CHARACTERIZATION AND FUNCTIONAL ANALYSIS OF SB-1 LECTIN FROM CULTURED SOYBEAN ROOT CELLS

by

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ABSTRACT

CHARACTERIZATION AND FUNCTIONAL ANALYSIS OF
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In an attempt to elucidate the mechanism of Rhizobial attachment to soybean roots, a model system was established using a cultured soybean cell line, SB-1, originally derived from roots of Glycine max. Incubation of Rhizobia with the SB-1 cells resulted in adhesion of the bacteria to the plant cells. This heterotypic interaction was strain specific for Rhizobium that normally infect soybean roots. Studying the inhibition of binding with various sugars suggested that the interaction of Rhizobium and SB-1 cells is mediated through a galactose specific recognition system.

A lectin, termed SB-1 lectin, was isolated from cultured SB-1 cells by affinity chromatography on a Sepharose column derivatized with N-caproyl-galactosamine, which is normally used for purification of soybean agglutinin (SBA), a galactose specific lectin found in soybean seeds. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting analysis of SB-1 lectin with anti-seed SBA antibody demonstrated a major polypeptide (M_r = 30,000) which co-migrated with seed SBA. SB-1 lectin was observed in fractions purified from culture medium of SB-1 cells or supernatant fractions of SB-1 cell suspension after enzymatic removal of the cell wall. Moreover, fluorescently-labeled rabbit anti-SBA antibody incubated with SB-1 cultured cells showed specific immunofluorescent staining on

the cell wall and plasma membrane of the SB-1 cells. These results suggest that SB-1 lectin, produced by SB-1 cells, may mediate the recognition between the Rhizobium and the soybean cells. This notion is supported by the observation that rabbit anti-seed SBA antibody blocked Rhizobium-soybean cell adhesion, whereas the control antibody did not.

Comparison of SB-1 lectin, derived from culture medium, and seed SBA by gel filtration and peptide mapping after limited proteolysis revealed no detectable difference between the lectins from the two sources. In addition, we found that both SBA and SB-1 lectin, under certain conditions, form highly stable dimers (M_r = 60,000) from their basic subunits (M_r = 30,000).

The polypeptide presentation of cell surface SB-1 lectin to the environment was probed by cell surface labeling of intact SB-1 cells with anti-peptide specific antibodies. The results suggest that the NH_2-terminal half of the SB-1 lectin is exposed, while the integration of SB-1 lectin in the cell wall may occur through interactions with the C-terminal half of the molecule. Combined results of fluorescence analysis of intact cell labeling and affinity chromatography methods suggest that the overall geometry of both SB-1 lectin anchored on the wall and SBA in solution may be similar.

DEDICATION

to my parents for their love

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BSA

PBS

HRP

AP

LIST OF ABBREVIATIONS

Con A	Concanavalin A
ANS	anilino-naphthalenesulfonic acid
TNS	toluidinylnaphthalenesulfonic acid
SBA	soybean agglutinin
WGA	wheat germ agglutinin
ABA	abscissic acid
2,4,D	dichlorophenoxy acetic acid
PHA	phytohemagglutinin
EPS	extracellular polysaccharide
LPS	lipopolysaccharide
Gal	D-galactose
GalNAc	N-acetyl-D-galactosamine
Gal-Sepharose	Sepharose derivatized with N-caproyl-galactosamine
Glc	D-glucose
SDS	sodium dodecyl sulfate
PAGE	polyacrylamide gel electrophoresis
BSA	bovine serum albumin
PBS	phosphate buffered saline
HRP	horseradish peroxidase
AP	alkaline phosphatase

Chapter I

LITERATURE REVIEW

REVIEW OF LITERATURE

Introduction to Lectins

A century ago, Stilmark made the startling observation that extract of castor bean agglutinated erythrocytes (1). This discovery marked the initiation of research in the field of agglutinins (see Ref. 2 for review). Subsequently, it was found that a large variety of plants contained such agglutinating activity. Because these agglutinins were found to be blood group specific (3), they were given the name "Lectins" from the Latin "Legere," to choose (3). Currently, the term lectin describes: "Proteins or glycoproteins of non-immunoglobulin nature capable of specific recognition and reversible binding to carbohydrate moieties of complex carbohydrates without altering covalent structure of any of the recognized glycosyl ligands" (4).

Even though lectins were originally found in plants, it is now firmly established that these proteins occur in a wide variety of organisms from microbes to humans. This thesis is concerned with plant lectins. The review will predominantly deal with their biochemistry, in particular the lectins derived from leguminous plants.

Specificity of Interaction of Lectins with Glycosylated Ligand(s)

The ability of lectins to bind saccharides and saccharide-containing proteins in a highly specific manner is one of the most

prominent characteristics of this group of proteins. Due to this unique property, lectins have provided investigators with a useful tool for isolating and characterizing carbohydrates and glycoproteins, as well as for probing the molecular architecture of the cell surface and the changes induced therein by transformation.

Sugar-lectin specificity is generally determined on the basis of hemagglutination reaction (5,6), affinity chromatography techniques such as passing glycoproteins of known glycoconjugate structure through a lectin column (7,8), or by polysaccharide precipitation (9,10). Lectins can be classified in terms of their specificity for monosaccharides as determined by hemagglutination inhibition or precipitation of carbohydrate-containing polymers (11). Such criteria put lectins into defined carbohydrate specificity groups, e.g. N-acetylgalactosamine/galactose-binding lectins, mannose/glucose-binding lectins, the N-acetylglucosamine-binding lectins, the L-fucrose-binding lectins, sialic acid-binding lectins, and those with complex binding sites (12). In general, lectins react with non-reducing, terminal glycosyl groups of polysaccharides or glycoprotein chain-ends. There are, however, a few exceptions to this. For example, the lectins from pea, lentil, and fava bean all react with reducing mannose units of N-acetylglucosamine β-1,2,mannose (13). Concanavalin A (Con A), the lectin from jack bean, in addition to its interaction with terminal groups, binds internal 2-O-α-mannopyranosyl residues (14). Lectins also exhibit a pronounced difference with regard to their anomeric specificity. Some show significant anomeric specificity (15,16), while other lectins seem to be anomerically indifferent (17). The carbohydrate-binding site of some lectins seems

to be complementary to a single glycosyl residue, while in some others, the combining site can accommodate between 2-6 sugar residues, or in other words, they have greater specificity for oligosaccharides (18). A few lectins are even more specific and recognize carbohydrate sequence, in conjunction with the linkage amino acid(s) (19).

Several lectins have also been observed to interact with non-carbohydrate ligands. This binding is independent of the carbohydrate-binding activity. Edelman and Wang (20) reported binding of Con A to plant auxin β-indoleacetic acid, a non-polar compound. Binding of several legume lectins to hydrophobic fluorescent molecule anilino-naphthalenesulfonic acid (ANS) and 2,6-toluidinylnaphthalene-sulfonic acid (TNS) has also been reported (21,22). The presence of binding sites for adenine and cytokinins has been demonstrated (13,23). The role of lectin binding to carbohydrates or hydrophobic ligands for cellular activity is another, predominantly, unanswered question.

Endogenous Ligands

To understand the biological functions of plant lectins, it is important to gain knowledge concerning potential endogenous ligands. Some early examples of the presence of endogenous lectin ligands were demonstrated by Gansera *et al*. (24) and also Gebauer *et al*. (25). They took advantage of affinity chromatography and could isolate components from the seeds of *Pisum sativum*, *Canavalia ensiformis*, *Vicia faba*, *Vicia sativa*, and *Ricinus communis* that bound to the appropriate homologous seed lectin attached to the affinity column. These seed components could be specifically eluted with saccharides.

Further investigations on the molecular properties of the isolated components revealed that they were glycoproteins.

Bowles and Marcus (26), using an approach similar to Gansera et al. (24) and Gebauer et al. (25), showed that endogenous lectins from soybean and jack bean are highly specific for the glycoproteins in the seed extracts of the homologous plants. Analysis of jack bean seed extract with gel electrophoresis and treatment of the gel with ^{125}I-labeled Con A indicated that the dominant endogenous Con A binding receptor was the heavy subunit of α-mannosidase (27). In soybean, Bond and Bowles (28) found some factor(s) in the lectin depleted extracts from cotyledon and axis which had the ability to interact with soybean agglutinin (SBA) and inhibit SBA-induced hemagglutination activity.

The presence of a receptor for SBA has been shown on the intact plasma membrane of the soybean protoplasts by Metcalf et al. (29) in studying mobility of membrane receptors (35). Despite these demonstrations of the presence of endogenous lectin-binding ligands, conclusive information about their function awaits further investigation.

Biosynthesis and Regulation of Lectins

A recurring theme in studying plant lectins has been the control of their synthesis and activity which may eventually shed light on a better understanding of the endogenous function of these proteins. Regulation of lectin biosynthesis and expression may occur at various levels ranging from gene transcription to post-translation. A comparison of amino acid sequence from several legume lectins (30)

strongly argues that these lectins are evolutionarily related. In a way, this suggests the possibility that synthesis of various legume lectins may be regulated via similar types of control mechanisms. Advances in DNA technology and cloning techniques have enabled investigators to provide more information about lectin genes during recent years.

Genetic studies using a cDNA probe specific for soybean seed lectin coding sequence have shown the presence of two related lectin genes, Le1 and Le2, in soybean plants (31). The Le1 gene was identified as the gene encoding for the prevalent soybean seed lectin. The function of the Le2 gene is unknown; however, it is expressed at a low level in embryo and roots. There are some soybean lines which apparently lack detectable seed lectin. Investigation of these lectin-negative soybean lines at the genetic level showed that they also contain both Le1 and Le2 genes (31,32). However, the Le1 gene is modified by a 3.4 kb DNA segment inserted within the coding region of this gene, which causes dramatic reduction in the transcription of the Le1 gene (31,32). The insert was shown to have structural features of a transposable element (32). Walling et al. (33) have clearly demonstrated that soybean seed Le1 lectin gene transcription is activated during early embryogenesis, maximized during mid-embryogenesis, and is repressed prior to dormancy. The Le1 gene appears to be also expressed at a low level in the roots of mature soybean plants (31,34), but the level of root lectin mRNA is lower (by a factor of 20,000) than that observed at mid-embryogenesis. In contrast, transcriptional activity of the soybean lectin gene in root and embryo differ only by a factor of 10 (34). These observations suggested that

post-transcriptional processing may be involved in the regulation of gene expression. Interestingly, Okamuro et al. (34) have shown soybean lectin gene transferred to tobacco plants undergo the same developmental regulation.

Studying the regulation of seed lectin gene transcript levels in Phaseolus vulgaris by hybridization of cotyledon RNA with lectin cDNA showed an accumulation of transcripts in the cotyledons during mid-maturation, and a decrease during late maturation (35). Studies of pea lectin gene transcript indicated the increase in lectin mRNA levels coincide with the time of maximal production of the seed lectin and that the accumulation of the lectin in the seed is regulated at the transcriptional level (36).

Both co- and post-translational processing has been shown to occur in many seed lectins. Comparison of the amino acid sequence of some lectins obtained by protein sequencing and those deduced from the nucleotide sequencing of cDNA revealed that primary translation products of mRNA contained a signal sequence at the NH_2-terminus which is absent in mature protein; the signal sequence is cleaved off post-translationally. Vodkin (37) found in vitro translation of soybean seed mRNA produced a major polypeptide precipitable with antibodies directed against soybean seed lectin. But the molecular weight of this polypeptide, 32,300, was several thousand daltons larger than that of the non-glycosylated soybean lectin subunit, 28,000. This implicated that processing of nascent polypeptide may be involved during in vivo synthesis.

In some other leguminous plants such as lentil, pea, and fava, it has been reported that seed lectin is composed of subunits, each of

which contains a short α-chain and a long β-chain. Basically, this type of two-chain lectin is synthesized as a single chain polypeptide precursor. The precursor chain is cleaved into α and β and held together by non-covalent forces. Combined results of *in vivo* pulse-chase labeling experiments and *in vitro* translation of mRNA from immature pea cotyledons (36,38) indicated that pea lectin is synthesized as a single 25,000 pre-pro lectin polypeptide in the form of NH_2-signal-β-chain-α-chain-COOH. This primary precursor is initially associated with rough endoplasmic reticulum and then sequestered into the lumen of endoplasmic reticulum where the signal sequence is removed co-translationally. The polypeptide, M_r 23,000, is then transported to the protein body where it is post-translationally cleaved to yield α (M_r = 6,000) and β (M_r = 17,000) chains. These results were further confirmed by sequencing of two overlapping cDNA clones complementary to pea lectin mRNA, which was shown to code for β and α subunits (36,38). Similarly, co- and post-translational processing has been shown to be involved in biosynthesis of favin, the lectin from *Vicia faba* (39). *In vitro* translation of fava bean mRNA showed that favin is first synthesized as a single polypeptide precursor of 29,000. Amino acid sequencing of this precursor indicated the presence of a 29 hydrophobic amino acid residue signal sequence at the NH_2-terminal, followed by the sequence of β-and α-chains (39).

Comparison of the complete amino acid sequence of several legume seed lectins, either deduced from their nucleotide sequences of cDNA obtained by reverse transcription of mRNA (pea (36), soybean (32), *P. vulgaris* (35, *Dolichos biflorus* (40)) or determined by classical

methods using various proteolytic cleavage and protein sequencer (fava bean (41), lentil (42), sainfoin (43), jack bean (44)), have shown extensive homology exists among these lectins from the same taxonomical grouping. However, to achieve maximum homology, the NH_2-terminal end of single chain lectins (soybean, sainfoin and Dolichos biflorus lectins) or the β-chain of two-chain lectins (fava bean, lentil and pea lectins) should be aligned with residue 120 of jack bean lectin (Con A). The α-chain of two-chain lectins is aligned with residues 70 through 119 of Con A. This relationship of the primary sequence of Con A with other legume lectins has been termed circular permutation (Figure 1) (45-47). The circular permutation of Con A may now be explained by the unique way that this protein is processed after synthesis to give rise to the mature lectin (48). The synthesis and processing of Con A was studied by metabolic labeling of the precursor and pulse-chase experiments. These investigations revealed this protein initially is synthesized in the form of a glycosylated precursor, which is then deglycosylated and cleaved into two smaller peptides of M_r 18,800 and 14,000. The two fragments are then re-annealed to form mature Con A, but the alignment of residues 1-118 and 119-237 is reversed as confirmed by NH_2-terminal sequencing of the precursor. The annealing of the fragments appears to be involved in a transpeptidation event, as suggested from the pulse-chase experiment.

Post-translational processing has also been demonstrated to occur in the biosynthesis of lectin from cereals and rice belonging to the Gramineae family (49,50). In these plants, lectin is synthesized as a 23,000 molecular weight precursor, which is post-translationally

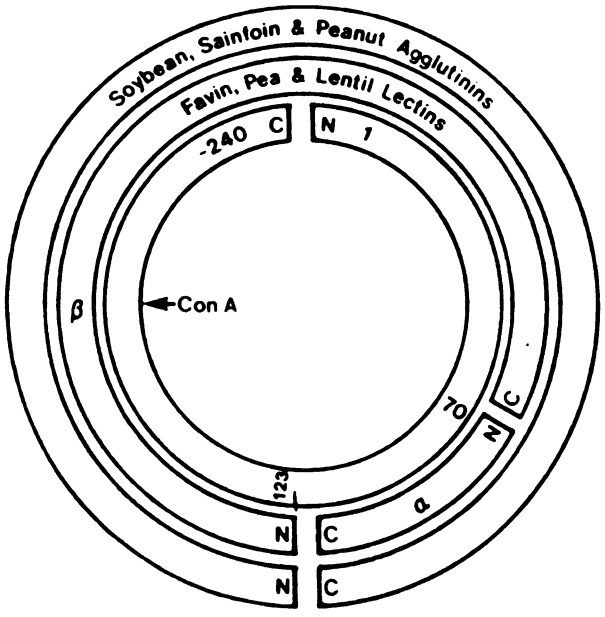

Figure 1. Diagrammatic representation of the circularly permuted sequence homology that relates concanavalin A to other leguminous lectins (from reference 30).

processed in a single step into an 18,000 molecular weight polypeptide, as was evident by *in vivo* pulse-chase labeling experiments (49,50). In cereals, the M_r 18,000 polypeptide is the final product, while in rice, the M_r 18,000 polypeptide is further cleaved in a second step into smaller molecular weight polypeptides of 10,000 and 8,000 (50a). Studying *in vitro* synthesis and the processing of these lectins in cell-free extracts derived from corresponding plants and in *Xenopus oocytes* have provided further support for the above synthesis and processing mechanism.

There is some evidence that the biosynthesis of lectins may be regulated via plant hormones, although the mechanism of such effects is not yet known. Such regulatory effect has been best studied in the biosynthesis of wheat germ agglutinin (WGA) from wheat belonging to Gramineae. Triplett and Quatrano (51) found that if young wheat embryos were removed from grain and cultured, they germinate precociously and concomitantly cease WGA synthesis as determined by measuring lectin levels using radioimmunoassay. The presence of 1-100 µM abscissic acid (ABA), a plant hormone, in the culture medium halts this precocious germination of embryos and not only was WGA synthesis initiated, but also the rate of synthesis was accelerated when compared with the level of lectin synthesis in the intact embryos. Raikhel *et al.* (52) provide evidence on the effect of ABA on the synthesis of WGA in "adult" wheat plants. These investigators found that wheat seedlings, grown under hydroponic conditions in the presence of ABA, showed 2- to 3-fold enhancement in lectin synthesis in the shoot, base and terminal portion of the root system. The effect of ABA on lectin synthesis and accumulation was also studied in

the callus culture derived from immature wheat embryo in conjunction with other plant hormones such as 2,4-dichlorophenoxy acetic acid (2,4-D) (52). The highest rate of lectin accumulation was observed in callus grown only in 10 μM ABA. The presence of 2,4-D seemed to have an antagonistic effect and decreased the amount of lectin in culture. Such negative effect of 2,4-D on lectin synthesis has also been reported for Dolichos biflorus (53). Callus cultures derived from hypocotyls of Dolichos biflorus plants was shown to produce lectins similar to those isolated from intact stems and leaves if grown in the culture medium deprived from 2,4-D. Lectin production was completely inhibited in the presence of the exogenous 2,4-D in the culture medium. In contrast, depression of endogenous levels of ABA in wheat seedlings resulted in lowering the level of WGA in shoot base and roots compared to control plants (52). Studies carried out on the effects of hormones on the synthesis of lectin in developing rice embryos grown in culture indicated ABA strongly promoted the level of lectin synthesis, while gibberellic acid suppressed the synthesis of lectin in rice embryos (50). There is no report of how and at which level these hormones exert their effects.

More recently, Raikhel and Wilkins (54), using a cDNA clone as a hybridization probe, have shown that treatment of excised wheat embryo with ABA changes the level of mRNA for WGA. They suggested exogenous ABA may play a regulatory role at the level of gene expression. Further investigation is necessary to explore the mechanism by which these hormones manipulate lectin synthesis and accumulation.

Multiple Forms of Lectin

A number of laboratories have provided evidence on the presence of multiple forms of lectins in plants. These multiple forms are called "isolectins" and usually can be identified by SDS-gel and isoelectric focusing. The basis for such variability is complex and could be due to different factors, including genetic polymorphism, species polymorphism and post-translational modification (36).

Lectins from Phaseolus vulgaris, phytohemagglutinin (PHA), contain two forms of subunits which are structurally different from one another by six residues at their amino-terminal sequence (55,56). These two subunits, which are termed E (erythrocyte-reactive) and L (lymphocyte-reactive), associate randomly and yield five different tetrameric isolectins: L_4, L_3E_1, L_2E_2, L_1E_3, and E_4 (65). In Vicia cracea seeds, two different lectins have been found, a mannose specific lectin and an N-acetyl-galactosamine specific lectin (57). Combined results from electrophoresis and N-terminal sequencing analysis of the two forms indicated that the mannose specific subunit is composed of two chains, while the N-acetyl-galactosamine specific subunit is a single chain. In soybean, the presence of two related lectin genes, Le1 and Le2, has been demonstrated (discussed earlier) (31).

