecipitates were analyzed using 7.5% (w/v) polyacrylamide gels run in the presence of sodium dodecyl sulfate according to Weber and Osborn (161). After the electrophoresis was complete, the gels were cut into 2 mm slices, and the radioactivity in each slice was determined by gamma counting.

Two dimensional gel electrophoresis, isoelectric focusing in the presence of urea in the first dimension, followed by discontinuous SDS PAGE in the second dimension, was done according to O'Farrell (162) as modified by Rubenstein and Spudich (97). The range of the pH gradient in the first dimension was 5-7 (LKB Ampholines; LKB, Bromma, Sweden) and the polyacrylamide concentration of the running gel in the second dimension was 10%. Following electrophoresis, the gels were fixed, stained, destained, and dried down as described above.

RESULTS

The Binding of Lectins to Soybean Photoplasts

As observed by fluorescence microscopy, protoplasts prepared from suspension cultures of soybean cells bound the lectins soybean agglutinin, concanavalin A and wheat germ agglutinin. Typical results obtained after incubation for 60 minutes at 25° are shown in Figure 11. The general labeling patterns of the cells showed a diffuse ring-like stain, suggesting that most of the receptors were uniformly distributed in the cell membrane (Figure 11a,d). However, in a number of protoplasts (accounting for about 15% of the population), the fluorescence was localized in patches spread over one hemisphere of the cell or completely segregated over one pole of the cell (Figure 11c,e,f). These patterns were observed for all lectins tested in our study.

The results of control experiments demonstrated that the observed fluorescence patterns were indeed due to binding of the lectin to carbohydrate structures on the plasma membranes (see Figures 12 and 13). First, protoplasts prepared from the SB-1 cell line (150) did not exhibit autofluorescence. Second, the majority (>95%) of the protoplasts used in our studies did not stain with the fluorescent dye, Calcofluor, which is commonly used to demonstrate the presence of cell wall material (163). Moreover, the simultaneous use of Calcofluor and rhodamine-conjugated reagents demonstrated that the lectins were bound in regions devoid of cell wall material.

Figure 11. Fluorescence patterns of soybean protoplasts treated for 1 hour at room temperature with various lectins. (a)-(d) Soybean agglutinin (50 µg/ml); (e) Con A (50 µg/ml); (f) Wheat germ agglutinin (50 µg/ml). ph, phase contrast microscopy; fl, fluorescence microscopy; magnification 250 x.

Figure 12

Control Experiments for the Binding of Soybean Agglutinin to Protoplasts

Treatment	Observed Fluorescence
None	-
Calcofluor	-
fl-G-anti-R-IgG	-
SBA + fl-G-anti-R-IgG	-
no SBA + R-anti-SBA-IgG + fl-G-anti-R-IgG	-
SBA + both antibodies	+
SBA + both antibodies + 0.2 M GalNAc	-

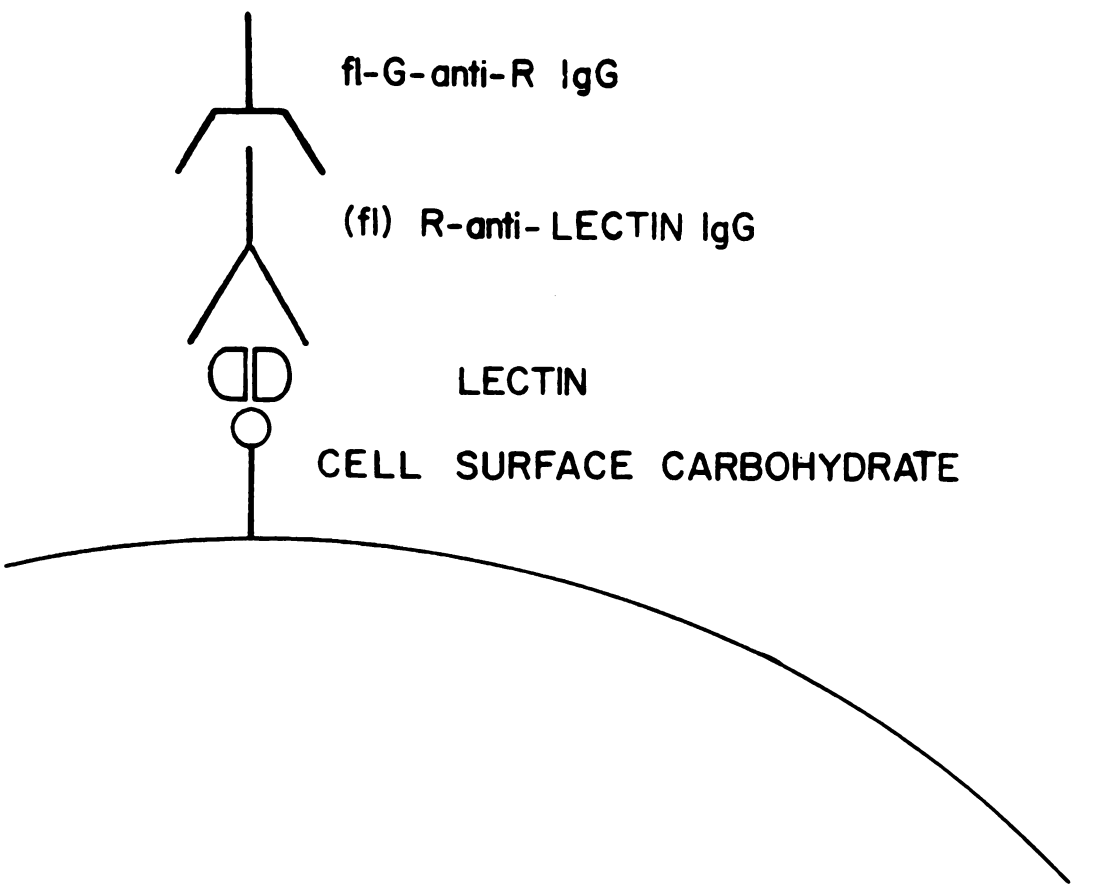
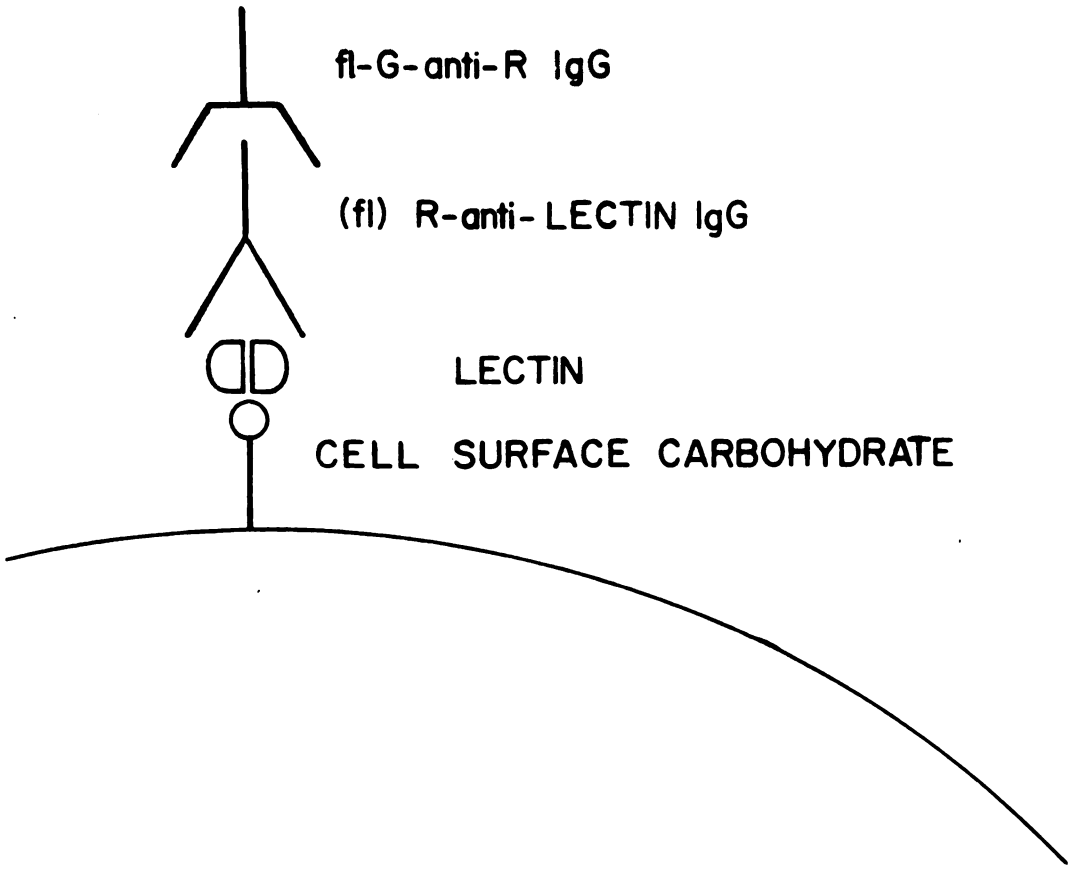