Heterogeneity in lectin subunits has also been shown to arise as a result of post-translational modification. For example, in some leguminous plants, seed lectin is composed of subunits of short α- and long β-chains (discussed earlier). The lectin from Dolichos biflorus seed is a tetramer composed of two subunit types. Structural studies of these two subunits have shown they are very similar and they only differ at their carboxy-terminal end (58,59). These two subunits have

been termed I and II. Recently, Schenell and Etzler (40) have shown by molecular cloning studies that both subunits are encoded by a single gene. These investigators provided evidence that subunit II arises by post-translational proteolytic cleavage of 10 amino acid sequences from the carboxy-terminus of subunit I. Materials cross-reactive to seed lectin have also been identified in the stems and leaves of Dolichos biflorus (60). Subunit I from the seed lectin apparently is shared by the lectin(s) identified in leaves and stems. Other kinds of known post-translational modifications, such as deamination of asparagine or glutamine side chains and glycosylation, may also occur after the synthesis of lectins, and lead to multiple forms of these proteins. Multiple forms of lectins have also been observed in plants from Gramineae. Three closely-related isolectins have been described in hexaploid wheat (Triticum aestivum) for WGA (61,62). Studies with wheat of different ploidy (diploid, tetraploid and hexaploid) have provided evidence for the existence of diverged triplicate genes for the lectins in hexaploid wheat (63,64); the synthesis of each of these lectins, which have been termed isolectin I, II, and III, is directed by a different genome, A, D, and B, respectively (64). The three isolectins have the same molecular weight, but have slightly different amino acid compositions (61,62). Amino acid sequences of these lectins have indicated that isolectin I and II are different at four residues (65,66). Isolectin III is different from isolectin I and II by 10 and 8 amino acids, respectively (54). Whether multiple forms of lectins in a plant have any physiological role or biological significance remains to be investigated.

Tissue Distribution and Subcellular Localization

Although lectins were originally observed in the seeds of leguminous plants, there are now numerous reports to support the ubiquitous occurrence of these proteins/glycoproteins in many plant species (for review see 67 and 68). Two major approaches have been employed for detection and/or localization of lectins in plants, biological assays and immunological assays. Biological assays basically involve measuring hemagglutination activity of lectins, since they can specifically react with simple or complex carbohydrate or with the carbohydrate moiety of glycoproteins. However, using such an assay as the sole method for detection and localization of lectins calls for caution for the following reasons: (a) It cannot detect inactive lectin, nor will it provide an accurate estimate of lectin if there are endogenous lectin receptors in the extract. (b) Monovalent lectins do not have hemagglutination activity. (c) It can sometimes produce false-positive results; for example, hemagglutination activity could be associated to a non-protein agglutinator such as tannic acid (69). Immunocytochemical techniques used for localization of lectins are based on the specificity of antibodies for antigen, in this case, lectin. But, there are some limitations to these techniques as well. For example, accessibility of the antigenic determinants to antibodies in vivo, as well as preservation of the determinant during preparation of samples for cytochemical staining or ultrastructural analysis, are extremely important for success by this method. The combination of both techniques would compensate for the limitations of either method alone. In this section, I will present some examples of lectins whose distribution or localization have been studied in leguminous plants.

Seed Lectins

Seed lectins constitute up to 10% of total soluble protein of the legume seeds (70). Studies on distribution of lectins in the legume seeds have indicated that in mature seeds most of the lectin is localized in the cotyledon cells in special storage organelles, commonly called protein bodies (69,71,72).

Subcellular localization of Dolichos biflorus seed lectin using a combined immunofluorescence and cell fractionation technique (73) revealed the majority of the seed lectin was associated with the protein bodies of cotyledon cells; although the lectin was also observed in the starch granules and cytoplasm of the cells. Localization of SBA in soybean seeds was studied at the ultrastructural level using anti-SBA antiserum labeled with gold particles (74). The lectin (SBA) was found uniformly distributed in the protein bodies; the labeling was also observed in the embryo axis. Recently, similar localization for SBA was reported by a different group, as studied by light level immunocytochemistry and indirect immunofluorescent labeling using anti-SBA antibodies (75). The lectins, Con A from jack beans (76) and PHA from Phaseolus vulgaris (77), have been localized in the matrix of protein bodies of cotyledon parenchyma cells of respective plants at the ultrastructural level with colloidal gold-labeled antibodies against corresponding lectins. PHA has also been localized in the cytoplasm of vascular cells and embryo axis cells (77). Similarly, favin (lectin from Vicia faba) was found in protein bodies as detected by radial immunodiffusion (78,79).

Lectins in Vegetative Plant Tissues

Several early investigators failed to detect any lectin in vegetative parts of plants using hemagglutination activity as a sole assay. Howard et al. (69) used a second assay, immunodiffusion, in addition to hemagglutination assay to follow the distribution of lentil lectin during the life cycle of the plant. They found only small amounts of lectin associated with the roots and stems in the early seedling. A lectin was isolated and purified by affinity chromatography from the root of Pisum sativum (80). The root lectin was shown to be identical to seed lectin in terms of molecular weight and immunological reactivity, but it had a different isoelectric point, saccharide specificity, and hemagglutination activity. A similar conclusion was obtained when lectins of Phaseolus vulgaris were compared using seed and non-seed tissues (81). Fluorescently-labeled antibodies to seed lectin from Pisum sativum showed the presence of the lectin on the surface of root hairs and in the root cortical cells of the plant (80,82). Buffard et al. (82a), using a pea (Pisum sativum) seed lectin cDNA as a hybridization probe, have now demonstrated the expression of a pea seed lectin gene in the pea roots, even though the level of lectin mRNA in the root is less than that in the seed by a factor of 4000. Investigations to detect lectin activity in vegetative parts of soybean have provided conflicting results. Pueppke et al. (72) have found lectin activity in soluble fractions from different soybean tissues, at different stages of growth, extracted in the presence of 0.03 M galactose. The lectin was detected by hemagglutination, radioimmunoassay, and isotope dilution assays. Lectin activity associated with the membrane fraction of

cotyledons from lectin positive (Le^+) soybean genotypes purified by the affinity chromatography, developed for soluble soybean seed lectin, was reported by Pueppke et al. (83). These investigators, however, failed to detect any hemagglutinin associated to roots of soybean seedlings. In contrast, Bowles et al. (84) did find hemagglutinin activity in soluble fractions of homogenates of leaves, shoots and roots (2- and 5-weeks-old) of soybean plants. Extraction of membrane pellets from all tissues, root, shoot and leaves, with 0.1 M of galactose or detergent (0.5% Triton X-100) at all stages of development, from seedling to maturity, yielded hemagglutinating activity. Based on inhibition of hemagglutination assays, the detergent extractable lectin appears to differ from the seed lectin in terms of carbohydrate-binding characteristics. These results led authors to suggest two possible classes of lectins: (a) those that are loosely attached to membrane and are similar to soluble seed lectin; and (b) those that are integrated in the membrane and require detergent for their solubilization. The conflict between these results reported by different groups may be explained by the use of different soybean genotypes or different growth conditions, ages of seedlings, and difference in other experimental conditions.

A lectin was isolated from soybean roots, cultivar Chippewa, and shown to be similar to seed lectin in terms of structural characteristics, immunological reactivity, and carbohydrate-binding specificity (85). In addition, the root lectin was shown to be endogenous to the tissue of analysis (86) because roots which have not been in direct contact with cotyledons were found to contain lectin at their surface; this eliminates the possibility that root lectin may be a contaminant

of seed lectin. Consistent with these results, soybean lectin activity has also been found in root exudates *in vivo* (87), in the cell surface of callus culture derived from soybean roots (88), and callus cultures enriched in root hair cells (89). Using immunological techniques, Stacey *et al.* (90) demonstrated the presence of lectin on the root hairs and epidermal cells of soybean roots.

Distribution of lectin in vegetative parts of *Phaseolus vulgaris* was studied using solid-phase enzyme immunoassay (81). All parts, including roots, stems and leaves, contained materials which cross-react with antibodies to seed lectin, even though at low levels (81,91). These lectins, however, showed some similarities and some differences when compared to seed lectin.

Lectin distribution in *Dolichos biflorus* was studied during the life cycle of plants using a radioimmunoassay (71). The results of this investigation revealed the presence of low level materials in the stem and leaves at all stages of the life cycle of the plant which cross-react with the antiserum to seed lectin. The cross-reactive material is presently referred to as DB58 (the protein is a dimer of 58,000 molecular weight) (92). Subcellular localization of DB58 in stems and leaves of *Dolichos biflorus*, as studied by immunofluorescence and cell fractionation, showed a significant portion of lectin is associated with the cell wall; the lectin was also observed in the cytoplasm (73). Treatment of cell wall pellets of leaves and stems with cellulase and pectinase could release significant amounts of lectin (73).

Hemagglutination activity was detected in the hypocotyl membranes of mung bean (*Vigna radiata*) by fractionation and sequential

extraction (93). Extraction of hemagglutinin from endoplasmic reticulum and Golgi apparatus, however, was possible only under conditions using EDTA-detergent solution for extraction of plasma membranes of these organelles (93). In addition to membranes, hemagglutination activity has also been detected in the cell wall fraction of mung bean (94,95).

Endogenous Function of Plant Lectins

During the past several years, a vast amount of information has been generated concerning the structure, specificity, localization and, more recently, genetics of lectins. Nevertheless, there is very little, if any, information available with regard to the endogenous physiological role and biological significance of this abundant group of proteins in plants. Several functions for plant lectins have been proposed, such as transport of carbohydrates, defense mechanisms, packaging and/or mobilization of storage materials, mitogenic stimulators of plant embryonic cells, cell recognition, cell wall extension, and attractants for rhizobial symbiosis (67). These functions are mostly speculative, and there is a paucity of supportive data for them. Among the proposed functions, however, the possible involvement of lectins in rhizobial-legume symbiosis, has received considerable attention within the past 15 years. Conflicting results, generated from various laboratories to support or refute such a role, have provoked a great deal of controversy. Due to the relevance of such function of lectins to this thesis, it is discussed in detail. Particularly, the molecular mechanism involved in the initial recognition between Rhizobium and the legume root is of major focus.

The recognition phenomenon has been well studied in R. trifolii-white clover, R. leguminosarum-pea, and R. japonicum-soybean (90,96, 97). When a Rhizobium strain is inoculated on a legume host, they initially clump at the tips of the root hair cells. This is followed by the attachment of the bacteria along the sides of the root hairs in a polar fashion. A marked root hair curling occurs, which results in a hook-like structure, termed the "Shepherd's crook". Bacteria entrapped within the curl induce the formation of a tubular structure, called the infection thread. Host specificity is most likely expressed during the initial attachment prior to the formation of the infection thread.

Rhizobia are slimy in nature, which may be due to the extracellular polysaccharide (EPS) and lipopolysaccharide (LPS) at the bacterial cell surface. These components would be strong candidates for the initial contact with the plant cell wall. In legumes, lectins have been proposed to impart the observed specificity of Rhizobium infection mediating cell-cell interactions (98,99,100). This is based on their carbohydrate-binding properties (discussed earlier), by which they can selectively interact with the carbohydrate receptors on Rhizobium. Much of the current interest in lectins was stimulated by discovery that some lectins could specifically interact with Rhizobium. Krupe first suggested a role for lectin in recognition (98). This suggestion was revised by Hamblin and Kent based on the observation of specific binding of bean lectin to R. phaseoli (99). Later, Bohlool and Schmidt showed that soybean lectin binds specifically to 22 of 25 nodulating strains of R. japonicum (100). Fluorescence and electron microscopic studies suggested that the lectin binding sites are polar

(100,101). This observation agrees with the end-on attachment of the bacteria to the root hair. This specific correlation became the basis for formulating the lectin recognition hypothesis which states that the recognition between Rhizobium and legume root involves a lectin on the plant root which binds to a unique carbohydrate moiety on the surface of the bacterial symbiont.

However, later studies have demonstrated that not all legume seed lectins specifically recognize the corresponding homologous Rhizobium. Lentil lectin bound to R. leguminosarum and R. japonicum (102), pea lectin and broad bean lectins bound to some, but not all, of the tested strains of R. leguminosarum (103) and jack bean lectin bound to all tested strains of R. leguminosarum, R. phaseoli, and R. japonicum that do not nodulate jack bean (104). Host specificity did not appear to correspond to the seed lectin binding properties in some strains of Rhizobium. Furthermore, the presence of the lectin at the sites of the infection has been questioned (105). Attempts to localize soybean lectin on soybean roots by immunological technique were unsuccessful (106). Results which confused the issue even more arose from the studies of several lines of soybean which contained undetectable lectin activities but could still be nodulated by R. japonicum (107).

Some of the controversies have been clarified by putting more emphasis on considering the root lectins rather than the seed lectins for bacterial recognition. Several studies have now confirmed the presence of lectin(s) in the roots of legume plants, even though, in some cases, it appears that root and seed lectins are identical and, in other cases, they show some differences in terms of their physico-chemical and/or immunological properties, as discussed earlier.

The lectin-binding ability of various Rhizobium strains is greatly dependent on the age of culture and growth environment (108,109). Some strains of Rhizobium which showed no lectin binding ability at any stage of growth in synthetic media might develop lectin receptors under the influence of root exudate (108). The involvement of lectin in nodulation was implicated by Halverson and Stacey by studying a mutant of R. japonicum that is defective in the rate of nodulation (110,111). This defect could be corrected by pretreatment of the mutant with the soybean seed lectin or root exudate obtained from various soybean varieties including the lectin-negative soybean line.

Consistent with the lectin recognition hypothesis, Rhizobium binding to its host can be inhibited by the corresponding hapten of its host lectin. Therefore, R. trifolii binding to clover could be inhibited by 2-deoxyglucose (112). Similarly, attachment of R. japonicum and R. leguminosarum to the root hairs of their hosts could be reversed by the corresponding hapten sugars of the host lectins (90,97). Fluorescently labeled capsular polysaccharide from the bacteria bound to the root hair tips, sites where the bacteria clumped and where root lectins were identified (113).

Dazzo has suggested a two-phase mechanism for the early recognition between R. trifolii and the clover (114). An initial non-specific adhesion of the bacteria to the root surface (Phase I attachment) is followed by a selective attachment of the rhizobial symbiont (Phase II attachment). Direct microscopic observation suggests that the firm and polar bacterial attachment of the host roots correlated well to host specificity. This host-specific

bacterial attachment has also been demonstrated in R. japonicum with soybean (90), and R. leguminosarum with pea (97). It has been suggested that this Phase II mechanism is the determinant for cellular recognition in the Rhizobium-legume symbiosis (114).

Rhizobium infection leading to nodule formation is highly regulated. Bhuvaneswari et al. have demonstrated the roots of soybean and cowpea are susceptible to nodulation only in a highly restricted area between the root tip and the small emergent root hair region during the time of inoculation (115). Host root cells within this region become progressively less susceptible to nodulation as roots grow. This rapid inhibitory response can be elicited by pretreatment with living or UV-inactivated Rhizobia strains that are homologous to the host. Unfortunately, there are no studies on the correlation between the transient susceptibility and the lectin distribution or differential bacterial binding throughout the length of the root. In another study, Gade et al. has quantitated the lectin content at different root segments of the five-day-old soybean seedlings (86). Significant quantities of lectin were found in the region of root hair development, which coincided with the region where Rhizobium binding occurred (90).

Several laboratories have attempted to establish in vitro symbiosis from legume culture system. Generally, infection thread-like structures are developed as bacteria penetrate within the intercellular spaces. Evidence from light microscopy suggested that some callus cells were infected. Holston et al. showed convincingly by electron microscopy the development of bacteroids within the infected cells (116). The development of nitrogenase activity as a

result of Rhizobium infection in callus culture is still a topic of controversy.

The lectin-recognition hypothesis is an appealing proposal due to the structural compositions of the bacterial surface and the carbohydrate-binding properties of the lectin. The uniqueness of a whole family of individual lectins synthesized by various legumes further suggest that this class of molecules may be important in controlling host specificity. This notion is further supported by the high correlation between the sites for Rhizobium binding and lectin expression domains in the roots. However, it must be stressed that the presence of the lectin on cell surface may be necessary but not sufficient to insure Rhizobium binding. A dual recognition mechanism involving another set of complementary molecules, is consistent with our current understanding of this system.

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Chapter II

ENDOGENOUS LECTINS FROM CULTURED SOYBEAN CELLS: ISOLATION OF A PROTEIN IMMUNOLOGICALLY CROSS-REACTIVE WITH SEED SOYBEAN AGGLUTININ AND ANALYSIS OF ITS ROLE IN BINDING OF *RHIZOBIUM japonicum*

ABSTRACT

Incubation of Rhizobium japonicum with the cultured soybean cell line SB-1, originally derived from the roots of Glycine max, resulted in specific adhesion of the bacteria to the plant cells. This binding interaction appears to be mediated via carbohydrate recognition, since galactose can inhibit the heterotypic adhesion, but glucose cannot. Affinity chromatography, on a Sepharose column derivatized with N-caproyl-galactosamine, of the supernatant fraction of a SB-1 cell suspension after enzymatic removal of cell wall yielded a single polypeptide (M_r ~ 30,000) on immunoblotting analysis with rabbit antibodies directed against seed soybean agglutinin. Fluorescently labeled rabbit anti-seed soybean agglutinin also yielded specific immunofluorescent staining on the cell wall and plasma membrane of the SB-1 cells. These results suggest that one likely candidate that may mediate the recognition between the Rhizobium and the soybean cells is the endogenously produced SB-1 lectin. This notion is supported by the observation that rabbit anti-seed soybean agglutinin blocked the Rhizobium-soybean cell adhesion whereas control antibodies did not.

INTRODUCTION

Many eukaryotic and microbial cells can recognize and interact specifically with other cells, either cells of the same type (homotypic) or cells of a different type (heterotypic). Our understanding of the molecular components mediating specific cell recognition and adhesion has advanced mainly as a result of several paradigms: (a) yeast sexual agglutination (28); (b) sea urchin fertilization (38); (c) slime mold aggregation (30); (d) cell-cell adhesion in embryonic tissues of the chick (33); and (e) recognition, by lymphoid cells, of target cells bearing foreign antigens (39). Among plant systems, one area that has attracted much attention is the binding of the bacterium Rhizobium to the root cells of leguminous plants, leading to the nitrogen-fixing symbiosis (2). Clearly, a key event in the establishment of the legume-Rhizobium symbiosis is the initial binding between the bacterium and the host cell.

Studies directed at analysis of this event have been guided, for the most part, by the "lectin recognition" hypothesis, first proposed by Krupe (20) and later revived by Hamblin and Kent (15) and by Bohlool and Schmidt (6). According to this hypothesis, legume lectins control host specificity by interacting with polysaccharide components on the bacterial symbiont. There have been many inconsistencies and experimental deficiencies in various studies that purport either to support or to refute the "lectin recognition" hypothesis (2, 29).

Many of these difficulties may be associated with the facts that the studies have been carried out, in general, with in vivo root systems in which the initial binding event may be difficult to assay and manipulate and that many of the assays are long term "end point" assays, i.e. successful nodule formation. While these studies are, for the most part, informative and directly relevant to biological nitrogen fixation, it seems that a defined tissue culture system is much more amenable to study the initial binding event.

I have identified and isolated a lectin produced endogenously by the soybean cell line, SB-1, originally derived from roots of Glycine max by Gamborg (12). Immunofluorescence and biochemical evidence indicated that the lectin may be localized on the cell wall and therefore is accessible to the external environment. These results prompted us to determine whether Rhizobium binds specifically to the cultured SB-1 cells and whether the endogenous lectin plays a role in this adhesion. The results of our studies on these issues are reported in the present communication.

MATERIALS AND METHODS

Cell Culture and Protoplast Isolation

The SB-1 cell line, derived from soybean roots (Glycine max (L.) Merr. cv. Mandarin) (12), was kindly provided by Dr. G. Lark (Department of Biology, University of Utah, Salt Lake City, UT). Cultures were grown in 125 ml Erlenmeyer flasks containing 30 ml of solution at 27°C on a gyratory shaker in the dark. Liquid cultures were subdivided every 3 to 4 days by transferring 10 ml of culture to 30 ml of fresh 1B5C medium (basic medium plus 1 ppm of 2,4-D and 2 g/l of casein hydrolyzate, pH 5.5). Suspensions of SB-1 cells were centrifuged for 4 min at 460 g; 1 ml of packed cells usually yielded ~ 10^7 cells by direct counting.