Table 13

Control Experiments for the Binding of Concanavalin A to Protoplasts

Treatment	Observed Fluorescence
None	-
Calcofluor	-
fl-G-anti-R-IgG	-
Con A + fl-G-anti-R-IgG	-
no Con A + R-anti-Con A-IgG + fl-G-anti-R-IgG	-
Con A + both antibodies	+
Con A + both antibodies + 0.2 M αCH_3-Man	-



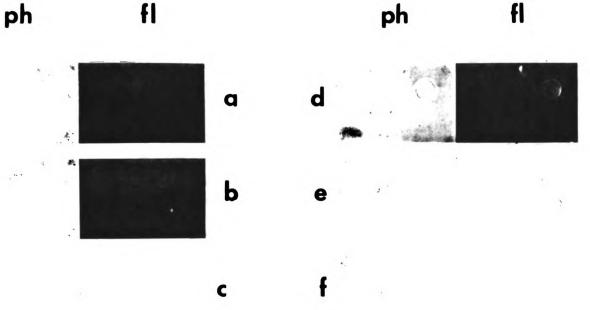
We have also found that the binding of soybean agglutinin, Con A and wheat germ agglutinin was inhibited by the monosaccharides N-acetyl-galactosamine, α-methyl-mannoside, and N-acetyl-glucosamine, respectively. Finally, incubation of protoplasts with fluorescein-labeled or rhodamine-labeled goat antibodies directed against rabbit immunoglobulin in the absence of lectins did not show any fluorescence. All of these results strongly suggest that the observed staining patterns for each of the lectins could be ascribed to the binding of the ligand to heterosaccharide structures of the plasma membrane.

The Binding of Soybean Agglutinin to Soybean Protoplasts

Detailed examination of the fluorescent patterns of cells treated with SBA showed that the labeling patterns were indeed dependent upon the conditions of incubation. Protoplasts which were incubated at 37° showed a bright ring-like staining pattern around the cell periphery, with very light and diffuse staining of the rest of the cell surface (Figure 13a). A similar staining pattern was observed in protoplasts incubated at 25°, with the exception that the staining intensity at the periphery was slightly greater than that seen at 37° (Figure 13b). In contrast to these results, the staining pattern at 4° showed a less intense ring staining (Figure 13c).

When the protoplasts were first treated for 15 minutes with colchicine at a concentration of 10^{-5} M, the resultant fluorescent staining pattern showed diffuse staining of the cell surface, surrounded by a faint ring stain at the cell periphery (Figure 13e). Similar results were observed when the protoplasts were pretreated with cytochalasin B (2.1×10^{-5} M; see Figure 13f).

Figure 14. Fluorescence patterns of soybean agglutinin bound to soybean protoplasts under various conditions. (a,b,c) Soybean agglutinin (50 µg/ml), 60 minutes at 37°, 25°, and 4°, respectively; (d) soybean agglutinin in (50 µg/ml), 60 minutes at 25°; (e) colchicine (10^{-5} M) for 15 minutes at 25°, then soybean agglutinin (50 µg/ml) for 60 minutes at 25°; (f) cytochalasin B (2.1×10^{-5} M) for 15 minutes at 25°, then soybean agglutinin (50 µg/ml) for 60 min at 25°. ph, phase microscopy; fl, fluorescence microscopy; magnification 250 x.


ph
fl
ph
fl
a
b
c
d
e
f

The differences in the fluorescent staining patterns at the various temperatures or after treatment with drugs cannot be ascribed to gross alterations in the number of lectin molecules bound per protoplast. This conclusion was deduced from a series of experiments which examined the binding of ^{125}I labeled soybean agglutinin ([^{125}I]SBA) to soybean protoplasts. As shown in Figure 15A, the binding of [^{125}I] SBA to protoplasts is concentration dependent. All subsequent experiments were done using 50 µg/ml [^{125}I]SBA. This concentration was chosen since it was the concentration of lectin used in the fluorescence studies.

Protoplasts were incubated with ^{125}I-labeled SBA at 37°, 25° and 4° and the results are presented in Table 5. The number of subunits of [^{125}I]SBA bound per protoplast did not change greatly due to different incubation temperatures. Moreover, when the protoplasts were preincubated with either colchicine or cytochalasin B, the total number of subunits bound per protoplast is similar to the number bound to an untreated protoplast at 25°. Therefore it appears that the difference in the staining patterns observed by fluorescent microscopy are due to differences in the distribution of the lectin receptors in the plasma membrane of the protoplasts.

The Binding of Concanavalin A to Soybean Protoplasts

In a second group of experiments, the binding of concanavalin A to soybean protoplasts was examined as an example of an heterologous system. Overall, the results obtained in the heterologous system are in agreement with those described above for the homologous system. The amount of Con A bound to the soybean protoplast was not affected by incubation temperature or by pretreatment of the protoplasts with drugs.

Figure 15. Dose response curve of the binding of ^{125}I-labeled lectin to soybean protoplasts. ^{125}I-labeled lectin was incubated at 1, 5, 20, 50 and 100 µg/ml with 5 x 10^5 cells/ml for 60 minutes at 25° in duplicate. Non-specific binding of ^{125}I-labeled lectin was determined by incubating the [^{125}I]lectin with the protoplasts in the presence of the competing sugar, and subtracted from total [^{125}I]lectin bound for each concentration. The bars represent the range of values obtained. Panel A - [^{125}I]soybean agglutinin; Panel B - [^{125}I]concanavalin.

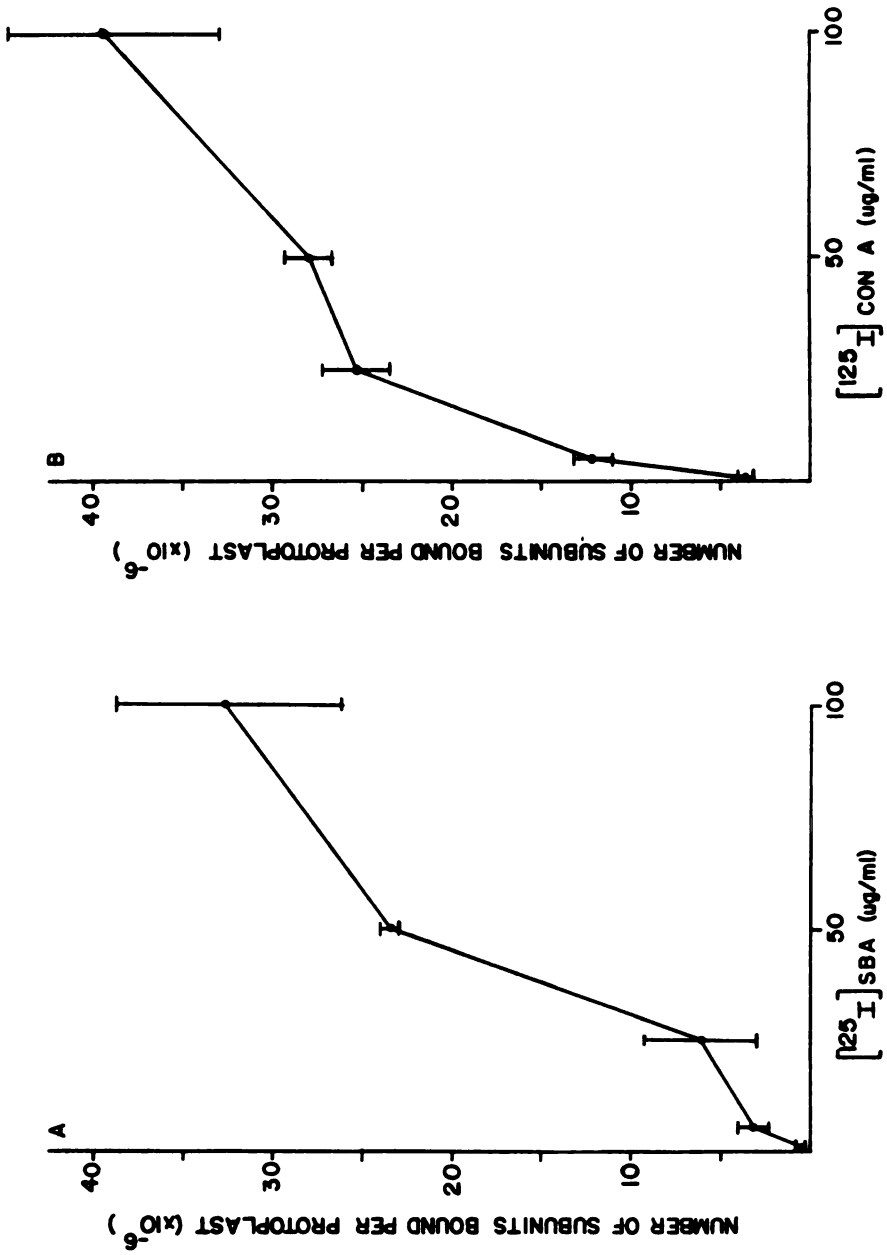

A
B
NUMBER OF SUBUNITS BOUND PER PROTOPLAST (x10^{-6})
40
30
20
10
50
100
[^{125}I] SBA (ug/ml)
[^{125}I] CON A (ug/ml)

Table 5

The Binding of [^{125}I]Soybean Agglutinin to Intact Protoplasts - Effect of Incubation Temperature and Drugs

Condition	Number of SBA Subunits Bound per Protoplast ($\times 10^7$) ± SEM*
37°	3.4 ± 0.3
25°	2.3 ± 0.04
4°	1.2 ± 0.2
Cytochalasin B	1.4 ± 2.0
Colchicine	1.6 ± 0.2

*The data represents the averages of duplicate determinations and are expressed as the number of monomeric subunits bound per protoplast ± standard error of the mean. The molecular weight of SBA used in calculations was 3.0×10^4. The specific activity of ^{125}-labeled SBA was 5.0×10^7 cpm/mg.

However, differences in the fluorescent staining patterns were observed and are described below.

When soybean protoplasts were incubated with Con A at 37°, the fluorescent staining observed is a generally bright, and diffuse pattern surrounded by a smooth ring at the cell's periphery (Figure 16c). At 25°, the ring labeling pattern persisted, but the staining within the peripheral ring often showed alternating bright and dark spots (Figure 16b). In marked contrast, the staining pattern at 4° showed pronounced spottiness over the entire surface of the protoplast (Figure 16a).

Protoplasts pretreated with colchicine (10^{-5} M) for 15 minutes, showed more spotty fluorescent staining pattern than was seen in untreated protoplasts (see Figure 16d). Treatment of protoplasts with cytochalasin B (2.1×10^{-5} M) also gave a more patchy distribution of fluorescence (Figure 16e). Both of these patterns were similar, but never identical to that observed with protoplasts incubated at 4°.