Protoplasts were prepared by a modified procedure of Constabel (9). Actively growing SB-1 cells (24-48 hours after transfer) were digested with an equal volume (20 ml) of enzyme solution containing 400 mg cellulysin (Calbiochem, La Jolla, CA), 200 mg pectinase (Sigma, St. Louis, MO) and 2 g D-sorbitol (Sigma), pH 5.5. After 2 hours, the protoplast suspension was filtered through a 48 μm nylon filter and pelleted by centrifugation for 4 min at 460 g. The pelleted protoplasts were washed by gentle resuspension and centrifugation using 5 ml of protoplast medium (9), which was modified by substituting 30 g D-sorbitol for glucose. After three washes, the protoplasts were resuspended in 5 ml of the same protoplast medium. Fluorescence

microscopy after Calcofluor staining and scanning electron microscopy of the protoplasts showed neither cell wall material nor cellulose microfibrils, respectively. The details of these analyses have been reported previously (25).

Seed Soybean Agglutinin and Antibody Reagents

Seed soybean agglutinin (SBA) was isolated and purified by affinity chromatography on Sepharose column derivatized with N-caproyl-galactosamine (Gal-Sepharose) (1). Soybean meal was defatted with petroleum ether (35-60°C; 1:10 w/v). The defatted meal was stirred with phosphate-buffered saline (PBS) overnight at 4°C, centrifuged at 9,000 g for 15 min and the supernatant was applied to the Gal-Sepharose column. Bound SBA was eluted from the column with 0.2 M D-galactose (Gal) in PBS at 4°C.

Antibodies directed against seed SBA were raised in rabbits (New Zealand White, female). The primary injection consisted of 1 mg of protein in Freund's complete adjuvant (Difco Laboratories, Inc., Detroit, MI). Booster injection of 1 mg protein in Freund's incomplete adjuvant were administered at weekly intervals. Antiserum was collected one week after boosting. Monospecific antibodies directed against seed SBA were isolated by affinity chromatography. Purified seed SBA (14 mg) was coupled to cyanogen bromide activated Sepharose 4B beads (3 ml; Pharmacia) and further cross-linked with 0.1% (v/v) glutaraldehyde (19). Antiserum raised against seed SBA was fractionated over this column; the bound fraction eluted with 0.1 M glycine-HCl, pH 3.0 was designated as rabbit anti-seed SBA.

The labeling of antibodies with ^{125}I was carried out with chloramine T following the procedure described by Ho et al. (17). Free ^{125}I was removed by passing the labeled material over a column (3.0 x 0.5 cm) of Dowex AG1x8 (BioRad Richmond, CA). The specific activities of the products were: rabbit anti-seed SBA (1.5 x 10^6 cpm/μg) and normal rabbit immunoglobulin (1.4 x 10^6 cpm/μg).

We have also generated a rabbit antiserum that binds to the cell wall of SB-1 cells; this antiserum serves as a control for certain experiments carried out with rabbit anti-seed SBA. SB-1 cells (15 g wet weight) were homogenized in 30 ml water at 4°C, using a Waring blender at maximum speed for 5 min. After centrifugation at 3,000 g for 5 min, the pellet was extracted three times with 30 ml of 1% SDS in 20 mM Tris-HCl, pH 7.4. The ruptured cells were homogenized in a Potter-Elvehjem tissue grinder and then extracted successively with methanol (three times, each with 30 ml) and water (three times, each with 30 ml). The final pellet was suspended in water as a 10% (v/v) suspension. Microscopic observation revealed cell wall fragments and no cytoplasmic organelles.

Equal volumes of a 2% (v/v) suspension containing cell wall fragments and complete Freund's adjuvant were sonicated and used for immunization of female New Zealand white rabbits. Booster immunizations were given at biweekly intervals using the same antigen fraction emulsified in incomplete Freund's adjuvant. Two months after the initial immunization, serum derived from the rabbit was fractionated on protein-A Sepharose; the immunoglobulin fraction obtained bound to intact SB-1 cells as well as to the fraction containing cell wall fragments as revealed by indirect immunofluorescence (see below).

This immunoglobulin fraction will be referred to as rabbit anti-cell wall fragments.

Polyacrylamide Gel Electrophoresis and Immunoblotting

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was done as described (21) using 10% and 4% (w/v) acrylamide concentrations in the running and stacking gels, respectively. Following electrophoresis, the proteins were revealed by staining with Coomassie Brilliant Blue or by immunoblotting after transfer to nitrocellulose paper. For immunoblotting, the proteins were transferred to nitrocellulose paper (Schleicher and Schuell, Keene, NH) by electrophoresis (200 MA, 3 h, 25°C) (36). After transfer, the blots were washed overnight in Tris-buffered saline (20 mM Tris, 500 mM NaCl, pH 7.5) plus 0.05% Tween 20 which contained 5% (w/v) bovine serum albumin. The nitrocellulose membrane was incubated with rabbit anti-seed SBA (40 µg/ml) for 6-8 hours and then washed three times with 10-min incubations in Tris-buffered saline. The membrane was then incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (BioRad; 1:2000 dilution, 90 min, 25°C) and washed three times with Tris-buffered saline. The immunoreactive material was revealed via horseradish peroxidase activity according to the BioRad procedure with 4-chloro-1-napthol and hydrogen peroxide as substrates.

Isolation of Antibodies by Specific Adsorption to a Polypeptide on Nitrocellulose

This procedure was used to isolate monospecific antibodies specifically directed against the 30 kD polypeptide of seed SBA. Purified seed SBA (0.5 mg) was subjected to SDS-PAGE and transferred to nitrocellulose membrane. The nitrocellulose membrane was incubated with rabbit anti-seed SBA (40 µg/ml) for 6-8 hours at 25°C. After washing, a strip of the membrane was cut out and used to determine the position of the 30 kD polypeptide band by immunoblotting as described above. The position corresponding to the 30 kD region was excised from the unstained membrane and the bound antibodies were eluted as described by Smith and Fisher (34). This preparation was designated monospecific antibodies directed against the 30 kD polypeptide of seed SBA.

Assays for SB-1 Cell Components Reactive with Rabbit Anti-Seed SBA

The presence of material reactive with rabbit anti-seed SBA on the surface of SB-1 cells or protoplasts was assayed as follows: cells (1 x 10^6/ml) were washed twice in 0.55 M sorbitol, 10 mM Tris-HCl, 10 mM $CaCl_2$ (Tris buffer), pH 5.5. The cells were then incubated with 1 ml of Tris buffer alone, or Tris buffer containing 0.1 M glucose (Glc), Gal, or N-acetylgalactosamine (GalNAc) for 10 min (3x) at room temperature. The cells were washed twice by centrifugation (460 g for 4 min) and resuspended in Tris buffer, and the extraction was repeated. After the final wash, the cells were resuspended in Tris buffer with 0.3% (v/v) normal goat serum, pH 7.2, and washed once. Monospecific rabbit anti-seed SBA or control rabbit immunoglobulin was

added to a final concentration of 30 µg/ml. The samples were incubated for 1 h at 4°C, washed three times in Tris buffer with serum (pH 7.2). Fluorescein conjugated goat-anti rabbit immunoglobulin (10 µl of 1:100 dilution; GIBCO, Grand Island, NY) was added to the cells and incubated for 30 min at 4°C. The cells were washed three times in Tris buffer with serum and the fluorescence staining observed under a Leitz fluorescence microscope, equipped with a Leitz KP 490 dichroic mirror. Micrographs were taken with Kodak Tri-X film, which was pushed to ASA 3200.

The binding of rabbit anti-seed SBA to SB-1 cells and protoplasts was also assayed with ^{125}I-labeled antibody. Cells (1 x 10^6/ml) were washed three times with 1 ml of Tris buffer, pH 7.2, containing 0.3% (v/v) normal rabbit serum, by centrifugation (460 g for 4 min) and resuspension. These samples were incubated on an orbital shaker (100 rpm) with ^{125}I-labeled rabbit anti-seed SBA or normal rabbit immunoglobulin for 2 h at 4°C. After three washes by centrifugation and resuspension of the cells in Tris buffer with rabbit serum, the radioactivity in the cells was determined. A similar binding experiment with ^{125}I-labeled antibodies was performed after SB-1 cells or protoplasts were extracted with 0.1M Glc, Gal, or GalNAc as described above.

Isolation of Lectin Activity from SB-1 Cells

The cell wall was degraded by a modified procedure of Constabel (9). Actively growing SB-1 cells (4 days old) were washed with fresh 1B5C medium by centrifugation (460 g, 4 min.) and resuspension. The pelleted cells were then readjusted to the same volume with fresh 1B5C

medium, and digested with an equal volume (100 ml) of enzyme solution containing 0.8 g pectinase (Sigma, St. Louis, MO), 1.6 g cellulysin (Calbiochem) and 10.0 g D-sorbitol (Sigma), pH 5.5. After 2 hours incubation at 37°C, the digestion mixture was centrifuged (10,000 g, 30 min, 4°C). The supernatant was adjusted to pH 7.4, and purified by affinity chromatography on Gal-Sepharose columns. Material bound to the column was eluted with 0.2 M Gal, concentrated by Amicon ultrafiltration and dialyzed against 0.1% SDS.

Rhizobium Culture and SB-1 Cell Binding

Rhizobium Strains: R. japonicum 110d was obtained from Dr. Barry Chelm, R. fredii PRC 205 str (a fast growing strain originally derived from R. japonicum) was obtained from Dr. Kenneth Nadler, and R. meliloti 102F28, R. leguminosarum 128C56, and R. trifolii 0403. were obtained from Dr. Frank Dazzo. Various Rhizobium strains were maintained on yeast extract-mannitol-sodium gluconate medium at 30°C as described previously (5). Inocula were grown to mid-exponential phase. The bacterial strains were centrifuged at 7,000 g for 15 min and washed once with 20 ml of sterile 1B5C medium. The concentration was adjusted to 0.8 unit (1 unit = absorbance of 1.0 at 620 nm, 0.03 unit = 1 x 10^8 cells).

SB-1 cells (2-day-old cultures) in 1 ml suspensions (5 x 10^6 cells) were placed in 25 mm culture dishes and 0.1 ml aliquots of the washed Rhizobium cultures (2.6 x 10^8 cells) were added. To examine the inhibition of Rhizobium binding by saccharides, a final concentration of 0.1 M of different saccharides in 1B5C medium was added to the

cell suspension. Similarly, inhibition studies were also carried out using various concentrations of rabbit anti-seed SBA, normal rabbit immunoglobulin, or rabbit anti-cell wall fragments.

The co-cultures of bacterial and soybean cells were incubated at 26°C for 2 h or 24 h in the dark without shaking. At the end of the incubation, the cell suspensions were transferred to polystyrene tubes. The SB-1 cells were washed three times by centrifugation (460 g for 4 min) with 2 ml of 1B5C medium to remove unbound bacteria. The binding of Rhizobium to SB-1 cells was observed with Leitz microscope with phase contrast optics.

Histological Studies of SB-1 Callus Infected with Rhizobium

SB-1 callus cultures were grown in 1B5C medium with 0.8% agar. One week after the transfer of the callus to new plates, it was inoculated with 50 µl of various Rhizobium strains (1.3×10^8 cells) that were grown to the mid-exponential phase. After incubation in the dark for 1 week at 26°C, the callus was transferred to another agar plate containing LNB5 medium with 0.8% agar. The LNB5 medium is the 1B5C medium with the omission of 2,4-dichlorophenoxyacetic acid and casein hydrolysate. The callus was further cultured for two weeks; then individual callus was taken out and fixed in FAA fixatives (37% formaldehyde: glacial acetic acid: 70% ethanol in ratio of 5:5:90) for 2 days at room temperature. The samples were dehydrated in a series steps of ethanol and xylene, and then embedded in paraffin. Sections of 8 µm thickness were obtained and stained with Gram stain (22) or hematoxylin-eosin stain (24). These approaches were developed and performed by Dr. John Ho (see Ref. 1, Chapter 3).

RESULTS

Characterization of Antibodies Against Seed SBA

Because the conclusions from our studies depended on the specificity of the rabbit anti-seed SBA antibodies used, extensive efforts were devoted to the characterization of the purity of the antigen used for immunization and of the specificity of the resulting antiserum. Seed SBA was isolated by affinity chromatography on Gal-Sepharose columns (1). On SDS-PAGE under reducing conditions, this purified preparation yielded a single band (M_r ~ 30,000) (Figure 1A, lane 2), corresponding to the molecular weight reported for the polypeptide subunit of seed SBA (23).

Rabbit antiserum was raised against this highly purified preparation of seed SBA. When extracts of soybean seeds were analyzed by SDS-PAGE and immunoblotting with rabbit anti-seed SBA, one major polypeptide band (M_r ~ 30,000) was observed (Figure 1B, lane 1). There was also a minor band (M_r ~ 60,000), which accounted for no more than 1% of the total material (Figure 1B, lane 1). These two bands were the only polypeptides detected by the antibody, among a host of many other polypeptides observed by Coomassie Blue staining (Figure 1A, lane 1). Similar results were obtained when purified seed SBA, the antigen used for immunization, was subjected to parallel immunoblotting analysis (Figure 1B, lane 2). No polypeptide was observed when preimmune serum was used for such an analysis.

Figure 1. SDS-PAGE analysis of extracts of soybean seeds, purified seeds SBA, and SB-1 lectin. (A) Polypeptides of the SDS gels were revealed by Coomassie Blue staining: lane 1, extract of soybean seeds (~ 100 μg total protein); lane 2, purified seed SBA (10 μg). (B) Polypeptides of the SDS gel were transferred to nitrocellulose paper and immunoblotted with rabbit anti-seed SBA and horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin: lane 1, extract of soybean seeds; lane 2, purified seed SBA; lane 4, SB-1 lectin derived from the cell wall of SB-1 cells (~ 20 g) purified by affinity chromatography on Gal-Sepharose column. In lane 3, purified seed SBA (10 μg) was subjected to SDS-PAGE and immunoblotted with monospecific antibodies directed against the M_r 30,000 polypeptide observed in lane 2. The arrows on the left indicate the positions of migration of polypeptides of M_r 30,000 and M_r 60,000, relative to known molecular weight markers.

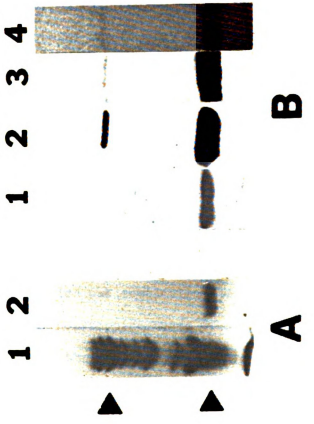


Figure 1

The position of migration of the predominant band (at 30 kD) in the immunoblots (Figure 1B, lanes 1 and 2) corresponded to the subunit molecular weight of seed SBA (M_r ~ 30,000). To test whether antibodies that bound to the 30 kD band also recognized the 60 kD polypeptide, the following experiment was performed. First, seed SBA was subjected to electrophoresis and transferred to nitrocellulose paper. The paper was incubated with rabbit anti-seed SBA. After washing to remove unbound antibodies, the region of the nitrocellulose paper corresponding to the 30 kD polypeptide was excised and those antibodies which bound the 30 kD polypeptide were re-eluted from the nitrocellulose strip. Finally, these monospecific antibodies directed against the 30 kD material were used to immunoblot another sample of seed SBA after SDS-PAGE. The results showed that antibodies bound and eluted from the 30 kD region of the original gel recognized both the 30 kD and the 60 kD polypeptides (Figure 1B, lane 3).

We have also carried out comparative peptide mapping analysis on the 30 kD and 60 kD polypeptide bands of seed SBA. Limited digestion with V-8 protease of the two polypeptide bands yielded identical peptide maps. Together with the immunoblotting results, these data strongly suggest that the 60 kD polypeptide is a dimeric form of the SBA subunit. More importantly, it does not appear that the 60 kD polypeptide was an irrelevant protein contaminating the SBA preparation. We concluded from these series of experiments that the seed SBA preparation used as immunogen was pure and that the rabbit anti-seed SBA antibody was highly specific for the lectin. This conclusion forms the basis for subsequent studies reported in this paper.

Binding of Antibodies Directed Against Seed SBA to SB-1 Cells

Incubation of SB-1 cells with rabbit anti-seed SBA resulted in the binding of the antibodies, as indicated by staining with fluorescein derivatized goat antibodies directed against rabbit immunoglobulin (Figure 2a). The fluorescence was localized around the outer periphery of individual cells, suggesting that the antibodies were bound to the outer surface. Parallel incubations of SB-1 cells with preimmune rabbit immunoglobulin, followed by fluorescein-labeled goat anti-rabbit immunoglobulin, failed to yield the same bright staining (Figure 2e).

A similar conclusion can be derived from studies in the binding of ^{125}I-labeled rabbit anti-seed SBA. In the experiments shown in Figure 3, monospecific affinity-purified rabbit antibodies directed against seed SBA were labeled with ^{125}I and used in the binding studies. The binding of rabbit anti-seed SBA to SB-1 cells was concentration dependent. The binding curve saturated at a concentration of about 10 µg/ml, suggesting that there was a finite number of antigenic sites exposed at the outer surface (Figure 3). These results suggest that a molecule, immunologically cross-reactive with seed SBA, was present on the cell wall of SB-1 cells.

When the binding studies were carried out on SB-1 cells preincubated with saccharides such as Gal and GalNAc (0.1 M), neither the indirect immunofluorescence (Figure 2b,c) nor the radiolabeled binding results (Table I) were affected, qualitatively or quantitatively. Similarly, preincubation of SB-1 cells with the saccharide Glc also failed to change the results (Figure 2,d and Table I). These data

Figure 2: Fluorescence staining patterns of SB-1 cells treated for 1 h at 4°C with rabbit anti-seed SBA (30 µg/ml) or normal rabbit immunoglobulin (30 µg/ml), followed by fluorescein-conjugated goat anti-rabbit immunoglobulin (1:100 dilution; 30 min at 4°C). (a)SB-1 cells treated with rabbit anti-seed SBA; (b)SB-1 cells washed with Gal (0.1 M), then treated with rabbit anti-seed SBA; (c)SB-1 cells washed with GalNAc (0.1 M), then treated with rabbit anti-seed SBA; (d)SB-1 cells washed with Glc (0.1 M), then treated with rabbit anti-seed SBA; (e)SB-1 cells treated with normal rabbit immunoglobulin. ph, phase contrast microscopy; fl, fluorescence microscopy; bar = 5 µm.

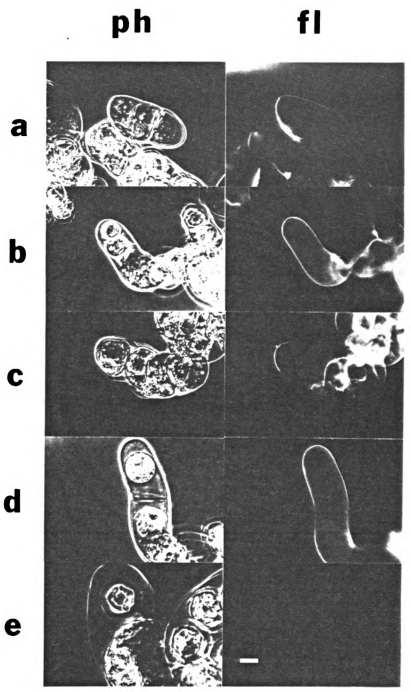


Figure 2

Figure 3. Dose-response curve for the binding of ^{125}I-labeled rabbit anti-seed SBA to SB-1 cells. The binding studies were carried out with 10^6 cells at 4°C for 2 h, as detailed in Materials and Methods. o, ^{125}I-labeled rabbit anti-seed SBA (1.5 x 10^6 cpm/μg); x, ^{125}I-labeled normal rabbit immunoglobulin (1.4 x 10^6 cpm/μg). Data points are the averages of triplicate determinations.