A series of binding experiments, using radioactive iodine labeled concanavalin A ([^{125}I]Con A) were conducted to ascertain that differences observed in the fluorescent micrographs were not caused by differential binding of the lectin by the protoplasts under the various incubation conditions. Various concentrations of [^{125}I]Con A were incubated with soybean protoplasts for one hour at room temperature. The plot of number of subunits specifically bound is shown in Figure 13B. Again, all further binding experiments were done using 50 µg/ml [^{125}I]Con A. The effect of incubation temperature upon the binding of [^{125}I]Con A to protoplasts is shown in Table 6. There was no difference detected in the number of subunits bound per protoplast under any condition examined. It should be noted that concanavalin A

Figure 16. Fluorescence patterns of concanavalin A bound to soybean protoplasts under various conditions. (a,b,c) Con A (50 µg/ml), 60 minutes at 4°, 25°, and 37°, respectively; (d) colchicine (10^{-5} M) for 15 minutes at 25°, then Con A (50 µg/ml) for 60 minutes at 25°; (e) cytochalasin B (2.1×10^{-5} M) for 15 minutes at 25°, then Con A (50 µg/ml) for 60 minutes at 25°; (f) Con A (50 µg/ml), 60 minutes at 4°. ph, phase contrast microscopy; fl, fluorescence microscopy; magnification 250 x.

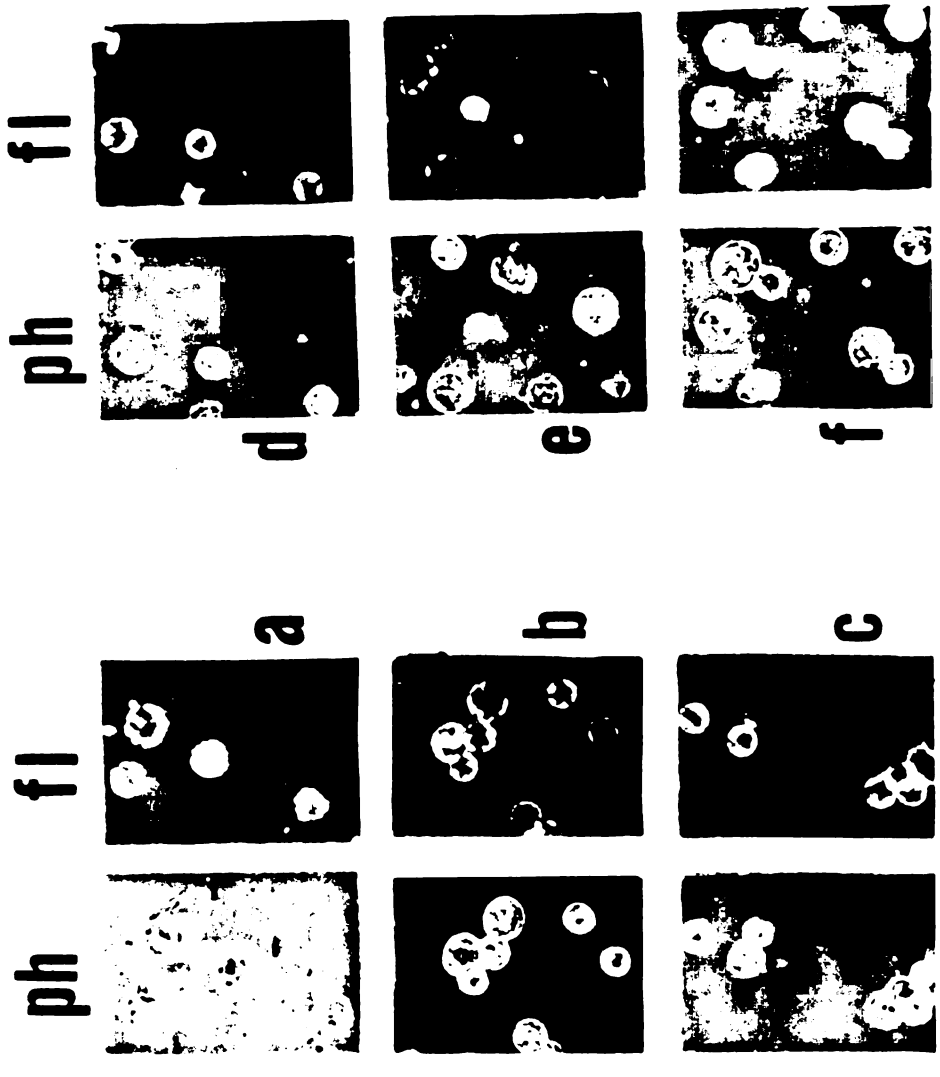

ph
fl
a
b
c
d
e
f

Table 6

The Binding of [^{125}I]Concanavalin A to Intact Protoplast - Effect of Incubation Temperature and Drugs

	Number of Con A Subunits Bound per Protoplast ($\times 10^8$) ± SEM*
37°	1.4 ± 0.1
25°	1.3 ± 0.02
4°	0.76 ± 0.02
Cytochalasin B	1.2
Colchicine	1.8

*The data represents the averages of duplicate determinations and are expressed as the number of monomeric subunits bound per protoplast ± the standard error of the mean. The drug treated samples were not done in duplicate. The molecular weight of Con A used in the calculations was 2.6×10^4. The specific activity of ^{125}I-labeled Con A was 1.9×10^6 cpm/mg.

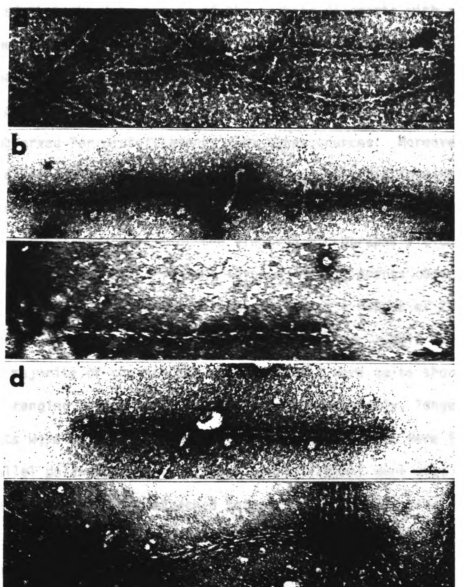
undergoes dissociation at low temperatures, changing from a tetramer (MW = 104,000) to a dimer (MW = 52,000) (164-166). Therefore, it was expected that there would be only 50% of the subunits bound at 4° as compared to 25° or 37°. Finally, the number of subunits bound did not change when the protoplasts were pretreated with either cytochalasin B or colchicine (Table 6). From these experiments it may be concluded that the differences observed in the fluorescent staining patterns reflect a change in the distribution of Con A binding sites on the cell surface of soybean protoplasts.

Ultrastructural Identification of Soybean Microfilaments

The above data provided evidence that lectin receptors were present at the plasma membrane of soybean protoplasts. In order to demonstrate that the postulated model of receptor-cytoskeletal interaction could occur in plants, we undertook a series of experiments to identify microtubules and microfilaments in soybean cells. The presence of cytoskeletal elements in plants has been demonstrated in ultrastructural studies (120-128). In particular, microfilaments, identified as 6-7 nm diameter filaments which will bind heavy meromyosin have been shown in numerous plant systems.

Liquid derived from squashes of soybean plant tissue, when examined by electron microscopy, contained filamentous structures which were decorated with heavy meromyosin (see Figure 17b,c,d). These filaments exhibited polarity, with the characteristic arrowhead pattern seen in rabbit muscle actin filaments decorated with heavy meromyosin (see Figure 17a). Several lines of evidence indicate that these structures represented microfilaments derived from the soybean tissues. First, control grids prepared in the absence of any soybean material showed no

Figure 17. Electron micrographs of heavy meromyosin decorated microfilaments. Representative micrographs from (a) rabbit muscle actin (115,830x); (b) roots of soybean plant (121,176x); (c) roots of soybean seedling (119,110x); (d) stem of soybean plant (118,542x); (e) roots of clover plant (111,160x). Grids were examined under a Philips 201 C electron microscope at 60 kV. The bar represents 100 nm.


b
d

decorated filaments, suggesting that the assembled structure was not derived from the rabbit heavy meromyosin preparation. Second, when the grids were treated with 1 mM ATP prior to staining with uranyl acetate, no decoration of the filamentous structures was observed. This indicated that the interaction of the soybean filaments with rabbit heavy meromyosin was sensitive to ATP, a characteristic well documented for other myosin-actin systems. Finally, measurements of the size of the filaments yielded values of 6-7 nm for the diameter, consistent with those observed for microfilaments from other sources. Moreover, the decorated filaments of soybean cells were almost indistinguishable from the microfilaments obtained with rabbit muscle actin (Figure 17).

More detailed analysis of the electron micrographs yielded the following characteristics for the soybean microfilaments observed in the present study: (a) the soybean filaments showed a repeat of 28 to 33 nm, compared to corresponding values of 30 to 34 nm for rabbit muscle actin; (b) the majority of the soybean microfilaments were quite short in length, ranging from 0.7 μm to 1.2 μm; (c) occasionally, longer filaments were observed (4 μm or longer), most of which were in bundles of parallel polarity; (d) the longer microfilaments were also observed to be associated with or attached to organelles and the heavy meromyosin decoration of these filaments always pointed toward the sites of attachment. Therefore, on the basis of these results we initiated a program to purify and characterize this actin-like protein from soybeans in order to conclusively demonstrate the presence of the cytoskeletal component, microfilaments, in soybeans.

Immunochemical Identification of an "Actin-Like" Protein in Soybean Seedlings

When extracts of soybean seedlings, clarified by centrifugation, were chromatographed on a column of DEAE cellulose and eluted with a linear gradient of KCl, five major fractions (fraction A-E, Figure 18) were obtained. SDS-PAGE of the material in these fractions showed that each consisted of highly heterogeneous populations of polypeptide chains (Figure 19).

Fraction C (Figure 19) was found to react with rabbit antibodies raised against purified calf thymus actin in Ouchterlony immunodiffusion tests; no precipitin line was observed when the same fraction was tested against preimmune serum (Figure 20). The material in Fraction C was labeled with ^{125}I and then subjected to immunoprecipition with rabbit anti-actin antibodies plus goat antibodies directed against rabbit immunoglobulin. Analysis of the precipitated radioactive protein components by SDS-PAGE showed a single major polypeptide chain, with an electrophoretic mobility identical to that of the calf thymus actin (Figure 21). The molecular weight of this protein was estimated to be 45,000. No ^{125}I-labeled protein was observed when preimmune serum was substituted for the rabbit anti-actin antibody.

When unlabeled calf thymus actin was added to the reaction mixture, the amount of ^{125}I-labeled soybean protein precipitated was reduced. Moreover, the decrease in precipitation of radioactivity was directly proportional to the amount of unlabeled actin added. These results strongly suggest that the precipitation of the 45,000 molecular weight component (Figure 21) was due to specific interactions with the anti-actin antibody.

Figure 18. Chromatographic profile of an extract of soybean seedlings fractionated on a column of DEAE cellulose. Column size: 4 x 25 cm. 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 liters. Each tube contained 10 ml of effluent.