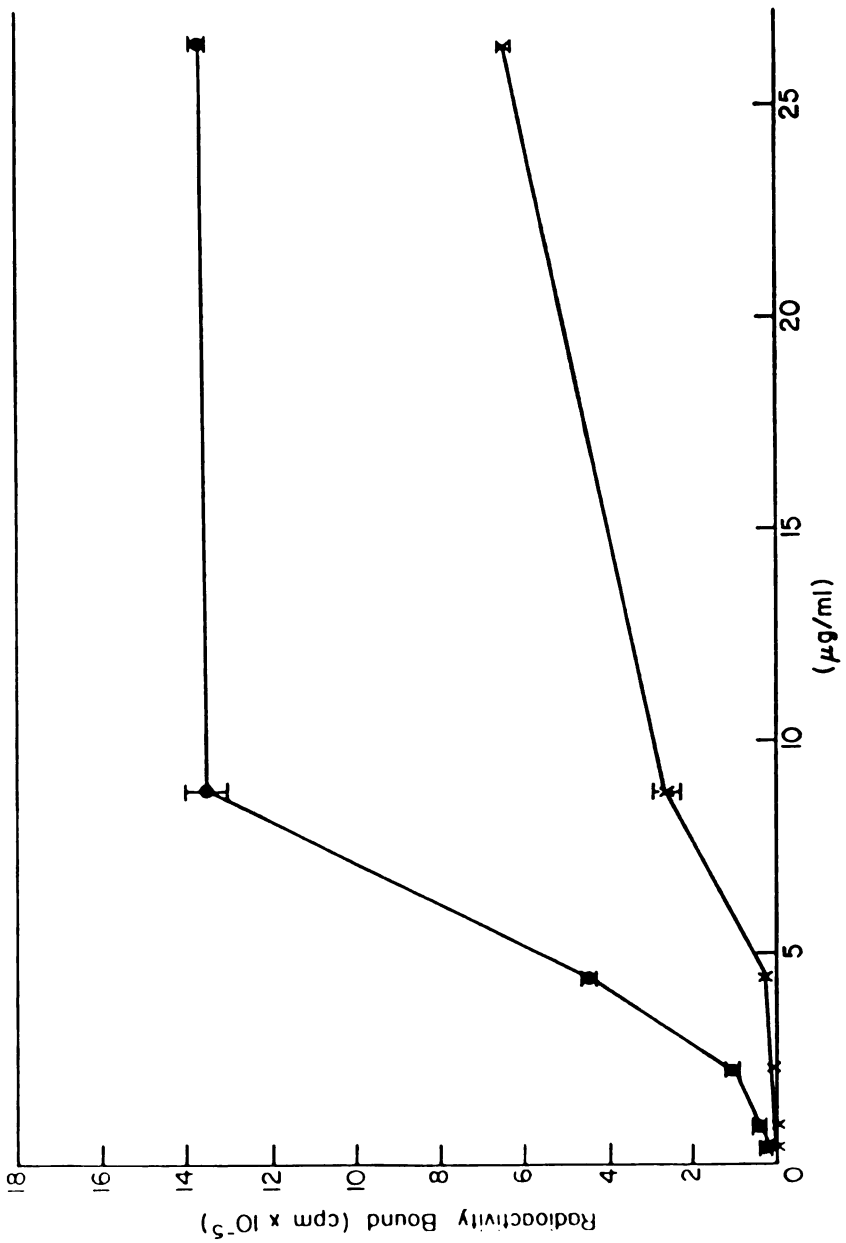

Radioactivity Bound (cpm x 10^{-5})
0
2
4
6
8
10
12
14
16
18
5
10
15
20
25
(µg/ml)

Figure 3

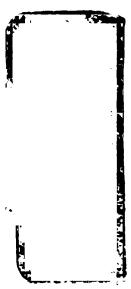


Table I. Binding of ^{125}I-labeled rabbit anti-seed soybean agglutinin to SB-1 cells and protoplasts prewashed with various saccharides*

Treatment	Cells	Protoplast
Control	100 ± 11	100 ± 6
D-glucose (0.1 M)	87 ± 3	102 ± 8
D-galactose (0.1 M)	89 ± 6	107 ± 7
N-acetyl-D-galactosamine (0.1 M)	80 ± 5	100 ± 9

* The binding experiments were carried out with 10^6 cells or protoplasts at 4°C for 2 h, as described in Materials and Methods. The data represent percent specific binding (binding observed for ^{125}I-labeled rabbit anti-seed soybean agglutinin minus the binding observed for ^{125}I-labeled normal rabbit immunoglobulin of the same specific activity and concentration). Averages of triplicate determinations ± standard error are shown for each ligand.

indicate that the molecule on the cell surface which binds anti-seed SBA was not anchored via its carbohydrate-binding properties.

Isolation of SB-1 Lectin After Cell Wall Digestion

SB-1 cells were digested with cellulase and pectinase to remove cell wall material. The digestion mixture was subjected to affinity chromatography on a Gal-Sepharose column. The bound material was eluted with 0.2 M Gal and, upon SDS-PAGE and immunoblotting, yielded a predominant band (M_r ~ 30,000) and a minor band (M_r ~ 60,000) (Figure 1B, lane 4). This pattern was identical to that obtained on immunoblots of seed SBA (Figure 1B, lane 2). Essentially the same results were obtained when the digestion mixture was subjected to affinity chromatography on columns derivatized with rabbit anti-seed SBA. The immunoreactive material yielded a predominant polypeptide band (M_r ~ 30,000) and a minor component (M_r ~ 60,000).

I designate this affinity purified material as SB-1 lectin. Preliminary studies indicate that SB-1 lectin is similar if not identical to seed SBA when compared by gel filtration under non-denaturing conditions and by peptide mapping analysis. These results suggest that SB-1 cells produce an endogenous lectin that binds to galactose-containing glycoconjugates and that the SB-1 lectin may be on the cell wall and could be released upon degradation of the wall. This conclusion is consistent with our observations on the immunofluorescence staining of the cell wall with rabbit anti-seed SBA.

Binding of Antibodies Directed Against Seed SBA to Protoplasts

Rabbit antibodies directed against seed SBA also bind to protoplasts derived from SB-1 cells. Indirect immunofluorescence revealed ring-like staining, outlining the periphery of the cell and characteristic of surface staining patterns obtained with other spherical objects (Figure 4a). This suggests that the antigenic determinant recognized by rabbit anti-seed SBA is diffusely distributed on the plasma membrane. Preincubation with saccharides failed to alter the staining pattern (Figure 4b,c,d). Preimmune rabbit immunoglobulin yielded little or weak staining (Figure 4e). Similar results were obtained using the radiolabeled antibody probes. In these respects, the molecule immunologically cross-reactive with seed SBA on the plasma membrane appears to be very similar to that found on the outside of the cell wall (see below, however, for possible differences in Rhizobium-binding properties).

Binding of Rhizobium to SB-1 Cells

When SB-1 cells were mixed with Rhizobium japonicum (R110d) at 26°C for several hours, washed, and sampled under a microscope, the bacteria adhered to certain soybean cells (Figure 5). Initially, there was little bacterial binding and the binding was limited to the tips of some plant cells. A representative photomicrograph, taken after 2 h of co-culture, is shown in Figure 5a. Between 12 and 24 h, however, there appeared to be a sorting out process. When the co-culture of SB-1 cells and Rhizobium was sampled after 24 h, a striking "polar" mode of binding was observed; the Rhizobium adhered to the plant cells in an "end-to-end" fashion (Figure 5b).

Figure 4. Fluorescence staining patterns of protoplasts derived from SB-1 cells treated for 1 h at 4°C with rabbit anti-seed SBA (30 µg/ml) or normal rabbit immunoglobulin (30 µg/ml), followed by fluorescein conjugated goat anti-rabbit immunoglobulin (1:100 dilution; 30 min at 4°C). (a) protoplasts treated with rabbit anti-seed SBA; (b) protoplasts washed with Gal (0.1 M), then treated with rabbit anti-seed SBA; (c) protoplasts washed with GalNAc (0.1 M), then treated with rabbit anti-seed SBA; (d) protoplasts washed with Glc (0.1 M), then treated with rabbit anti-seed SBA; (e) protoplasts treated with normal rabbit immunoglobulin. ph, phase contrast microscopy; fl, fluorescence microscopy; bar = 5 µm.

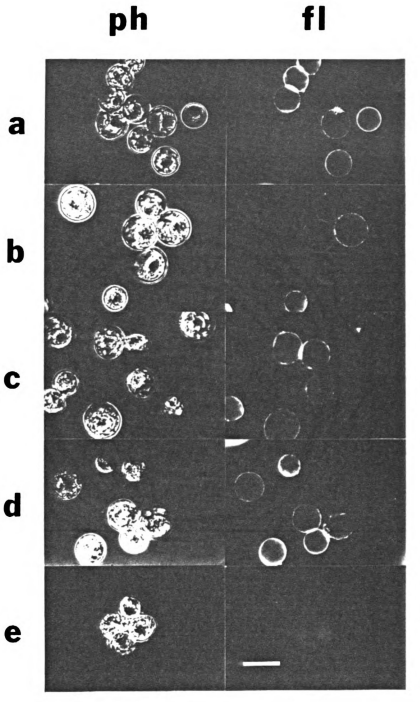
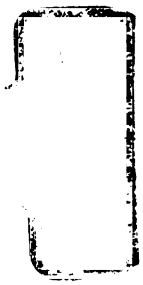


Figure 4

Figure 5. Representative photomicrographs showing the adhesion of Rhizobium japonicum (R110d) to SB-1 cells after (a) 2 h and (b) 24 h of co-culture at 26°C in the dark. Bar = 10 μm. Arrows indicate bacteria.



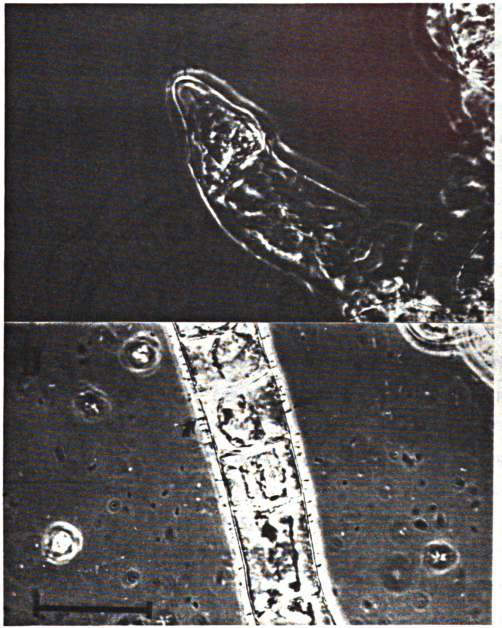


Figure 5

To test whether this interaction between Rhizobium and SB-1 cells leads to penetration into the soybean cell and infection by the bacteria, we carried out histological staining on SB-1 cells in callus culture that had been incubated with Rhizobium for three weeks. Using the Gram stain to reveal the presence of bacteria, we observed staining within the cell wall of certain cells (indicated by the dark arrow in Figure 6a), suggesting bacterial infection of these cells. In addition, there was also staining in the interstitial spaces between cells (indicated by the open arrow in Figure 6a). These observations, particularly the presence of bacteria in areas between adjacent cells, are reminiscent of similar observations on pseudo-infection threads in the establishment of Rhizobium-soybean symbiosis (18). Control sections, derived from cultures without Rhizobium, failed to show bacterial staining (Figure 6b).

We have also stained sections of the Rhizobium-SB-1 callus co-culture with hematoxylin-eosin. These sections showed the positions of the nucleus and cytoplasm of the plant cell instead of the bacteria. In cultures containing Rhizobium, there were focal regions containing many cells stained with the reagent, revealing prominent nuclei (Figure 6c). In contrast, control sections contained many areas that were not stained, most probably because these areas of the cell were filled with vacuoles (Figure 6d). These observations are similar to those reported previously on the in vivo infection of soybean roots by Rhizobium (2), in which the infection process stimulated cell division.

Figure 6. Histological staining of sections derived from callus cultures of SB-1 cells with and without Rhizobium japonicum (R110d). The SB-1 callus was cultured with the bacteria for three weeks, fixed, sectioned, and stained as described in Materials and Methods. (a) SB-1 callus culture plus Rhizobium stained with Gram stain. The black arrows point to infected cells, containing bacteria within the cell wall. The open arrow shows bacteria in the interstitial space between cells, mimicking a pseudo-infection thread. (b) Control SB-1 callus culture without Rhizobium stained with Gram stain. (c) SB-1 callus culture plus Rhizobium stained with hematoxylin-eosin. The black arrow highlights a focal region of proliferative cells. (d) SB-1 callus culture without Rhizobium stained with hematoxylin-eosin. Bar = 10 μm.

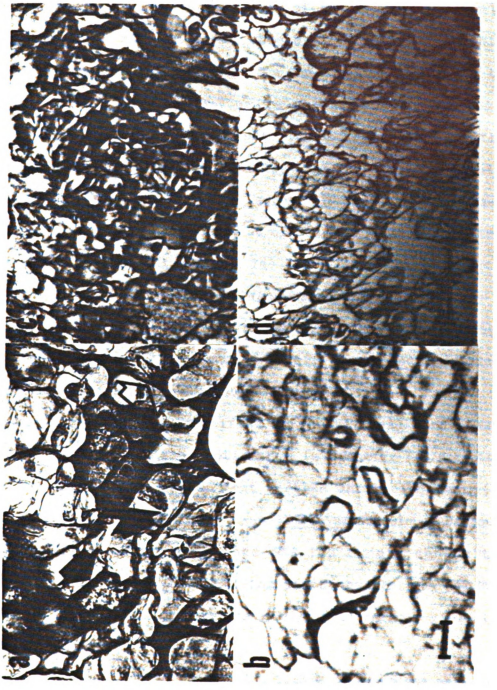


Figure 6

Evidence for Specificity and Role of Lectin in Rhizobium Binding to SB-1 Cells

Several aspects of the specificity of the binding interaction between Rhizobium and SB-1 cells were checked. First, the inclusion of certain saccharides such as Gal during the co-culture inhibited the binding of the Rhizobium to SB-1 cells when the observed polar adherence was assayed at 24 h (Figure 7, a and b). This inhibition was observed at a Gal concentration as low as 3 mM. Similar results were observed with the disaccharide lactose (Table II). In contrast, other saccharide epimers of Gal, such as Glc (0.2 M), failed to yield the same inhibitory effect (Figure 7c). Melibiose, the α anomer of lactose, did not show inhibition. GalNAc was also not inhibitory at the concentration tested (Table II). These results raise the possibility that the adhesion of Rhizobium to SB-1 cells may be mediated via a highly specific carbohydrate recognition system.

Second, since we have identified on the cell wall and plasma membrane of the SB-1 cells a lectin that is specific for galactose residues, it was of interest to test whether antibodies reactive against the cell wall lectin could block Rhizobium adhesion. We found that inclusion of rabbit anti-seed SBA (10 μg/ml) during the co-culture inhibited the polar binding of the bacteria to the SB-1 cells (Figure 7d). Normal rabbit immunoglobulin did not yield the same effect (Figure 7e).

We also wished to test whether any ligand bound to the cell wall of SB-1 cells would block Rhizobium adhesion. To accomplish this, we took advantage of the availability of rabbit anti-cell wall fragments. This immunoglobulin fraction showed immunofluorescence staining of

Figure 7. Representative photographs showing the adhesion of Rhizobium japonicum (R110d) to SB-1 cells after 24 h of co-culture at 26°C in the dark. (a) co-culture; (b) co-culture in the presence of Gal (0.1 M); (c) co-culture in the presence of Glc (0.1 M); (d) co-culture in the presence of rabbit anti-seed SBA (10 µg/ml); (e) co-culture in the presence of normal rabbit immunoglobulin (1 mg/ml); and (f) co-culture in the presence of rabbit anti-cell wall fragments (1 mg/ml). Bar = 10 µm.

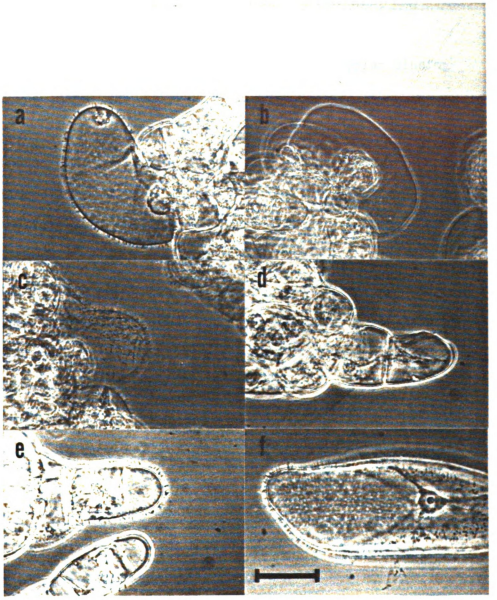


Figure 7

Table II. Saccharide inhibition of Rhizobium japonicum binding to SB-1 cells.

Saccharide*	Inhibition of polar binding to SB-1 Cells
Control	-
Galactose+	+
N-acetyl-galactosamine	-
Lactose+	+
Galacturonic acid+	+
Gluconic acid	-
Mannose	-
Glucose	-
Melibiose	-
Glucuronic acid	-
Xylose	-

* Saccharide concentrations varied from 3 mM to 0.2 M.

+ At a saccharide concentration of 3 mM or above, polar binding of Rhizobium to SB-1 cells was inhibited (see text).

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both intact SB-1 cells and the fraction containing cell wall fragments but it did not yield any positive reaction with seed SBA or SB-1 lectin on immunoblots. More importantly, the binding of this immunoglobulin on the outer surface of SB-1 cells did not inhibit Rhizobium binding (Figure 7f).

These results provide strong evidence for the specificity and the role of the SB-1 lectin in mediating the initial recognition and adhesion between the Rhizobium and SB-1 cells. However, it should be noted that not all of the soybean cells bound Rhizobium. For example, Figure 7a shows one cell with many bacteria bound, but several adjacent cells devoid of any Rhizobium. In addition, we also found that Rhizobium did not bind to protoplasts derived from SB-1 cells after cell wall removal. Therefore, even though the plasma membrane of SB-1 protoplasts contained a lectin reactive with rabbit anti-seed SBA, no binding of Rhizobium was observed.

Correlation Between Rhizobium Binding and Establishment of in vivo Symbiosis

The polar binding of bacteria to the SB-1 cells was also specific in terms of the bacterial cells used in the co-culture (Table III). Rhizobium japonicum bound, but Escherichia coli did not. Moreover, the binding was restricted to Rhizobium japonicum and Rhizobium fredii, two strains of bacteria that normally infect soybean roots to form a nitrogen fixing symbiosis. In contrast, Rhizobium meliloti, Rhizobium trifolii, and Rhizobium leguminosarum did not bind to the SB-1 cells.

Table

Table III. Correlation between bacterial binding and symbiotic infection.

Bacterium	Normal Host	Polar binding to SB-1 cells
E. coli	?	-
R. japonicum R110d	soybean	+
R. fredii PRC 205 str	soybean	+
R. meliloti 102F28	alfalfa	-
R. trifolii 0403	clover	-
R. leguminosarum 128C56	pea	-

DISCUSSION

The data documented in the present study indicate: (a) Incubation of *Rhizobium* with a cultured cell line derived from roots of *Glycine max* (SB-1) results in specific adhesion of the bacteria to the plant cell. (b) This binding interaction appears to be mediated via carbohydrate recognition, since Gal can inhibit the heterotypic adhesion whereas Glc failed to inhibit. (c) One likely candidate that may mediate such an interaction is a lectin identified on the cell wall and plasma membrane of the SB-1 cells. This notion is supported by the observation that rabbit anti-seed SBA blocked the *Rhizobium*- soybean cell adhesion whereas control rabbit immunoglobulin did not.

These results are consistent with the "lectin recognition" hypothesis that suggests carbohydrate recognition as a basis for determining legume host-bacterial symbiont interactions (6,15,20). This hypothesis has been supported by experiments carried out in the soybean system (2,3,13,14,35) and in the clover system (10). There are, however, a number of experiments from various laboratories arguing against the acceptance of the hypothesis that lectins play a specific and indispensable role in legume-*Rhizobium* symbiosis; in the case of soybeans, this viewpoint has been put forth succinctly by Pueppke (29). In light of these circumstances, it is important to

discuss our data on the SB-1-*Rhizobium* interaction with respect to the following key points.

First, we have obtained definitive evidence for the presence of a lectin in the SB-1 cells. This endogenously produced lectin has been purified to apparent homogeneity on the basis of its carbohydrate-binding activity. Immunofluorescence and binding studies carried out with ^{125}I-labeled antibodies indicate that the lectin is found on the cell wall. Treatment of SB-1 cells with the haptens for seed SBA, Gal and GalNAc, failed to remove the soybean lectin from the cell wall. This implies that the lectin may be anchored on the cell wall with its carbohydrate-binding sites unoccupied and therefore is capable of mediating recognition and binding of external ligands (e.g. *Rhizobium*). Therefore, the requirement for the presence of lectin molecule at the proximal point of interaction has been fulfilled.