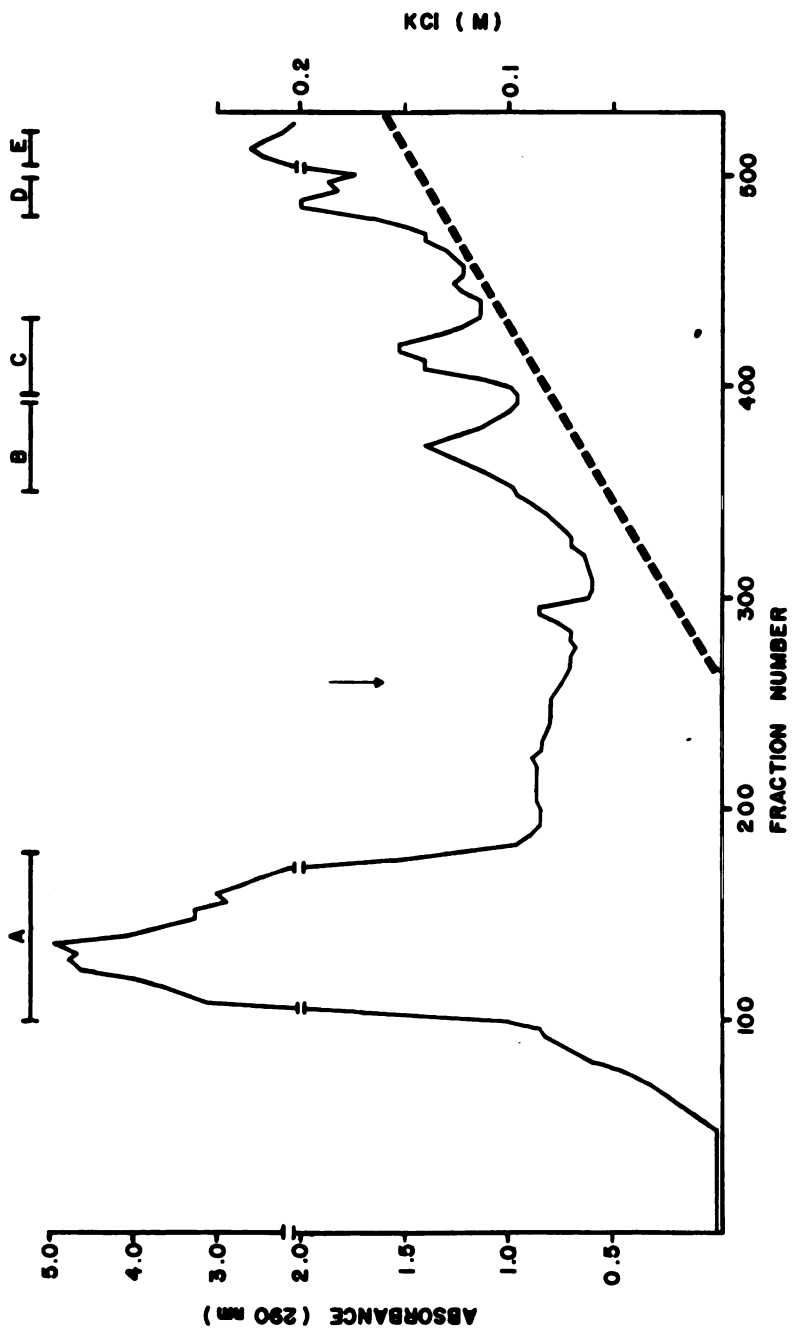

KCl (M)
0.2
0.1
A
B
C
D
E
100
200
300
400
500
FRACTION NUMBER
ABSORBANCE (280 nm)
5.0
4.0
3.0
2.0
1.5
1.0
0.5

Figure 19. SDS polyacrylamide gel electrophoresis of fractions derived from DEAE cellulose chromatography and from affinity chromatography using rabbit anti-actin antibodies. (a) calf thymus actin; (b) rabbit muscle actin; (c)-(g) Fractions A-E from Figure 18; (h) material from Fraction C (Figure 18) purified by affinity chromatography on columns containing rabbit antibodies directed against calf thymus actin; (i) calf thymus actin. The acrylamide composition of the gel was 7.5% (w/v). The arrows on the right indicate the positions of migration of molecular weight markers: bovine serum albumin (68,000); glutamic dehydrogenase (53,000); ovalbumin (43,000); pancreatic deoxyribonuclease I (33,000) and the dye bromophenol blue. Approximately 10-30 µg of protein was loaded in each lane of the gel.


a b c d e f g h i

Figure 20. Ouchterlony double immunodiffusion plate demonstrating reactivity between rabbit anti-calf thymus actin serum and Fraction C (Figure 18) from DEAE cellulose fractionation of soybean seedling extracts. Immunodiffusion plate was developed as described in Materials and Methods. Fraction C was lyophilized, then dissolved in distilled water at a concentration of 10 mg/ml. CT - calf thymus actin. PI - preimmune serum. SB - Fraction C (Figure 18). RaCT - rabbit anti-calf thymus actin serum.

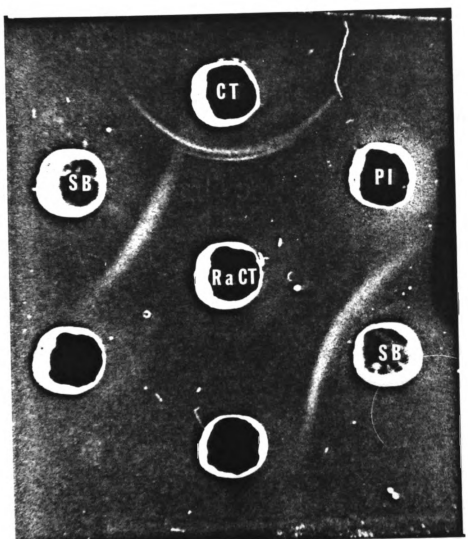
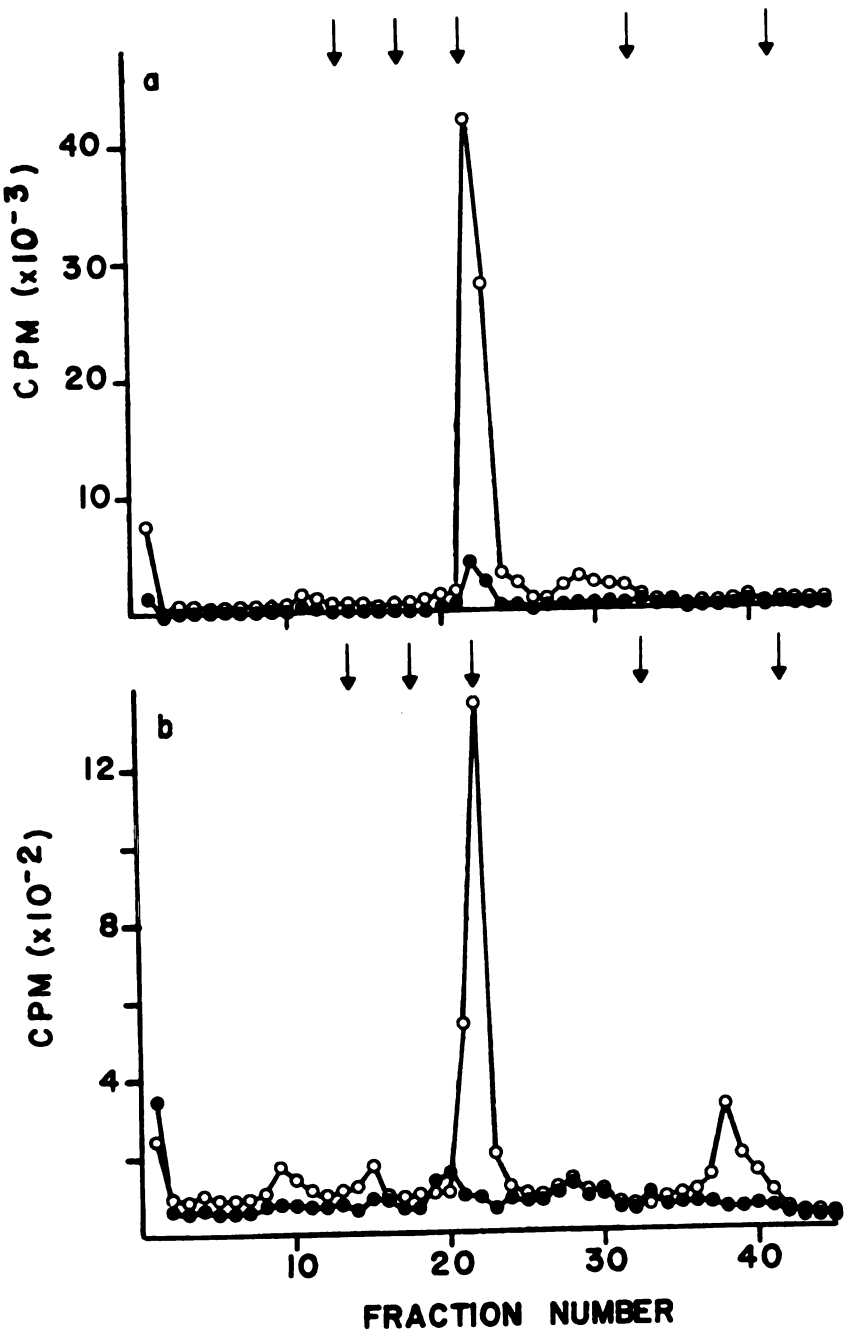

CT
SB
PI
RaCT
SB

Figure 21. SDS polyacrylamide gel electrophoretic analysis of ^{125}I-labeled material immunoprecipitated using rabbit anti-calf thymus actin serum plus goat antibodies directed against rabbit immunoglobulin. (a) ^{125}I-labeled calf thymus actin (1.7×10^5 cpm/μg); (b) ^{125}I-labeled Fraction C (Figure 18) from soybean cells (8.3×10^3 cpm/μg). For both (a) and (b): o——o, radioactivity profile of immune precipitates obtained using rabbit anti-actin antiserum; o——o, radioactivity profile of immunoprecipitates obtained using preimmune serum. The arrows indicate the positions of migration of molecular weight markers (see legend to Figure 19).


a
CPM (x10⁻³)
40
30
20
10
b
CPM (x10⁻²)
12
8
4
10
20
30
40
FRACTION NUMBER

Inasmuch as the specificity of the antiserum is a key factor in the interpretation of our results, the following experiments were performed to ascertain that the antibodies were indeed specific for the actin polypeptide chain. First, calf thymus actin was shown to be pure by SDS PAGE and by two dimensional gel electrophoresis. Second, extracts of calf thymus tissue were labeled with ^{125}I and then precipitated using the rabbit anti-actin antiserum plus goat antibodies directed against rabbit IgG. Analysis of the immunoprecipitate by polyacrylamide gel electrophoresis indicated that only a single ^{125}I-labeled polypeptide chain was precipitated. The electrophoretic mobility of this material was identical to that obtained with purified calf thymus actin (Figure 21), the antigen used for immunization. Moreover, parallel experiments using preimmune serum showed no precipitation of any ^{125}I-labeled protein. Finally, the rabbit anti-actin antiserum produced a single precipitin line in Ouchterlony immunodiffusion tests against both the crude calf thymus extract and the purified calf thymus actin; no reaction was observed with a variety of other irrelevant proteins such as chicken muscle myosin, bovine serum albumin, fetuin and deoxyribonuclease I.

The availability of an immunochemical reagent capable of selectively binding to a single component of soybean cell extracts immediately suggested affinity chromatography as a purification step for the actin like protein. Fraction C (Figure 18) was chromatographed on a Sepharose column containing covalently-coupled rabbit anti-actin antibodies; the bound protein material was eluted with 1 M acetic acid and subjected to gel electrophoresis in sodium dodecyl sulfate. The gel pattern showed one predominant Coomassie blue-stained band (Figure 19, lane h), with an electrophoretic mobility corresponding to a protein of molecular weight

45,000. All of these results suggest that an actin-like protein may be found in Fraction C (Figure 18) derived from extracts of soybean cells.

Colchicine Binding Activity in Soybean Protoplasts and Extracts

Finally, we have preliminary evidence for the presence of microtubules in soybean cells. When soybean protoplasts were incubated with [^{3}H]colchicine at 37° and 25°, appreciable amounts of the label were bound to the protoplasts (see Table 7). In contrast, when the incubation was carried out at 4°, much less colchicine was retained by the protoplasts. Similar results were obtained when an extract of soybean cells was centrifuged at 100,000 x g for one hour and the supernatant fluid was incubated with [^{3}H]colchicine at various temperatures. Again, it was found that there was appreciable colchicine binding activity at high temperatures and no activity at the low temperature.

When the binding studies were carried out in the presence and absence of lumicolchicine (0.5 mM), identical levels of binding were observed. Lumicolchicine is a photoinactivated derivative of colchicine that does not bind to tubulin (66,167). Therefore, in the presence of lumicolchicine, one would expect that non-specific binding by [^{3}H]colchicine is minimized and that the radioactivity bound represented colchicine associated with the tubulin dimer. All of these results indicate that the observed colchicine binding activity may be due to plant tubulin.

Table 7

The Binding of [^{3}H]Colchicine to Intact Protoplasts and Extracts of Soybean Cells at Different Temperatures

Temperature	Protoplasts [^{3}H]colchicine bound/mg protein ± SEM*	Cell Extracts
4°	390 ± 630	0
25°	2250 ± 590	1050 ± 450
37°	2340 ± 450	8850 ± 430

*The data represents the averages of triplicate determinations and are expressed as counts per minute of [^{3}H]colchicine bound per mg of total protein ± the standard error of the mean. The concentrations of colchicine used were: (a) 8 μM (specific activity 1.4 x 10^7 cpm/μmole) for the protoplasts; (b) 20 μM (specific activity 7.0 x 10^7 cpm/μmole) for the cell extract.

DISCUSSION

The work described in this thesis demonstrated that: (a) there are lectin receptors on the plasma membrane of the soybean protoplasts; (b) the existence of an actin-like protein in soybean cells; (c) preliminary evidence for the presence of the microtubule subunit, tubulin, in soybean cells. The most detailed biochemical studies in the present thesis have been carried out on the actin-like protein in soybean cells. This identificiation has been based on three lines of evidence: (a) molecular weight (M_r 45,000) by gel electrophoresis in the presence of sodium dodecyl sulfate; (b) immunologocial reactivity with rabbit anti-serum directed against calf thymus actin, and (c) the presence of filaments which form arrowhead complexes upon treatment with heavy meromyosin.

Actin has been isolated and characterized in a variety of animal muscle and non-muscle cells (37,79-81,103), as well as in other organisms such as Mycoplasma pneumonia (92), Chlamydomonas (93), Physarum (88-90), Acanthamoeba (82-84), Amoeba (85), Saccharomyces cerevisiae (91) and Dictyostelium (86,87). In practially all cases studied to date, the various actins are remarkably similar to each other and to muscle actin, all consisting of globular subunits of approximately 45,000 molecular weight. The present identification of an actin-like protein from soybean cells with a polypeptide chain of molecular weight 45,000 further extends the list of similar proteins.

The usefulness of an immunological reagent is directly related to the purity of the antigen used. In this study calf thymus actin, an example of non-muscle or cytoplasmic actin, was used as antigen. It was demonstrated to consist of a single Coomassie blue stained band when analyzed by SDS gel electrophoresis. The purity of the antigen was further demonstrated by two dimensional gel electrophoresis in which the sample is subjected to isoelectric focusing in urea in the first dimension followed by gel electrophoresis in sodium dodecyl sulfate in the second dimension. Using this technique, one is able to differentiate between the various forms of actin which comigrate in SDS gel electrophoresis. Muscle actin has the lowest isoelectric point, while the non-muscle or cytoplasmic forms of actin have more basic isoelectric points (94-98). When calf thymus actin was analyzed in two dimensional gel, the only polypeptides detected were the cytoplasmic actins. As shown in Figures 6 and 7, these proteins can be distinguished from muscle actin when these samples are analyzed together by two dimensional gel electrophoresis.