Second, it should be noted that the mere presence of the lectin is not sufficient for *Rhizobium* binding. Two observations make this point particularly clear. The lectin of SB-1 cells is found on the cell wall of all cells examined by immunofluorescence. Yet, only certain cells out of a given population have *Rhizobium* adsorbed on them after co-culture of the plant cells and bacterium. This may be related to the growth phase of the SB-1 cells in culture or other phenomena associated with transient susceptibility of root cells to be nodulated by *Rhizobium in vivo* (4). In addition, protoplasts also have SB-1 lectin exposed outside the plasma membrane but these protoplasts do not bind *Rhizobium* at all under conditions used to assay the adhesion of the bacteria to SB-1 cells. These results suggest that lectin-carbohydrate interactions may be a necessary but not sufficient

condition for adhesion of the cells. A requirement for dual recognition (involving another set of complementary molecules) has been persuasively demonstrated in the interaction between lymphoid cells and target cells bearing foreign antigens (39).

Third, it is important to realize that lectin-carbohydrate binding need not be the only, or even the main, determinant of specificity in soybean root cell-Rhizobium interactions. The notion of dual recognition, invoking other sets of complementary molecules, is consistent with the less absolute "lectin recognition" hypothesis. In any case, the demonstration of saccharide and antibody specificity in blocking Rhizobium adhesion to SB-1 cells strongly suggest that at least one required component is a carbohydrate-binding protein. In this connection, it should be noted that GalNAc, a known hapten for seed SBA (23), did not inhibit Rhizobium adhesion to SB-1 cells. This may reflect a difference between SB-1 lectin and seed SBA. Alternatively, it may reflect the fact that the lectin anchored on the cell wall does not bind GalNAc.

Because our studies have been carried out in a defined cell culture system, one issue is whether this Rhizobium-SB-1 cell binding is relevant to the in vivo symbiosis. Several phenomenological observations suggest that our system mimics at least the early phase of the process of nodule formation in soybean roots. First, the binding of Rhizobium is polar as had been observed in a number of systems of Rhizobium binding to root cells (7,11,37). Second, there is preliminary evidence, based on histological staining, for the presence of bacteria in the interstitial spaces mimicking a pseudo-infection thread (18). The staining with hematoxylin and eosin

also suggest an increase in the size of the nucleus and possibly cell division (2). These observations at the light microscope level must now be extended to the ultrastructural level to confirm that the Rhizobium initially bound to SB-1 cells actually penetrate and infect the target cells. Finally, correlative studies between Rhizobium binding to SB-1 cells and establishment of in vivo symbiosis indicate the specificities of the Rhizobium strains and their hosts.

There have been several previous reports on the binding of Rhizobium to cultured cells derived from callus of soybean roots (8,16,18,26,27,31,32). In some of these systems, the interaction of Rhizobium with the soybean cells ultimately led to infection of the plant cell and the generation of a nitrogen-fixing symbiosis, as characterized by ultrastructural studies and enzymatic assays. It remains to be demonstrated that our present Rhizobium adhesion to SB-1 cells will lead to a symbiosis and activation of nitrogenase.

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CHAPTER III

ENDOGENOUS LECTIN FROM CULTURED SOYBEAN CELLS:

CHEMICAL CHARACTERIZATION OF THE LECTIN OF SB-1 CELLS

ABSTRACT

A lectin has been identified in the cell line, SB-1, originally derived from the roots of Glycine max. This lectin, which we shall refer to as SB-1 lectin, was isolated on the basis of its carbohydrate-binding activity (affinity chromatography on Sepharose column derivatized with N-caproyl-galactosamine) and its immunological cross-reactivity (immunoblotting with rabbit antibodies directed against seed soybean agglutinin (SBA)). SDS-PAGE and immunoblotting analysis of SB-1 lectin revealed a major polypeptide (M_r = 30,000) which co-migrated with seed SBA. This form of the lectin was observed in fractions purified from culture medium of SB-1 cells or supernatant fraction of SB-1 cell suspension after enzymatic removal of cell wall. Extracts of SB-1 cells under some other conditions yielded a major band (M_r = 60,000) as revealed by SDS-PAGE and immunoblotting with rabbit anti-seed SBA; prolonged incubation of these samples in the presence of SDS resulted in the appearance of the 30 kD polypeptide. It appeared that the 60 kD band represented a highly stable, even under SDS-PAGE conditions, dimeric form of the 30 kD subunit. The SB-1 lectin derived from the culture medium was compared with seed SBA by gel filtration and by peptide mapping after limited proteolysis; no difference between the lectins from the two sources was found. Extracts of soybean roots fractionated on N-caproyl-galactosamine Sepharose affinity columns yielded, upon elution with galactose,

polypeptides of M_r 30,000 and M_r 60,000. These results suggest that soybean roots contain a lectin whose polypeptide composition corresponds to that of seed SBA and SB-1 lectin.

INTRODUCTION

In the previous studies, we have shown that incubation of Rhizobium japonicum with the cultured soybean cell line SB-1, originally derived from the roots of Glycine max, resulted in specific adhesion of the bacteria to the plant cells (1). We had also shown that rabbit antibodies directed against seed soybean agglutinin (SBA) blocked the Rhizobium-soybean cell adhesion, whereas control antibodies did not. These results prompted the hypothesis that one likely candidate that may mediate the recognition between the Rhizobium and the soybean cells is a lectin produced endogenously by the SB-1 cells. Consistent with this hypothesis, fluorescently-labeled rabbit anti-seed SBA yielded specific immunofluorescent staining on the cell wall and plasma membrane of the SB-1 cells and a lectin-like activity could be identified in the cell wall fraction of the same cells.

Because of its implicated role in Rhizobium-soybean cell adhesion, it was of interest to characterize this lectin in some detail. In particular, we wished to determine the relationship of the SB-1 lectin to seed SBA. The present paper documents that the SB-1 lectin is similar, if not identical, to seed SBA on the basis of the following criteria: (a) carbohydrate-binding activity; (b) immunological cross-reactivity; and (c) peptide mapping patterns after limited proteolysis.

MATERIALS AND METHODS

Seed SBA and Anti-Seed SBA Antibodies

Seed SBA was isolated and purified by affinity chromatography on Sepharose column derivatized with N-caproyl-galactosamine (Gal-Sepharose) (2). The details of this procedure, as well as the characterization of the purified lectin, have been documented previously (1). The generation of antibodies directed against seed SBA and the characterization of the specificity of these antibodies have also been described (1).

Seed SBA was labeled with ^{125}I using the chloramine T procedure described by Ho *et al.* (3). Free ^{125}I was removed by passing the labeled material over a column (3 x 0.5 cm) of Dowex AG1x8 (Bio Rad, Richmond, CA). The specific activity of the ^{125}I-labeled seed SBA was approximately 2 x 10^7 cpm/μg. The same procedure was used to label the lectin isolated from SB-1 cells.

Polyacrylamide Gel Electrophoresis and Immunoblotting

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was done as described (4) using 10% and 4% (w/v) acrylamide concentrations in the running and stacking gels, respectively. Following electrophoresis, the proteins were revealed by staining with Coomassie Brilliant Blue, by fluorography of radioactive samples, or by immunoblotting after transfer to nitrocellulose

paper. Fluorography was carried out according to the method of Bonner and Laskey (5), using XAR-5 film (Kodak, Rochester, NY) and an exposure time of three weeks.

For immunoblotting, the proteins were transferred to nitrocellulose paper (Schleicher and Schuell, Keene, NH) by the method of Towbin et al. (6) and the immunoreactive material was revealed either via horseradish peroxidase (HRP) conjugated or alkaline phosphatase (AP) conjugated secondary antibodies. In the former case, the immunoblotted nitrocellulose membrane was incubated with HRP-conjugated goat anti-rabbit immunoglobulin (Bio Rad; 1:2000 dilution), followed by development with 4-chloro-1-napthol and hydrogen peroxide (1). In some experiments, the sensitivity of the detection and stability of immuno-reactive materials were enhanced by the use of AP-conjugated goat anti-rabbit immunoglobulin (Sigma, St. Louis, MO; 1:1000 dilution). The nitrocellulose membrane was then developed for alkaline phosphatase activity following the procedure described by Blake et al. (7) using 5-bromo-4-chloro-3-indolyl phosphate and Nitro Blue Tetrazolium as substrates.

Culture of SB-1 Cells

The SB-1 cell line, derived from soybean roots (Glycine max (L.) Merr. cv. Mandarin) (8), was kindly provided by Dr. G. Lark (Department of Biology, University of Utah, Salt Lake City, UT) and cultured as previously described (1). For experiments on the metabolic labeling of SB-1 cells with $^{35}SO_4^{2-}$, the 1B5C medium was modified as follows: (a) casein hydrolysate was omitted; (b) magnesium sulfate and ammonium sulfate were replaced with magnesium chloride and

ammonium chloride, respectively. Actively growing SB-1 cells were washed and resuspended in the sulfate-free medium described above. $H_2{}^{35}SO_4$ (0.5 mCi/35 ml of medium) was added and the culture was carried out for four days.

Isolation of Lectin Activity from SB-1 Cells

Two protocols were used to identify and isolate lectin activity from SB-1 cells: (a) culture medium, and (b) digestion mixture after enzymatic removal of the cell wall. Each procedure is briefly described below.

(a) Culture medium: Suspension culture of SB-1 cells were grown in 1B5C medium for 3-4 days. The medium was separated from the cells by filtration through a Whatman filter paper. The pooled medium was adjusted to pH 7.4, then fractionated on Gal-Sepharose column (2). The material eluted with 0.2 M Gal was concentrated by Amicon ultrafiltration (PM 10 membrane).

(b) Supernatant fraction after cell wall removal: The cell wall was degraded by a modified procedure of Constabel (9). Actively growing SB-1 cells (4 days old) were washed with fresh 1B5C medium by centrifugation (460 g, 4 min) and resuspension. The pelleted cells were then readjusted to the same volume with fresh 1B5C medium and digested with an equal volume (100 ml) of enzyme solution containing 0.8 g pectinase (Sigma), 1.6 g cellulysin (Calbiochem, CA) and 10.0 g D-Sorbitol (Sigma), pH 5.5. After 2 hours incubation at 37°C, the digestion mixture was centrifuged (10,000 g, 30 min, 4°C). The supernatant was adjusted to pH 7.4 and fractionated on a Gal-Sepharose

column followed by elution with 0.2 M Gal. The eluted material was concentrated by Amicon ultrafiltration and dialyzed against 0.1% SDS.

Isolation of Lectin Activity from Soybean Seedlings

Soybean seeds, variety Williams, were germinated at room temperature in the dark for four days. The primary root tips including root hair regions were excised, frozen in liquid N_2, and lyophilized. Dried sample was then extracted in PBS buffer (10 mM sodium phosphate, 0.14 M NaCl, 4 mM KCl, pH 7.4) (50 mg/ml) containing 5% 2-mercaptoethanol and the clarified supernatant was obtained by centrifugation in a microfuge (Beckman).

Isolation of Polypeptides from SDS-PAGE

Purified seed SBA (8 mg) was subjected to preparative SDS-PAGE. Proteins were revealed in the gel by a short period of Coomassie-blue staining and destaining. The regions corresponding to the 60 kD and 30 kD polypeptides were sliced from the gel and then the proteins were eluted by incubation in 0.1% SDS (0.5 ml/1 cm slice) on a rotary shaker overnight at room temperature. The eluted material was either maintained in the SDS solution (room temperature) or was dialyzed against 50% methanol to remove SDS. In the latter case, methanol was evaporated and the dried sample was dissolved in PBS buffer and stored at 4°C.

Comparative Peptide Map Analysis

Seed SBA and SB-1 lectin were compared after partial proteolysis following the procedure of Cleveland *et al.* (10). The isolated

proteins were subjected to SDS-PAGE in separate gels. Each individual gel was then soaked for 30 min in 15 ml of 125 mM Tris, pH 6.8, 0.1% SDS, and 1 mM EDTA. The gel was next placed horizontally at the top of a new slab of SDS-PAGE (15% acrylamide). Staphylococcus aureus V-8 protease (Miles Laboratories, Elkhart, IN; 250 µl at a concentration of 32 µg/ml) was overlayed on this and the digestion was allowed to continue for 30 min at room temperature. Electrophoresis was then continued subsequently. The protein fragments were revealed by Coomassie Blue staining. This yielded a comparison of the polypeptides migrating in the M_r 30,000 region of the gel as only this region showed detectable dye staining.

To compare the polypeptides migrating in the M_r 60,000 region of the gel, seed SBA and SB-1 lectin were first labeled with ^{125}I (3) as described above. The radioactive samples were subjected to SDS-PAGE and regions of the gels corresponding to polypeptides of M_r 30,000 and M_r 60,000 were excised from the gel using a gel slicer (2 mm per slice). The proteins in the gel slices were eluted with 0.1% SDS as described above. Samples containing ^{125}I-labeled material from the 60 kD region of gels derived from seed SBA and SB-1 lectin were digested with V-8 protease (32 µg/ml) for 30 min at 37°C (10). After digestion, the samples were boiled for 10 min and then subjected to SDS-PAGE (15% acrylamide). The gel was dried and the radioactive polypeptides were revealed by autoradiography.

The gel slices containing ^{125}I-labeled 30 kD polypeptides from seed SBA and SB-1 lectin were used for two-dimensional peptide mapping analysis following the procedure of Elder et al. (11), with slight modifications. The SDS was first removed from the gel slices by three

washes with 50% methanol and then the gel slices were dried by a heat lamp. Each dried slice was treated with 0.25 ml trypsin (1 mg/ml in 50 mM NH_4HCO_3, pH 8) (Millipore Corporation, Freehold, NJ; 240 units/mg) at 37°C overnight. The supernatant fractions were separated from the gel pieces and evaporated to dryness. The dried samples were dissolved in 10 µl of buffer I (acetic acid/formic acid/water, 15:5:80) and 5 to 10 µl (0.2-10 x 10^5 cpm) was spotted onto Avicel TLC plates (Analtech, Inc., Newark, DE). Electrophoresis was carried out at room temperature on a high voltage electrophoresis apparatus (Brinkmann, Westbury, NY) in buffer I at 1 KV for about 30 min. The plates were then dried and the peptides were chromatographed in a second dimension in buffer II (butanol/pyridine/acetic acid/water, 32.5:25:5:20) with 7% (w/v) 2,5-diphenyloxazole. The plates were again dried and exposed on XRP-5 film (Kodak) for one week.

Gel filtration and Radioimmunoassay

Protein samples containing lectin activity were subjected to gel filtration on a column (1.5 x 54 cm) of Sephadex G-200 equilibrated with 10 mM sodium phosphate, 0.1 M NaCl, 0.2 M Gal, pH 7.4 at 4°C. Fractions of 2 ml were collected and were assayed for material reactive with rabbit anti-seed SBA by a solid phase radioimmunoassay.

Microtitre wells (Immulon-2, Dynatech Labs, Alexandria, VA) were coated with 200 µl of rabbit anti-seed SBA (1 µg/ml in 0.1 M $NaHCO_3$, pH 8.3) for 18 h at 4°C. The wells were washed twice with Tris-buffered saline (20 mM Tris, 500 mM NaCl, pH 7.5) containing 0.05% Tween 20. Samples (100 µl) were then added to the wells, followed by ^{125}I-labeled seed SBA (1 x 10^5 cpm) and incubated overnight at 4°C.

The wells were washed three times with Tris-buffered saline to remove unbound radioactivity, and the amount of radioactivity bound in each well was determined in a gamma counter. The presence of unlabeled seed SBA in the sample results in a decreased binding of [^{125}I]seed SBA to the well. Immunoreactive SB-1 lectin could then be quantitated by the ability to compete with the labeled SBA for binding by the solid phase immunoglobulin. Seed SBA was used as a standard.

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RESULTS

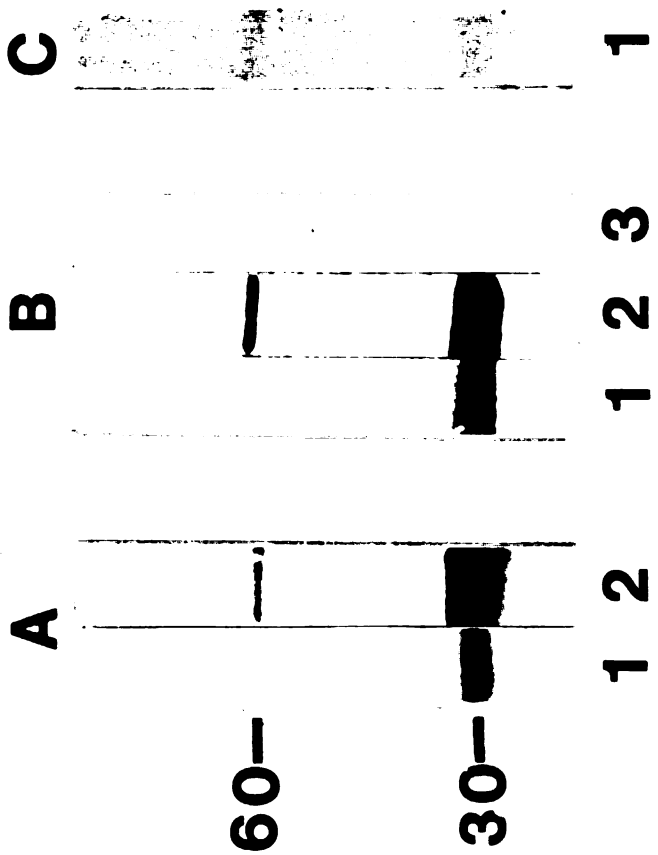
Isolation of a Lectin from SB-1 Cells

In previous studies (1), we had demonstrated the specificity of a rabbit antiserum directed against seed SBA. Using this highly specific antibody, we had shown, by immunofluorescence and by radioactive binding studies, the presence of a molecule cross-reactive with rabbit anti-seed SBA on the cell wall and plasma membrane of the cultured soybean cell line, SB-1. Moreover, a lectin-like activity could be identified in the supernatant fraction after SB-1 cells were digested with cellulysin and pectinase to remove cell wall material. In order to obtain sufficient material for more extensive characterization, we surveyed a number of conditions to identify a source readily amenable to our purification procedures. We found that culture medium which had been exposed to SB-1 cells contained immunoreactive material.

The culture medium of SB-1 cells (72-hour collection) was subjected to affinity chromatography on Gal-Sepharose columns. The material bound by the affinity column was eluted by Gal (0.2M). Upon SDS-PAGE analysis, this material yielded a single band (M_r ~ 30,000) (Figure 1B, lane 1), corresponding to that obtained with the 30 kD polypeptide of seed SBA (Figure 1A, lane 1).

We have also cultured SB-1 cells in the presence of $^{35}SO_4^{2-}$ for 96 h to label the cellular components. The culture medium was then

Figure 1. SDS-PAGE analysis of seed SBA and SB-1 lectin purified by affinity chromatography on Gal-Sepharose column. (A) Purified seed SBA (~ 10 μg of sample were electrophoresed in each lane): Lane 1, Coomassie Blue staining; lane 2, immunoblotting with rabbit anti-seed SBA and horseradish peroxidase-goat anti-rabbit immunoglobulin. (B) SB-1 lectin: Lane 1, Coomassie Blue staining; lane 2, immunoblotting with rabbit anti-seed SBA and horseradish peroxidase-goat anti-rabbit immunoglobulin (~ 50 μg of sample were electrophoresed in these lanes); lane 3, fluorogram of purified SB-1 lectin (medium) derived from the medium of ^{35}S-labeled SB-1 cells. Approximately 2000 cpm (0.1 μg protein) were electrophoresed and the fluorogram was exposed for three weeks. (C) Extract of soybean root (~ 130 μg of total protein was loaded in this lane) immunoblotted with rabbit anti-seed SBA and alkaline phosphatase-goat anti-rabbit immunoglobulin. The numbers on the left indicate the positions of migration of polypeptides of M_r 30,000 and 60,000, relative to known molecular weight markers.



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collected and fractionated by affinity chromatography. The material bound by the Gal-Sepharose column was subjected to SDS-PAGE and fluorography. The results showed one predominant radioactive polypeptide (M_r ~ 30,000; Figure 1B, lane 3). All of these data provide strong evidence that SB-1 cells synthesize a lectin with a subunit molecular weight and carbohydrate-binding capacity similar to seed SBA. We shall hereafter designate the affinity purified material as SB-1 lectin.

When purified seed SBA was subjected to immunoblotting analysis with rabbit anti-seed SBA, one major polypeptide band (M_r ~ 30,000) and one minor band (M_r ~ 60,000) were observed (Figure 1A, lane 2). The position of migration of the predominant band at 30 kD corresponded to the subunit molecular weight of seed SBA (M_r ~ 30,000) (12) and will be hereafter designated seed SBA (30 kD). The material corresponding to the minor band (M_r ~ 60,000), which accounted for about 1% of the total protein, will be designated seed SBA (60 kD).

Immunoblotting analysis of SB-1 lectin with rabbit anti-seed SBA yielded one predominant band (M_r 30,000) and one minor band (M_r 60,000) (Figure 1B, lane 2); this pattern was identical to that seen on immunoblotting analysis of purified seed SBA (Figure 1A, lane 2). For consistency of nomenclature, the material corresponding to the M_r 30,000 and M_r 60,000 region of the gel will be designated SB-1 lectin (30 kD) and SB-1 lectin (60 kD), respectively.

Comparative Peptide Map Analysis of Seed SBA and SB-1 Lectin

Seed SBA and SB-1 lectin were subjected to SDS-gel electrophoresis. The individual lanes for the seed SBA and SB-1 lectin were

overlaid on a second polyacrylamide gel (15% acrylamide). The proteins separated in the original gels were subjected to limited proteolysis with V-8 protease and then electrophoresed and stained with Coomassie Brilliant Blue. This yielded a comparison of the peptide maps of seed SBA (30 kD) and SB-1 lectin (30 kD) since only this region of the gel showed detectable dye staining. The results showed that, in addition to residual undigested material (band e, M_r ~ 30,000, Figure 2A, lanes 1 and 2), both seed SBA (30 kD) and SB-1 lectin (30 kD) yielded four comparable fragments (bands a-d, M_rs 10,500 to 15,000, Figure 2A, lanes 1 and 2).

There was too little material in the M_r 60,000 region of the gel to allow a comparison of seed SBA (60 kD) and SB-1 lectin (60 kD) by the above procedure. To increase the sensitivity of detection, seed SBA and SB-1 lectin were first radiolabeled with ^{125}I. The radioactive samples were subjected to SDS-PAGE and ^{125}I-labeled SBA (60 kD) and SB-1 lectin (60 kD) were extracted from the gel. After V-8 protease digestion and SDS-PAGE (on a 15% acrylamide gel), autoradiographic analysis revealed essentially identical patterns for seed SBA (60 kD) and SB-1 lectin (60 kD) (Figure 2B, lanes 1 and 2). Both samples yielded four peptide fragments below the M_r 30,000 region (bands a-d, Figure 2B, lanes 1 and 2), similar to those fragments generated from the 30 kD material (bands a-d, Figure 2A, lanes 1 and 2). Both seed SBA (60 kD) and SB-1 lectin (60 kD) samples also yielded a M_r 30,000 band (band e, Figure 2B, lanes 1 and 2), which most probably corresponded to the undigested 30 kD polypeptide (band e in Figure 2A, lanes 1 and 2). Finally, both samples yielded a band migrating in the M_r 48,000 region (band f, Figure 2B, lanes 1 and 2).

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Figure 2. (A) Comparative peptide map analysis of purified seed SBA and SB-1 lectin after limited V-8 protease hydrolysis and SDS-PAGE. Approximately 30 µg of protein were electrophoresed in a 10% acrylamide gel; the 30 kD region was then excised and subjected to the protease V-8 (8 µg) and electrophoresed in a 15% acrylamide gel. The polypeptides were revealed by Coomassie Blue staining. (B) Peptide map analysis of radioiodinated peptides of 60 kD isolated from seed SBA (lane 1) and SB-1 lectin (lane 2). The labeled peptides (10,000 cpm) were subjected to limited V-8 protease (32 µg/ml) hydrolysis in a test tube; the digestion mixtures were electrophoresed in a 15% acrylamide gel and revealed by autoradiography. The numbers indicate the molecular weights of the peptide fragments. The individual peptides were assigned with letters from a to g.

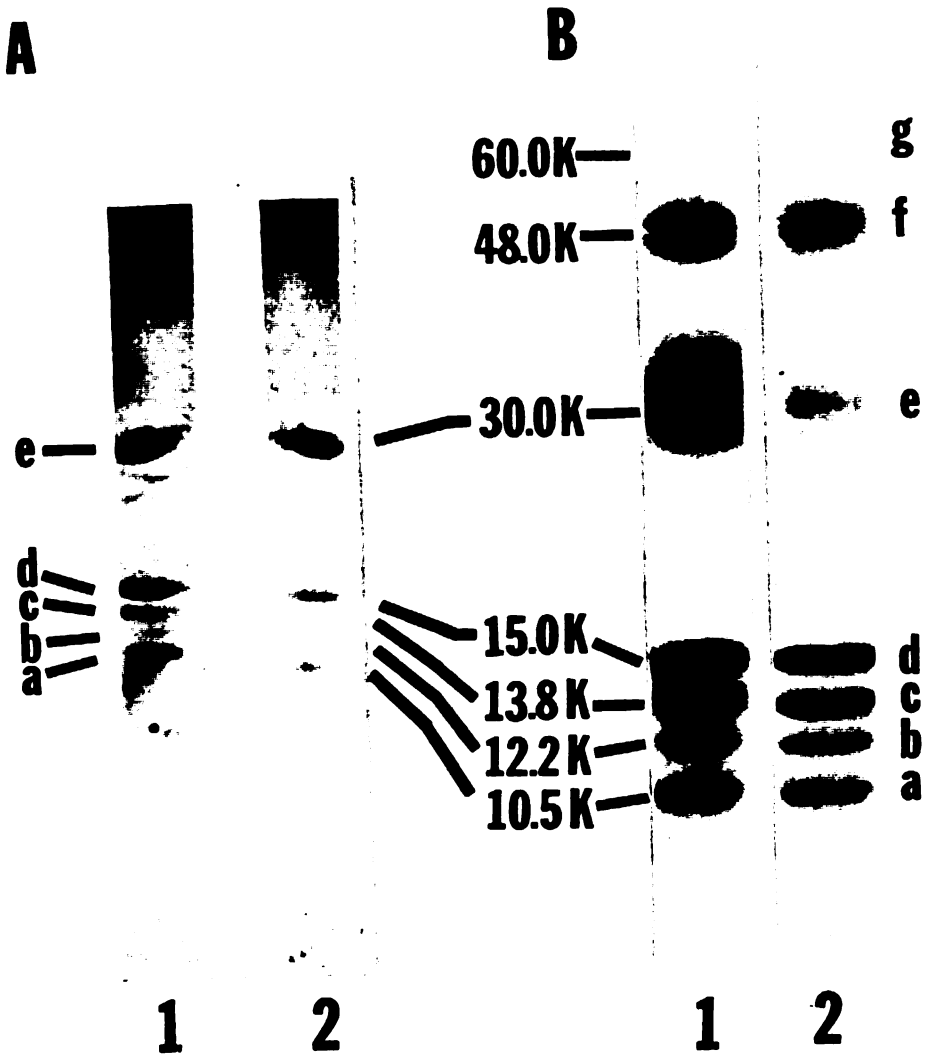

Figure 2

These results indicate that, at least at the one-dimensional peptide mapping level, there is no detectable difference in the polypeptides of seed SBA and SB-1 lectin.

Seed SBA (30 kD) and SB-1 lectin (30 kD) were further compared by two-dimensional peptide mapping after tryptic hydrolysis. ^{125}I-labeled seed SBA and SB-1 lectin were subjected to SDS-PAGE. Radioactive seed SBA (30 kD) and SB-1 (30 kD) were extracted from the gel and exhaustively digested with trypsin. The tryptic peptides were separated on thin layer chromatography plates by electrophoresis in the first dimension and chromatography in the second dimension. The separated radioactive peptides were detected by autoradiography (Figure 3, A and B). A detailed analysis of the films of the peptide maps, after long and short autoradiographic exposures, indicated that the number and position of radioactive spots produced from both samples were identical. These results strongly suggest that seed SBA (30 kD) and SB-1 lectin (30 kD) were, in fact, identical.

Interconversion of the 30 kD and 60 kD Forms of the Lectin

In previous studies (1), we had shown that rabbit anti-seed SBA antibodies, affinity purified on the basis of binding to seed SBA (30 kD), also recognized seed SBA (60 kD). In the present study, limited digestion of seed SBA (60 kD) with V-8 protease yielded peptide fragments similar to those derived from similar treatment of seed SBA (30 kD) (lane 1 in Figure 2A and 2B). Together, these data strongly suggested that seed SBA (60 kD) was a dimeric form of seed SBA (30 kD). The question is now raised as to whether this represents a

Figure 3. Two-dimensional peptide map of radioiodinated 30 kD polypeptides from seed SBA and SB-1 lectin. Radioiodinated proteins were separated in a 10% SDS-PAGE and the 30 kD region was sliced from the gel. After removal of SDS from each gel slice by 50% methanol, the polypeptides in the gel slice were digested with trypsin (0.25 ml, 1 mg/ml). The digestion mixtures (~ 10^5 cpm) were subjected to separation by electrophoresis in the first dimension and chromatography in the second dimension as described in Experimental Procedures. The radioactive peptides were visualized by autoradiography. (A) seed SBA, (B) SB-1 lectin.

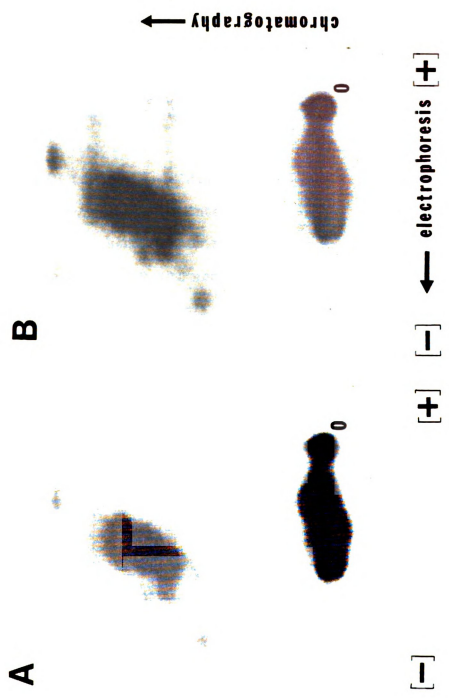

Figure 3

covalent dimerization or non-covalent association of the 30 kD subunit.

The frequent occurrence of the 60 kD material, even under strong denaturing conditions such as heating in buffers containing SDS, β-mercaptoethanol, and urea, suggested a possible covalent dimerization. The fact that V-8 digestion products of seed SBA (60 kD) yielded a band with a M_r - 48,000 (band f, Figure 2B, lane 1) is consistent with the covalent dimer hypothesis. In more detailed analysis, however, we observed interconversion of the two forms of the lectin. For example, seed SBA was subjected to SDS-PAGE and seed SBA (30 kD) and seed SBA (60 kD) were separately isolated from their corresponding regions of the gel. When seed SBA (30 kD) was maintained in 0.1% SDS (24 h at room temperature) and subjected to re-electrophoresis, immunoblotting analysis revealed only a single band (M_r 30,000) (Figure 4, lane 2). In contrast, when seed SBA (30 kD) was incubated in PBS (4-5 days at 4°C), re-electrophoresis and immunoblotting yielded bands at both M_r 30,000 and 60,000 (Figure 4, lane 1).

Conversely, seed SBA (60 kD) yielded predominantly a M_r 60,000 band after incubation in PBS (4-5 days at room temperature) followed by SDS-PAGE and immunoblotting (Figure 4, lane 3). After prolonged incubation in 0.1% SDS (4-5 days at room temperature), however, there was a significant amount of conversion into the M_r 30,000 polypeptide (Figure 4, lane 4). These results indicate that seed SBA (60 kD) most probably represent non-covalently associated dimers of the 30 kD subunit.

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Figure 4. Interconversion of 60 and 30 kD polypeptides from seed SBA and purified SB-1 lectin analyzed by SDS-PAGE and immunoblotting. The samples were electrophoresed, transferred to nitrocellulose paper, and immunoblotted with rabbit anti-seed SBA and alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin. Lane 1, seed SBA (30 kD) (= 3 μg) stored in PBS at 4°C. Lane 2, seed SBA (30 kD) (= 3 μg) incubated in 0.1% SDS at room temperature. Lane 3, seed SBA (60 kD) (= 0.5 μg) stored in PBS at 4°C. Lane 4, seed SBA (60 kD) (= 2 μg) incubated in 0.1% SDS at room temperature. Lane 5, SB-1 lectin (100 ng) derived from the cell wall of SB-1 cells purified by affinity chromatography on Gal-Sepharose column and stored in PBS. Lane 6, the same material as in lane 5 (100 ng) after prolonged incubation in 0.1% SDS at room temperature. The numbers on the left indicate the positions of migration of polypeptides of M_r 30,000 and 60,000 relative to known molecular weight markers.

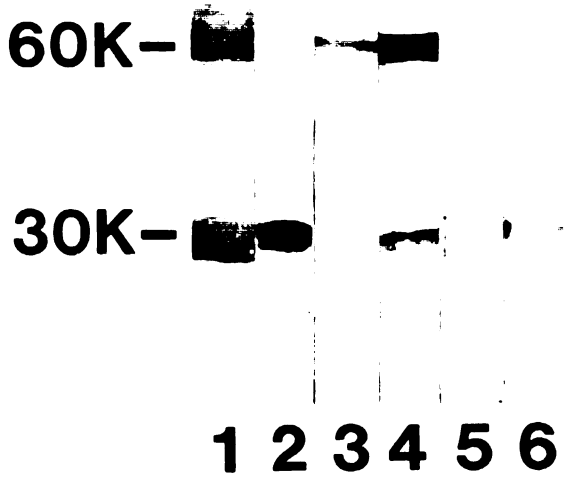

Figure 4

In a similar fashion, SB-1 lectin also appears to exhibit non-covalent association of its subunits, even under denaturing conditions of SDS-PAGE. SB-1 lectin stored in PBS yielded predominantly a M_r 60,000 band upon subsequent SDS-PAGE and immunoblotting (Figure 4, lane 5). The same preparation of SB-1 lectin, after prolonged incubation (4-5 days at room temperature) in 0.1% SDS, yielded a M_r 30,000 polypeptide (Figure 4, lane 6). SB-1 lectin isolated from the medium, from the supernatant fraction after cell wall digestion, and from detergent extracts of SB-1 cells all yielded similar results.

Characterization of SB-1 Lectin

When purified SB-1 lectin was subjected to gel filtration on Sephadex G-200 equilibrated with 10 mM phosphate buffer containing 0.1 M NaCl and 0.2 M Gal, a single component was observed using a radioimmunoassay for seed SBA. The position of migration of the component corresponded to the tetrameric form of seed SBA (M_r ~ 110,000) observed previously under non-denaturing conditions (12). These results suggest that SB-1 lectin also forms tetrameric structures of the basic subunit.

In order to probe the saccharide-binding specificity of SB-1 lectin, various sugars were tested for their capacity to elute the lectin bound in the Gal-Sepharose affinity column. When the column was developed sequentially with mannose, glucose, and GalNAc, the lectin was observed only upon the addition of GalNAc (Figure 5B, lane 1-3), as revealed by SDS-PAGE and immunoblotting analysis. After the elution with GalNAc, no additional lectin was eluted upon

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Figure 5. SDS-PAGE and immunoblotting analysis of seed SBA and SB-1 lectin obtained from Gal-Sepharose column upon sequential elution with various saccharides. Samples were electrophoresed, transferred to nitrocellulose paper, and immunoblotted with rabbit anti-seed SBA and alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin. (A) Seed SBA: lane 1, material eluted with 0.2 M mannose; lane 2, material eluted with 0.2 M glucose; lane 3, material eluted with 0.2 M GalNAc; lane 4, material eluted with 0.2 M Gal. Equal volume from each sample was loaded in the gel. (B) SB-1 lectin derived from the cell wall of SB-1 cells (- 15 g) purified on Gal-Sepharose column: Lane 1, material eluted with 0.2 M mannose; lane 2, material eluted with 0.2 M glucose; lane 3, material eluted with 0.2 M GalNAc; lane 4, material eluted with 0.2 M Gal. The numbers on the left indicate the positions of migration of polypeptides relative to known molecular weight markers.

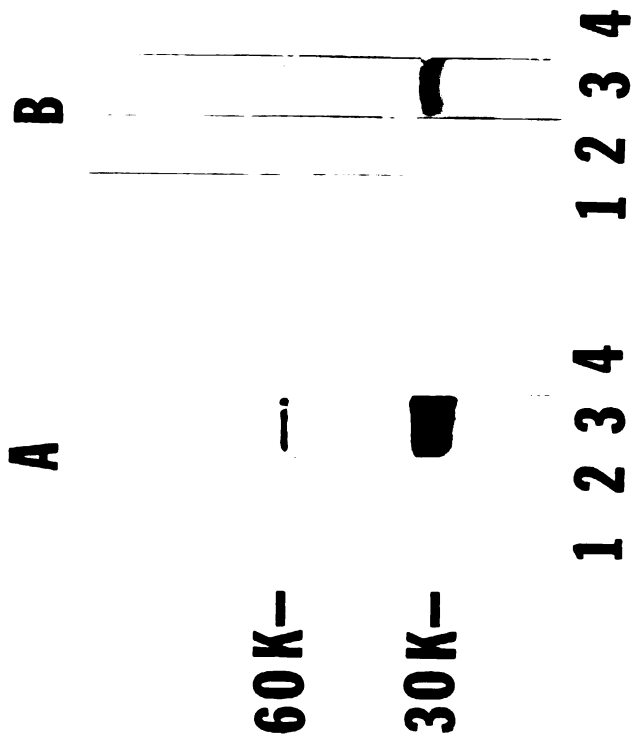

Figure 5

further development of the column with Gal (Figure 5B, lane 4). Inasmuch as Gal was used in the original isolation of SB-1 lectin, these results suggest that the material eluted with GalNAc was the same as that eluted with Gal.

More importantly, the results of using different saccharides to elute the lectin bound on the Gal-Sepharose column were identical to those obtained when seed SBA was subjected to parallel analysis (Figure 5A, lanes 1-4). These results indicate, therefore, that the carbohydrate-binding specificity of seed SBA and SB-1 lectin were the same.

Identification of the Lectin in Soybean Roots

Although the SB-1 cell line was derived originally from roots, the expression of the lectin molecule that appears to be identical to seed SBA may only reflect the dedifferentiated state of the cell rather than the state of true lectin expression in root tissues. Therefore, we have carried out a parallel analysis for the presence of the lectin in soybean roots. Homogenates of root tissue were subjected to SDS-PAGE and immunoblotting analysis with rabbit anti-seed SBA. The immunoblots revealed two bands (M_r 30,000 and 60,000), which co-migrated with seed SBA (30 kD) and seed SBA (60 kD), respectively (Figure 1C). In contrast, preimmune serum failed to blot either the 30 kD or the 60 kD bands in root extracts. These results suggest that root tissues contain a lectin whose polypeptide composition corresponds to that of seed SBA and SB-1 lectin. This conclusion was supported by affinity chromatography studies. Root extracts fractionated on Gal-Sepharose columns yielded, upon

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specific elution with Gal, polypeptides of M_r 30,000 and 60,000 corresponding to seed SBA (30 kD) and seed SBA (60 kD).

DISCUSSION

Soybean agglutinin is a well-characterized glycoprotein lectin from seeds of the cultivated soybean, Glycine max (L.) Merr. The lectin (M_r = 120,000) is a tetramer with identical subunits (M_r = 30,000). It displays carbohydrate-binding specificity for GalNAc and, to a lesser extent, for Gal. The concentration of SBA in the seed can be quite substantial (13-15), but the distribution and origin of the lectin in other organs and tissues, particularly the roots, is a subject of much controversy (15,16).