The specificity of the antiserum was demonstrated by Ouchterlony double immunodiffusion and by immunoprecipitation. In immunodiffusion plates, rabbit antiserum against calf thymus actin gave a single precipitin line when tested against either purified calf thymus actin or extracts of calf thymus tissue. Moreover, no precipitin lines were seen when immune serum was replaced by preimmune serum. Furthermore, when ^{125}I-labeled calf thymus actin was immunoprecipitated using the rabbit antiserum and goat antibodies against rabbit IgG, and analyzed by SDS gel electrophoresis, a single peak of radioactivity was found. The electrophoretic mobility of this peak corresponded to a molecular weight

of 45,000. No radioactivity was precipitated when preimmune serum was substituted for the rabbit immune serum in the immunoprecipitation. Finally, identical results were obtained when ^{125}I-labeled extracts of calf thymus tissue were subjected to immunoprecipitation.

Having demonstrated the specificity of the antiserum, we used immunological reactivity to identify an actin-like protein in extracts of soybean seedlings which had been fractionated by DEAE cellulose chromatography. A single precipitin line was seen in Ouchterlony double immunodiffusion of Fraction C, while no precipitin line was seen with preimmune serum. Analysis of immunoprecipitates of ^{125}I-labeled Fraction C by SDS gel electrophoresis demonstrated that a single radioactive species had been precipitated. Furthermore, the electrophoretic mobility of this species corresponded to a molecular weight of 45,000, identical to that of actin.

The above results was further supported by the use of rabbit anti-actin IgG affinity column. Electrophoretic analysis of the affinity purified material showed the predominant component to be identical to actin with respect to molecular weight. This result obivated any problems associated with the use of ^{125}I-labeled proteins (such a preferential labeling of a single polypeptide).

Electron microscopic analysis of soybean extracts have demonstrated the presence of filamentous structures, which yielded arrowhead complexes upon treatment with heavy meromyosin. We have, in fact, observed a differential distribution of microfilaments in the tissues of soybean plants as well as seedlings. Microfilaments were found in all regions except the cotyledons of seedlings and the petioles of plants. The frequency of microfilaments was found to be the highest in the

root-tip region, where one grid square may contain 30 or more filaments. Samples prepared from stems or leaves contained very few filaments per grid square. In some cases, several preparations were required before decorated microfilaments were observed in squashes of those tissues.

In order to account for the differential distribution of microfilaments in soybean tissues, mechanisms at two different levels must be considered. The first hypothesis is that actin subunits are actually present in all tissues but are only assembled into filaments in the root cells. This regulation of assembly hypothesis can be tested by quantitating the amount of actin subunits in different tissues using radioimmunoassay, utilizing for example antibodies which react with soybean actin (168). A second hypothesis is that the amount of actin subunits is different in various tissues due to differential levels of mRNA coding for actin. This regulation of transcription hypothesis can be studied by quantitating the amount of actin specific mRNA in tissues using cDNA probes generated by recombinant DNA techniques (169-172).

Thus far, we have been unable to detect microfilaments in DEAE cellulose fractionated extracts of soybean seedlings. The reason for this failure to detect microfilaments is unclear since one or more of the following situations may exist: (a) presence of a factor which inhibits polymerization (173-175); (b) concentration of actin is below the critical concentration required for polymerization (81); (c) a cofactor, required for polymerization, has been removed or denatured; or (d) denaturation of actin itself during the fractionation procedure.

Immunological reactivity, the assay used to detect actin in the fractionation of soybean seedlings extracts, does not require the plant actin to be biologically active. Therefore, future research

efforts will be directed at the isolation of a biologically active form of actin in order to demonstrate that it possesses the biological activities which are characteristic to other actins: polymerization into 6-7 nm filaments, stimulation of muscle myosin Mg^{2+}-dependent ATPase activity and stoichiometric binding of ATP.

We have also obtained evidence to suggest that soybean cells contain another cytoskeletal element, tubulin. This evidence is based on the finding that soybean protoplasts contained colchicine binding activity at a level comparable to that observed in mouse splenic lymphocytes, although far below that seen with brain cells (36). It has been reported that tubulin from a number of plant cells have lower affinity for the drug colchicine than tubulin from animal cells (105,176). It was particularly significant that the activity was observed at 25° and 37° and was greatly reduced at 4° because previous experiments have shown that colchicine binds to animal cell tubulin at 37° but not at 4°. This data is of a preliminary nature, and we have not pursued this line of research.

It has been previously documented that a variety of lectins bind and agglutinate protoplasts of soybean (177), broad bean (178) and tobacco (178) cells. In agreement with Williamson and co-workers (177) we have observed that the binding of lectins to soybean protoplasts yielded patches of stain. This was observed in both the homologous system (SBA binding to soybean cells) and in the heterologous system (Con A or wheat germ agglutinin binding to soybean cells). We have observed different staining patterns of the bound lectin by changing the temperature of incubation or by treatment of the protoplast with either colchicine or cytochalasin B. These changes in fluorescent staining

were seen in both the homologous as well as the heterologous systems. On the basis of binding of ^{125}I-labeled lectins, it appears that the changes in fluorescent staining patterns may reflect a redistribution of lectin receptor in the plasma membrane as opposed to a gross alteration in the number of lectin subunits bound.

However, there are several differences between the two systems which were examined. First, the number of Con A subunits bound are 10-fold higher than the number of soybean agglutinin subunits bound under identical conditions. Secondly, the fluorescent staining patterns of the two lectins responded differently as the incubation conditions were changed. With the lowering of temperature, the Con A staining pattern became progresively more patchy. The staining patterns in the homologous system exhibited a maximal intensity at 25°, with the intensity decreasing as the temperature was changed.

The findings described in this thesis concerning the identification of the microfilament and microtubule subunits in soybean cells lend strong support to the notion that cytoskeletal proteins such as tubulin and actin are ubiquitous. These results, coupled with the evidence demonstrating the presence of lectin receptors at the plasma membrane of soybean protoplasts, are particularly relevant to the generality of hypotheses concerning the submembranous macromolecular assemblies and their role in mediating receptor-cytoplasmic interactions (36,41,179).

But more importantly, we have acquired both the materials (antisera and purified proteins) and techniques (electron and fluorescent microscopy, two-dimensional gel electrophoresis) which are necessary tools for the next level of analysis. Specifically, the future research

goals include: (a) isolation of native actin from soybeans; (b) study of the assembly properties of soybean actin *in vitro* and *in vivo*; (c) study of the regulation of assembly of microfilaments in higher plants by associated proteins (e.g., villin (180,181), α-actinin (182-184), profilin (173-175) vinculin (185-188)); (d) search for the direct connection between the lectin receptor and the cytoskeletal elements; and finally (e) identification and characterization of the root hair cell lectin receptor in order to demonstrate whether they are anchored on the plasma membrane.

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