There are several reports that crude extracts of soybean roots contain hemagglutinating activity (14,17,18); such activity is insufficient reason, however, to conclude that the roots contain SBA. Gade et al. (19) isolated and characterized a lectin from Chippewa soybean roots. This lectin was shown to be similar to seed SBA in terms of structural characteristics, immunological reactivity, and carbohydrate-binding specificity. Although there was some question concerning the original source of the root lectin, Gade et al. (20) have provided additional evidence that the root lectin is endogenous to the tissue of analysis. These results are consistent with observations that a soybean lectin can be found in root exudates in vivo (21), that callus cultures derived from soybean roots express SBA-like material on the cell surface (22) and callus cultures enriched in root hair cells elaborate appreciable amounts of soybean

lectin (23). Attempts to detect SBA on the surfaces of soybean roots and root hairs by immunochemical techniques have yielded conflicting results; some groups have found cross-reactive polypeptide(s) (24-26), while other have failed to detect it or any immunologically reactive molecule (13,16).

The results of our present experiments document the properties of a lectin produced endogenously by the cultured SB-1 cell line, which was originally derived from soybean roots (8). We had shown previously that fluorescently labeled rabbit anti-seed SBA yielded specific immunofluorescent staining on the cell wall and plasma membrane of the SB-1 cells, implicating the presence of a SBA-like molecule (1). We had also shown that the same rabbit anti-seed SBA can block the specific adhesion of Rhizobium japonicum to the cultured SB-1 cells. It was important, therefore, to characterize the structure and activities of the SB-1 lectin, one likely candidate that may mediate the recognition between Rhizobium and the SB-1 cells.

Because lectins in soybeans belong to multi-gene families (27), it was particularly important to establish the relationship between SB-1 lectin and seed SBA, whose gene structure and expression has been analyzed in detail (27,28). SB-1 lectin appears to be identical to seed SBA on the basis of the following criteria: (a) peptide mapping of the polypeptide subunit (M_r 30,000); (b) subunit structure and oligomerization; (c) carbohydrate-binding specificity; and (d) immunological cross-reactivity.

In the course of our studies, we found that both seed SBA and SB-1 lectin yielded two bands (M_r's 30,000 and 60,000) in immunoblotting experiments after SDS-PAGE. More strikingly, the M_r 30,000 and M_r 60,000 bands were interconvertible. The conditions of incubation were important in determining the extent of interconversion. Thus, seed SBA (60 kD) maintained in PBS yielded predominantly a M_r 60,000 band upon SDS-PAGE and immunoblotting. In contrast, the same sample of seed SBA (60 kD), after prolonged incubation in SDS, yielded both M_r 30,000 and M_r 60,000 bands. These results suggest that seed SBA (60 kD) represents a dimer consisting of the non-covalently associated M_r 30,000 subunits. Aggregation and dimerization of seed SBA has also been observed previously (29). Similar results were obtained for SB-1 lectin. The stability of the 60 kD dimer, even under strong denaturing conditions such as heating in buffers containing SDS, β-mercaptoethanol, and urea, was surprising.

These observations on the SB-1 lectin of a cultured soybean cell line and seed SBA need to be put in perspective in the context of other studies on comparing lectins from seeds and roots. In Pisum sativum, the root lectin yielded polypeptides that had similarities (molecular weights and immunological reactivity) and differences (isoelectric points, saccharide specificity, and hemagglutination activity) with the seed lectin (30). A similar conclusion was obtained when lectins of Phaseolus vulgaris were compared for seed and non-seed tissues including roots (31). By contrast, two distinct lectins in terms of polypeptide composition, native molecular weight, carbohydrate-binding specificity, and

reactivity with antibodies were isolated from the roots and seeds of Lotononis bainesii (32). There was no evidence of any relatedness between the lectins from the two different organs/tissues of the same plant. Finally, Etzler and co-workers (33-35) have carried out an extensive analysis of the lectins derived from the Dolichos biflorus at the molecular, subcellular, and systemic levels. This plant contains at least two lectins which are structurally related. Callus cultures from the epicotyl and leaves, hypocotyl, and roots showed no seed lectin; in contrast, lectins that were isolated from these cultures had molecular properties similar to those of the lectin isolated originally from stems and leaves (36).

In the soybean system, a lectin has been isolated from roots on the basis of its carbohydrate-binding activity and has been shown to be similar to the well-characterized seed SBA (19,20). More recently, Vodkin and Raikhel (26) have demonstrated that a protein (M_r 33,000), reactive with antibodies directed against seed SBA, can be found in the roots of four soybean varieties, regardless of whether SBA is present (Le^+) or absent (Le^-) in the seed. The 30 kD seed SBA subunit, however, was not detectable in the roots. These results should be contrasted with our present studies, in which we do detect polypeptides corresponding to seed SBA (30 kD) and seed SBA (60 kD) in extracts of roots. In this connection, it should be noted that in our SDS-PAGE analysis of root extracts, the M_r 33,000 region represented a section of the gel that contained large amounts of protein (detected by silver or Coomassie Blue staining). Under these conditions, a 33 kD band can be observed in certain immunoblots, including those with preimmune serum. This calls for caution

in interpreting the significance of the M_r 33,000 band in immunoblots, as it may represent non-specific detection of a polypeptide in root extracts. In any case, it does not appear that the M_r 33,000 polypeptide in soybean roots represents a gene product of the Le 1 gene, which encodes the M_r 30,000 seed SBA subunit (37). The relationship of the M_r 33,000 polypeptide with the M_r 30,000 seed SBA subunit remains, therefore, to be established.

In our previous studies (1), we had noted that most saccharides known to bind to seed SBA also inhibited the binding of Rhizobium japonicum to SB-1 cells. The lone exception was GalNAc, which bound to seed SBA but did not inhibit Rhizobium-SB-1 cell adhesion. One possible explanation was that SB-1 lectin was different from seed SBA, despite their immunological cross-reactivity. The results of our present paper indicate, however, that the lectins from the two sources were the same, even in terms of their carbohydrate-binding specificity. In light of these observations, we are now forced to face alternative hypotheses to account for the failure of GalNAc to inhibit Rhizobium adhesion to SB-1 cells: (a) lectin anchored on the cell wall may bind saccharides with different specificity than solubilized lectin; (b) the saccharide specificity in inhibition of Rhizobium-SB-1 cell binding may reflect additional recognition components which may exhibit Gal-binding characteristics. The latter notion of dual recognition, involving other sets of complementary molecules, would be greatly advanced if a protein molecule mediating such interactions could be identified, isolated, and characterized.

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Chapter IV

ENDOGENOUS LECTIN FROM CULTURED SOYBEAN CELLS: SB-1 LECTIN ON THE CELL WALL

ABSTRACT

Digestion of seed soybean agglutinin (SBA) with V-8 protease yielded seven distinct peptides (M_r 10,000 - 20,000) that were well-resolved by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Each individual peptide (F_1 through F_7) was isolated; determination of the amino acid sequence at the NH_2-terminal portion of each peptide established its position in the sequence of SBA. The isolated peptides were used as affinity adsorbents to obtain anti-peptide antibodies (anti-F_1 through anti-F_7). These anti-peptide antibodies were used in a comparative study to explore: a) potential conformational differences between SBA in solution and the structurally identical soybean cell lectin SB-1 attached to the cell wall surface, and b) examine the polypeptide presentation of cell surface SB-1 lectin to the environment. Results of fluorescence analysis of whole cell labeling and affinity chromatography methods suggest that the peptide sites available for antibody binding in non-denatured SBA are exposed and demonstrate similar antibody reactivity for cell surface anchored SB-1 lectin. In addition, the pattern of anti-peptide reactivity demonstrated for SB-1 lectin in the cell wall suggest that the C-terminal half of the molecule is involved in cell surface anchoring.

INTRODUCTION

The cultured soybean cell line, SB-1, synthesizes a lectin designated the SB-1 lectin (9). In previous studies (11), we demonstrated that SB-1 lectin was identical to seed soybean agglutinin (SBA) in terms of: (a) molecular weight of the polypeptide (M_r 30,000), determined by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE); (b) molecular weight of the native protein (M_r 120,000 tetramer); (c) carbohydrate-binding specificity; and (d) immunological reactivity with a rabbit antiserum raised against seed SBA. This lectin was detected on the cell wall of intact SB-1 cells by the binding of fluorescently-labeled or radioactively-labeled rabbit anti-seed SBA. Moreover, quantitative studies showed that the amount of SB-1 lectin detectable at the cell surface was not affected by the addition of specific saccharide ligands (9). These data indicated that the molecule was not anchored on the cell surface via its carbohydrate binding properties, but by some other mechanism.

A question raised at this point was one of integration and presentation of the SB-1 polypeptides on the cell surface and, in particular, what regions of the polypeptide chains serve to hook the lectin to the cell wall. One approach to this problem is to probe the cell surface with SBA sequence specific antibodies and examine extents of reactivity. Since, in the course or our previous studies (11), we had observed that V-8 protease digestion of SBA and SB-1 lectin

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resulted in a number of peptides (M_r 10,000 - 20,000), we initiated an effort to prepare anti-peptide antibodies purified by immunoaffinity from polyclonal anti-denatured SBA antibodies. These antibodies were used to examine the exposed sequences of SB-1 lectin on soybean cells.

MATERIALS AND METHODS

Preparation of Seed SBA and Anti-SBA Antibodies

Seed SBA was isolated and purified by the procedure of Allen and Neuberger (1) on galactosamine-Sepharose affinity column. The details of this procedure, as well as the characterization of the purified lectin, have been reported previously (9).

Seed SBA was first denatured by boiling in 0.1% SDS for 5 min. Antiserum to the denatured SBA were raised in rabbits (New Zealand, white, female). The primary injection consisted of 0.2 mg of protein in Freund's complete adjuvant (Gibco Laboratories). Booster injections of 0.2 mg denatured protein in Freund's incomplete adjuvant were administered at weekly intervals. Antiserum was collected three days after the last booster, and the immunoglobulin fraction was purified by affinity chromatography on a protein A-Sepharose (Pharmacia) column. This is designated as anti-seed SBA.

Polyacrylamide Gel Electrophoresis and Immunoblotting

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed by the method of Laemmli (10) using 12.5 and 4% (w/v) acrylamide in the running and stacking gels, respectively. Following electrophoresis, the proteins were revealed either by staining with Coomassie brilliant blue, with the silver reagent (6,17), or by immunoblotting. For immunoblotting, the

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proteins were electrotransferred to nitrocellulose paper (Schleicher and Schuell) by the method of Towbin et al. (15). The immunoreactive material on nitrocellulose membrane were revealed via alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin (Sigma, 1 to 1000 dilution) following the procedure described by Blake et al. (3) using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Sigma) as substrates.

Enzymatic Digestion of Seed SBA

Purified seed SBA was digested with Staphylococcus aureus V-8 protease (Miles Laboratories) as described by Cleveland et al. (4). The digestion was carried out with 32 µg/ml enzyme for 2 hr and then reaction was terminated. The digests were kept at -80°C.

Peptides generated via V-8 hydrolysis were separated by SDS-PAGE. The gel was sliced into 1-2 mm sections, which were then rinsed for 10 min with water. The peptides were eluted from the gel slices by soaking in 0.05 M Tris-HCl, pH 7.9, containing 0.1% SDS, 0.1 mM EDTA, 0.15 M NaCl, 5.0 mM dithiothreitol (0.4 ml/slice) for at least 4-6 h at 25°C as described by Hager and Burgess (8). Proteins were precipitated by acetone. Four volumes of cold acetone (-20°C) were added to one volume of the gel eluate in a silanized 30 ml Corex tube and allowed to precipitate for 30 min in a dry ice-methanol bath. The samples were next centrifuged for 10 min (9,000 x g, 4°C). This procedure, almost completely removed Coomassie blue, as well as SDS. The supernatant was discarded and the tubes were tilted to allow the evaporation of residual acetone and water. Dried samples were resuspended in 0.1 ml of 0.05% SDS and sonicated for 2 min. These

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samples were analyzed by re-electrophoresis and by amino acid sequence determination.

Amino acid sequence analyses were performed on a Beckman Instrument 890M (Michigan State University, Macromolecular Structure Facility) by the Edman degradation method (5). Phenylthiohydantoin amino acids were identified by high pressure liquid chromatography (13).

Preparation of Anti-Peptide Antibodies by Specific Adsorption to Polypeptides Immobilized on Nitrocellulose Membrane

To prepare anti-peptide antibodies, seed SBA was digested with V-8 protease and then subjected to SDS-PAGE to separate the resulting peptides. They were then electrotransferred to nitrocellulose paper. The transferred proteins on nitrocellulose were stained with amido black for 2 minutes. This revealed the exact position of each peptide. Nitrocellulose strips containing individual peptides were carefully cut out and incubated for 4 h with Tris-buffered saline (20 mM Tris, 500 mM NaCl, pH 7.5) containing 0.05% Tween-20 and 5% bovine serum albumin (BSA) in order to block nonspecific sites. This procedure also removed most of the amido black stain from the membrane. Each of the peptide-containing nitrocellulose strips was then incubated overnight with 1.0 ml of Tris buffered saline containing anti-seed SBA (0.5 mg/ml) overnight. Unbound antibodies were removed by three washes with Tris buffered saline containing 0.05% Tween-20. The bound antibodies were then eluted from each peptide containing nitrocellulose by the method described by Smith and Fisher (14), with the modification that only 5 mM glycine, pH 2.3 was used for elution. The

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eluted antibody preparations were immediately neutralized by addition of 2 M Tris-base, pH 7.5, and dialyzed against water extensively. These samples were then lyophilized and redissolved in 50 µl of phosphate buffered saline, pH 7.4.

Culture and Immunolabeling of SB-1 Cells

The SB-1 cell line, derived from soybean roots (Glycine max (L.) Merr. cv. Mandarin) (7) was kindly provided by Dr. Lark (Department of Biology, University of Utah, Salt Lake City, UT) and cultured as described previously (9).

Labeling of SB-1 cells with various anti-peptide antibodies was performed using the assay described previously (9). SB-1 cells (1 x 10^6/ml) were washed three times by centrifugation (460 g, 4 min) and resuspension in Tris buffer (10 mM CaCl and 10 mM Tris-HCl, pH 5.5). The pellet was then resuspended in the Tris buffer, pH 7.2, containing 0.3% BSA. The cells were incubated with the primary antibody preparation (50 µg/ml) on a rotary shaker for 2 h at 4°C and then washed three times in Tris buffer with 0.3% BSA, pH. 7.2. Fluorescein-conjugated goat anti-rabbit in Tris-BSA buffer (1:100 dilution; Gibco) was added to the cells and incubated for 30 min at 4°C on a rotary shaker. The cells were washed three times in the same Tris buffer containing 0.3% BSA and the fluorescence staining observed under a Leitz fluorescence microscope, equipped with a Leitz KP 490 dichoric mirror. Micrographs were taken with Kodak Tri-X film, which was pushed to ASA 3200.

RESULTS

Digestion of Seed SBA by V-8 Protease

In a previous study (9), we had reported the presence of a molecule, designated as the SB-1 lectin, that was immunologically cross-reactive with antibodies directed against seed SBA. We had also shown that SB-1 lectin was chemically similar, if not identical, to seed SBA on the basis of comparative peptide maps after limited protease treatment (11). In the course of these studies, we observed that V-8 protease digestion of either seed SBA or SB-1 lectin resulted in peptides (M_r 10,000 - 20,000) that could be distinguished and separated in SDS-PAGE.

We have now optimized the conditions for digestion of SBA and separation of the peptides. Treatment of SBA with V-8 protease at 37° for 2 h produced seven major polypeptide bands as revealed by SDS-PAGE (Figure 1A). These are designated F_1 through F_7 in order of descending apparent molecular weights. In the 12.5% acrylamide gels, the individual bands were distinct; this allowed us to cut the gel into slices (1-2 mm width). The polypeptide(s) in these slices were eluted and then concentrated by acetone precipitation. When the material derived from each of the individual bands of Figure 1A was re-electrophoresed and subjected to silver staining, each yielded again a single peptide band (Figure 1B), with the possible exception of F_4 (see below).

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Figure 1. SDS-PAGE analysis of peptides from seed SBA obtained via digestion with V-8 protease. A, purified seed SBA (= 100 µg) was subjected to limited V-8 hydrolysis (32 µg/ml) in test tube. Digestion mixture was electrophoresed in a 12.5% acrylamide gel and polypeptides were revealed by staining with Coomassie Blue. B, individual representative peptides in lane A (from 2-4 mg of SBA digest) were excised and eluted from the gel. The eluates were concentrated to a final volume of 100 µl as described in the Methods. An aliquot of 2 µl from each sample was used for SDS-PAGE analysis. Peptides in the gel were revealed by silver staining. Numbers 1 through 7 at the top refer to the peptides in order of descending apparent molecular weights. For example, No. 1 denotes the largest peptide and No. 7 denotes the smallest peptide. Numbers on the left indicate the range of molecular weights.

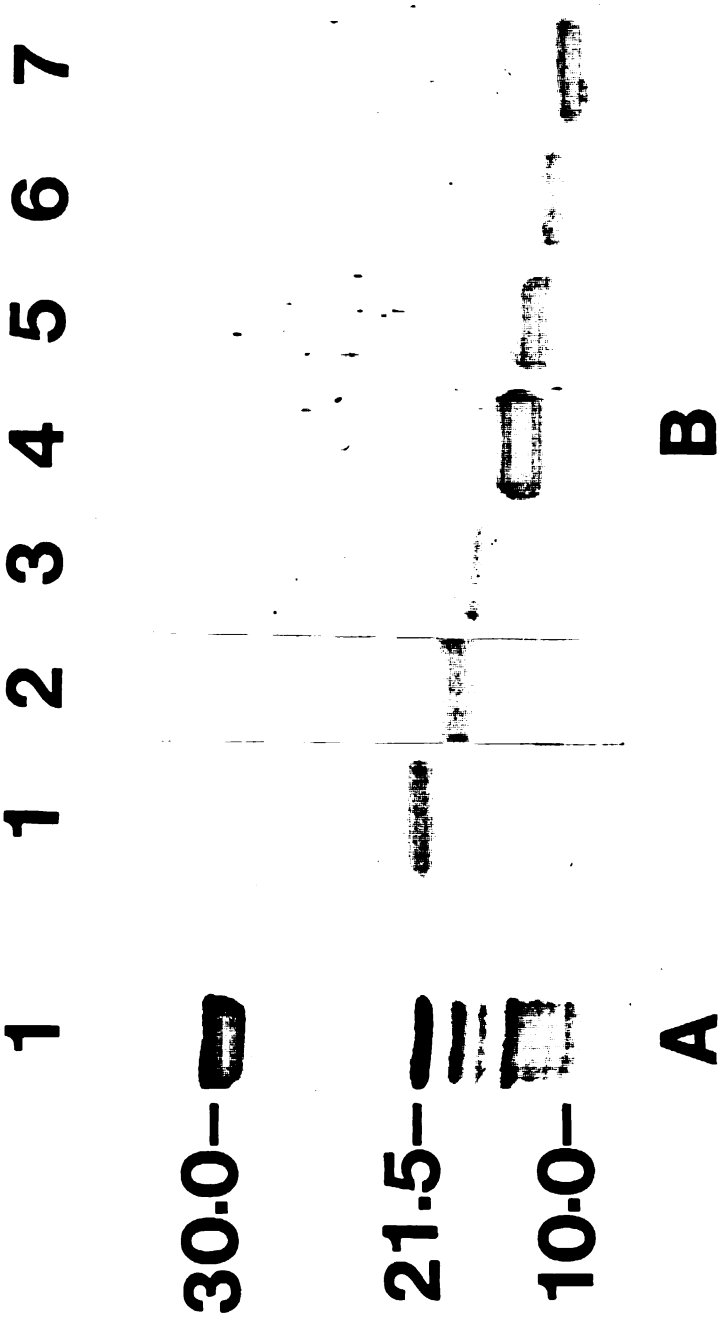

Figure 1

On the basis of the mobility of the peptide bands on 12.5% acrylamide gels, the apparent molecular weights estimated for each of the V-8 protease digestion products of seed SBA were: F_1 ~ 21.5 kD; F_2 ~ 20 kD; F_3 ~ 18 kD; F_4 ~ 15 kD; F_5 ~ 13.8 kD; F_6 ~ 12.2 kD; F_7 ~ 10 kD.

Placement of the Peptides in the Primary Structure of Seed SBA

The material from F_1, F_4-F_7 (Figure 1B) were subjected to amino acid sequence analysis. With the exception of F_4, each sample yielded a single amino acid per turn of the Edman cycle, providing evidence that these samples were relatively homogeneous. The first ten amino acid residues from the NH_2-terminus of each sample were identified. This information, together with the previously published complete amino acid sequence of SBA (16), enabled us to establish the position of the V-8 generated proteolytic peptides (F_1, F_4-F_7) in the primary sequence of SBA (Figure 2).

The NH_2-terminal sequence of F_1 was identical to that of the NH_2-terminal sequence of SBA polypeptide. The COOH-terminus of F_1 was predicted on the basis of: (a) the specificity of V-8 protease, which cleaves on the carboxyl side of Glu and Asp residues in polypeptides (4); and (b) the apparent molecular weight of the polypeptide on SDS-PAGE (Figure 1B). By these criteria, we believe that F_1 spans residues 1 through 169 of the SBA polypeptide (Figure 2). This region includes residue Asn 75, which is glycosylated in seed SBA (2) and, therefore, could affect the apparent molecular weight of the polypeptide as estimated by mobility on SDS-PAGE.

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Figure 2. Mapping of SBA peptides in the linear structure of SBA polypeptide. The origin of peptide F_1 and peptides F_4 through F_7 (see Figure 1B) was established in the primary sequence of intact SBA by NH_2-terminal sequencing. The written sequences at NH_2-terminal portion represent actual data, the C-terminal for each peptide was determined based on criteria explained in the text. Residues 1 to 169 represent peptide 1 (F_1); residues 40 to 155 (F_4A) and 124 to 253 (F_4B) represent peptide 4 (F_4); residues 124 through 240, 222 and 202 represent sequence of peptides 5, 6, and 7 (F_5, F_6, F_7), respectively. The - line indicates amino acid was not identified.

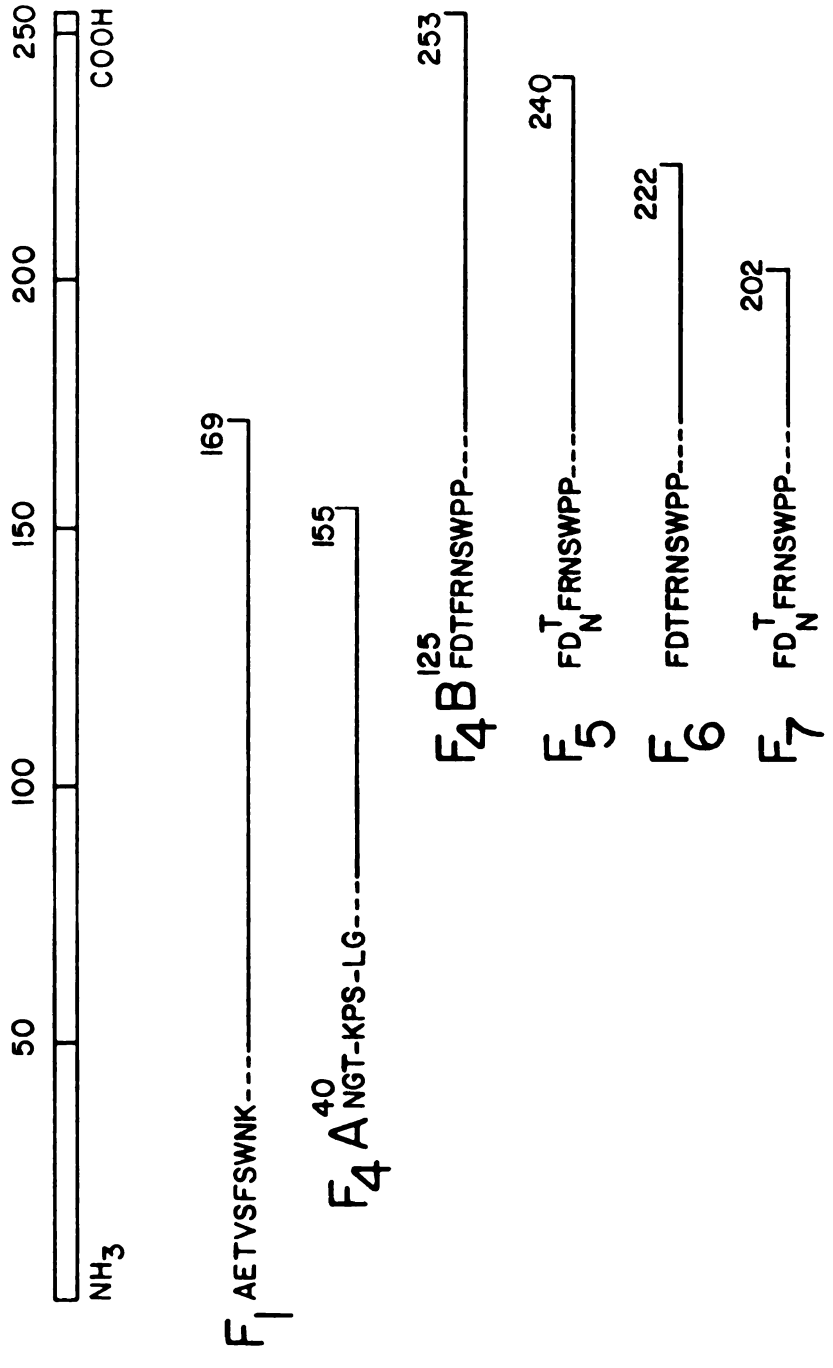
NH3
COOH
50
100
150
200
250
F1 AETVSFSWNK----169
F4A 40 NGT-KPS-LG----155
F4B 125 FDTFRNSWPP----253
F5 FDTFRNSWPP----240
F6 FDTFRNSWPP----222
F7 FDTFRNSWPP----202

Figure 2

The NH_2-terminal sequence of F_5, F_6 and F_7 were identical and indicated that each of these peptides started at Phe 125 in the SBA polypeptide chain. This is consistent with the cleavage by V-8 protease on the carboxyl side of Glu 124. Again, the specificity of the V-8 protease cleavage site and the apparent molecular weights of the individual polypeptides were used to predict the carboxy terminus of fragments F_5, F_6 and F_7 (Figure 2).

The material isolated from band F_4 yielded two amino acids per turn of the Edman cycle. Although this indicated that F_4 consisted of a mixture of at least two peptides, the sequence results nevertheless provided information on the location of these peptides in the amino acid sequence of SBA. One of the peptides, designated F_4A, begins at Asn 40 and extends to Asp 155 (Figure 2). The other peptide, designated F_4B, yielded NH_2-terminal sequence identical to those of F_5, F_6 and F_7; therefore, fragment F_4B begins at Phe 125. Fragment F_4B could encompass Phe 125 through Ile 253, the COOH-terminal amino acid of SBA polypeptide chain. Alternatively, F_4B could stop at Asp 240 and would simply represent contamination of F_4A by fragment F_5. This latter case would be consistent with the observation that the material in F_4, upon re-electrophoresis, yielded a gel pattern that did not show distinct separation of F_4 from F_5 (Figure 1B).

Antibodies Reactive with Peptides F_1 - F_7

A polyclonal rabbit antiserum was raised against seed SBA that had been denatured by treatment with boiling SDS prior to immunization. The immunoglobulin fraction of this antiserum was then incubated with individual peptides, derived from V-8 protease digestion of seed SBA

that had been electrophoresed and transferred onto nitrocellulose strips. Antibodies bound to each peptide were then eluted and were designated anti-F_1, anti-F_2, etc. (see Materials and Methods).

It should be noted that this designation simply indicates the particular peptide to which a given preparation of antibodies was bound. It does not imply, for example, that anti-F_7 reacts only with peptide F_7. On the contrary, immunoblotting analysis of a V-8 digest of seed SBA showed that anti-F_7 reacted with all seven peptides, as well as the denatured SBA polypeptide (Figure 3). Therefore, although anti-F_7 antibodies presumably recognized antigenic determinants spanning residues 125-202 (Figure 2), they would be expected to bind other peptides that overlapped in any portion of this region. Similar results and conclusions were derived from immunoblotting analysis using anti-F_1 through anti-F_6 (Figure 3). In general, all of the anti-peptide antibodies reacted most strongly with F_1 and F_2, most probably because these contained a greater proportion of the intact polypeptide chain.

Because the isolation of antibodies bound to individual peptides on nitrocellulose strips involved procedures such as exposure to low pH (pH 2.3) and detergent (Tween-20), we also prepared in parallel antibodies that bound to the intact subunit (~ 30 kD) that was not digested by the V-8 protease. This antibody preparation was designated as anti-SBA (30 kD) to emphasize the fact that it was eluted from the 30 kD polypeptide band of SBA. The anti-SBA (30 kD) serves as a reference antibody (positive control) in subsequent immunofluorescence experiments.

Figure 3. Reactivity of V-8 generated SBA peptides with anti-SBA (30 kD) and various anti-peptide antibodies (see text) as analyzed by immunoblotting. SBA digest (= 20 μg/lane) was electrophoresed in 12.5% SDS-polyacrylamide gel. The peptides were electrotransferred to nitrocellulose paper. The nitrocellulose-containing peptide strips were then incubated with antibodies. A, anti-SBA (30 kD). B, anti-peptide antibodies: Lane 1, anti-F_1; lane 2, anti-F_2; lane 3, anti-F_3; lane 4, anti-F_4; lane 5, anti-F_5; lane 6, anti-F_6; lane 7, anti-F_7. Immunoreactive material was detected by alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin. Markers indicate the position of each fragment.

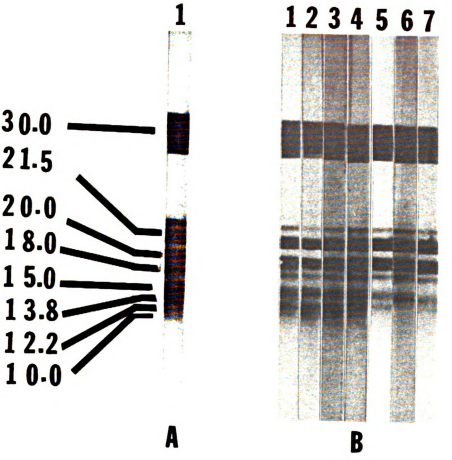

Figure 3

Reactivity of Peptides (F_1-F_7) with Antibodies Raised Against Undenatured SBA

Antibodies raised against undenatured seed SBA were purified by affinity chromatography either on undenatured SBA coupled to cyanogen bromide-activated Sepharose beads (9) or on a protein A affinity column. These antibody preparations were tested for their reactivity with V-8 generated SBA peptides (F_1-F_7) by immunoblotting of the V-8 digest of seed SBA. The anti-SBA antibodies fractionated on the protein A column showed immunoreactivity with all peptides (F_1-F_7) (Figure 4A). In contrast, antibodies fractionated on the SBA column, reacted most strongly with F_1 and F_2, while revealing very weak reactivity with F_4-F_7 (Figure 4B).

Immunofluorescence Staining of SB-1 Cells with Anti-Peptide Antibodies

SB-1 cells (10^6 cells/ml) were incubated with anti-SBA (30 kD) or the various anti-peptide antibody preparations. All these incubations were carried out at the same immunoglobulin concentration (50 µg/ml). The binding of the antibodies to cells was detected, in turn, by staining with fluorescein-conjugated goat antibodies directed against rabbit immunoglobulin (Figure 5). Using this procedure, anti-SBA (30 kD) yielded bright staining on the cell wall (Figure 5a), consistent with our previous observation that SB-1 lectin was exposed on the cell wall (9). Similarly, preparations of anti-F_1, anti-F_2, anti-F_3, and anti-F_4 all yielded staining of the cell wall (Figure 5, b-e). The level of staining was comparable to that of anti-SBA (30 kD) (Figure 5a).

Figure 4. SDS-PAGE and immunoblotting analysis of V-8 peptides from seed SBA with different preparations of anti-SBA (undenatured) polyclonal antibodies. Lane A: SBA digest (40 µg) was electrotransferred onto nitrocellulose paper. The nitrocellulose containing fragments was incubated with anti-SBA antibodies which was purified on a protein A column. Lane B: Same as Lane A, except the nitrocellulose containing SBA-proteolytic peptides was incubated with anti-SBA antibodies purified on SBA column. Immunoreactive material was detected by alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin. Numbers on the left indicate the range of molecular weights.

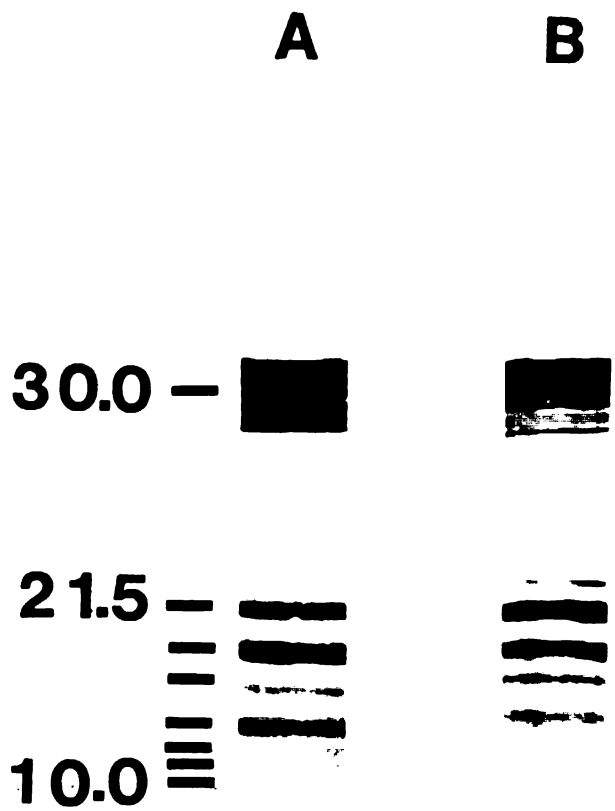

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Figure 5. Fluorescence staining pattern of intact SB-1 cells with anti-SBA (30 kD) or anti-peptide antibodies prepared by specific adsorption to nitrocellulose paper (14). SB-1 cells were treated with either anti-SBA (30 kD) or anti-peptide antibodies (50 µg/ml) for 2 h at 4°C. Control cells were incubated with normal rabbit immunoglobulin (50 µg/ml) under the same conditions. Binding of antibodies to the cells was detected following incubation with fluorescein-conjugated goat anti-rabbit immunoglobulin (1:100 dilution; 30 min at 4°C). (a) cells treated with anti-SBA (30 kD); (b) cells treated with anti-F_1; (c) cells treated with anti-F_2; (d) cells treated with anti-F_3; (e) cells treated with anti-F_4; (f) cells treated with anti-F_5; (g) cells treated with anti-F_6; (h) cells treated with anti-F_7; (i) cells treated with normal rabbit immunoglobulin. All micrographs were taken at 2 min exposure and printed with the same setting. Bar, 50 µm.

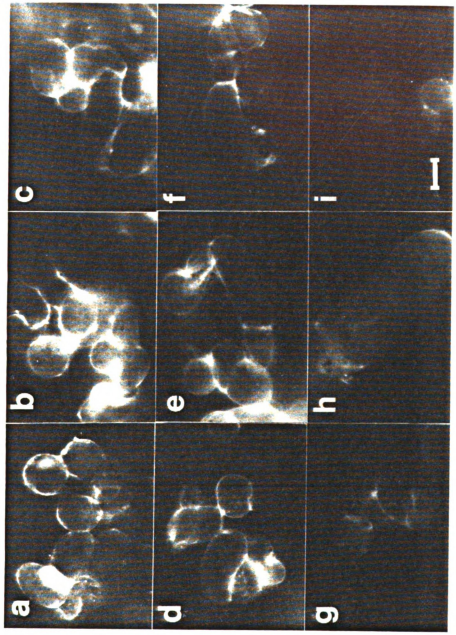

Figure 5

In contrast to these results, the staining obtained with preparations of anti-F_5, anti-F_6, and anti-F_7 was considerably weaker (Figure 5, f-h). Staining with anti-F_7 was most comparable with preimmune immunoglobulin control (Figure 5i).

DISCUSSION

In this study, we have taken advantage of the previous observation (11) that digestion of seed SBA with V-8 protease yielded peptides (M_r 10,000 - 20,000) that were distinctly separated by SDS-PAGE. The individual peptides were isolated and subjected to amino acid sequence analysis. Comparison of the NH_2-terminal sequence of each peptide with the complete amino acid sequence of the intact SBA polypeptide (16) enabled us to establish the position of the individual peptides in the sequence of the SBA chain. The isolated individual peptides (F_1 - F_7) were then used as affinity adsorbents to sub-fractionate the anti-seed SBA immunoglobulin fraction, yielding antibodies that bound to the individual peptides (anti-F_1 through anti-F_7). Because the affinity adsorbent consisted of polypeptide(s) subjected to SDS-PAGE and transfer to nitrocellulose paper, it appears most likely that the antibody preparations of anti-F_1 through anti-F_7 were sequence specific.

By immunofluorescence experiments, we found that antibody preparations that recognized the NH_2-terminal portion of the SBA polypeptide (residues 1-124 (determined by comparing reactivity of anti-F_1 with anti-F_5, F_6, and F_7), for example) bound to intact SB-1 cells. In contrast, those antibody preparations that recognized sequences between residues 125-240 (anti-F_5, anti-F_6, and anti-F_7, for example) showed little or no binding to intact SB-1 cells. These results were

obtained through qualitative observations of fluorescent staining of cells on photomicrographs. It is important to further note that the results presented in Figure 4 suggest that undenatured SBA shows the same pattern of reactivity to anti-peptide antibodies observed for cell surface bound SB-1 polypeptide. These patterns of anti-peptide reactivity serve as evidence that the NH_2-terminal is available for antibody binding, while the COOH-tail appears to be masked by the folding of the molecule.

Our conclusions at this point must be limited to the following: a) The anti-peptide pattern of reactivity of SB-1 lectin bound to the cell surface of soybeans in culture mimics the pattern of peptide reactivity observed for undenatured SBA; SBA epitopes available for antibody binding in undenatured SBA are also available for solid-phase (cell wall localized) SB-1 lectin; this implies that the overall conformation of both lectins, as probed by our antibodies, is the same; b) Furthermore, it appears that the N-terminal half of the SB-1 molecule is not directly involved in the attachment of SB-1 molecule to the cell wall. Future work will now focus on the C-terminal for possible sites of non-carbohydrate mediated SB-1 attachment to the cell wall.

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CLOSING STATEMENT

This thesis aimed to examine the chemical properties of SB-1 lectin derived from cultured soybean root cells. In addition, attempts were made to study its role as the recognition molecule in the heterotypic interaction between *Rhizobium* and root cells. To pursue these investigations, we employed a defined tissue culture system using an established soybean cell line, SB-1, originally derived from the roots of *Glycine max*. The results of our studies may be summarized as follows:

(a) *Rhizobium* bind to SB-1 cells in culture and this binding is strain specific for *Rhizobium* that normally infect soybean roots.

(b) Adhesion of *Rhizobium* to SB-1 cells is mediated via a carbohydrate recognition system.

(c) A lectin (SB-1 lectin) endogenously produced by SB-1 cells and also identified on the SB-1 cell surface appears to be an element of the recognition between *Rhizobium* - SB-1 cells.

(d) Biochemical characterization of SB-1 lectin demonstrated that SB-1 lectin is similar, if not identical, to seed soybean agglutinin (SBA) on the basis of the following criteria:

  (i) peptide mapping of polypeptide subunit, M_r 30,000;

  (ii) subunit structure and oligomerization;

(iii) carbohydrate binding specificity;

(iv) immunological cross-reactivity.

(e) Our results from fluorescence analyses of intact SB-1 cell surface labeling with various anti-peptide antibodies, specific for certain sequences of SBA, and affinity chromatography methods suggest that:

(i) SB-1 lectin is not anchored to the cell wall by galactose binding.

(ii) SBA epitopes available for antibody binding in solution are also available for solid phase (cell wall localized) SB-1 lectin.

(iii) It appears that the NH_2-terminal portion of cell-wall anchored SB-1 lectin (anchored in the wall) is exposed to its environment and is not directly involved in the attachment of the SB-1 molecule to the cell wall.