Immunofluorescent localization of sainfoin lectin
by Sharon Jo Fitzwater Solomon

A thesis submitted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Biochemistry
Montana State University

Abstract:
Immunofluorescent staining procedures were developed and applied toward the localization of lectin in the leguminous plant Sainfoin (Onobrychis viciifoliae, Scop.). Tissue sections from root, seed, and nodule were microscopically examined with the "indirect" immunofluorescent staining technique. Specific lectin antiserum was produced in rabbit. Goat anti-rabbit immunoglobulin labelled with rhodamine isothiocyanate was used as the secondary stain.

Immunofluorescent stains with comparative histological stains showed lectin to be present in the cell wall area of the aleuron layer of the seed as well as in the protein matrix surrounding the starch granules. In the nodule, positive staining was found in the cell wall area of the outer cortical cells. Lectin was localized in the cell wall areas of the central vascular elements of 10, 20, and 30 day old root as well as in the cell wall area of the outer epidermis. There was no significant staining of the cortical parenchyma of root tissue in these age groups. In young (96 hr) root tissue, lectin was localized in the wall areas of all cells, including the outer epidermis. Cells near the extreme root tip also contained lectin localized in the cytoplasmic regions. In contrast, older tissue showed no cytoplasmic staining.

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SHARON JO SOLOMON

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ABSTRACT

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INTRODUCTION

Phytolectins can be defined as proteins or glycoproteins from plants that are capable of binding animal erythrocytes and other cells due to their specificity towards saccharide receptors present on the cell surface (1). This selective binding is presumably the basis of certain cell responses such as agglutination, lysis, mitosis, and induced contact regulation of growth in malignant cells (2). Therefore, lectins provide a fruitful area of study not only to the protein biochemist and plant physiologist but also to the immunologist, cell biologist and other researchers involved in cell phenomena.

Since the late 1800's lectins have been studied as model systems analogous to the antigen-antibody reaction and to investigate specificity of erythrocyte agglutination (3). In the 1940's Concanavalin A (Con A) was isolated and to date it has been the most thoroughly studied phytolectin. Edelman's group at Rockefeller University has recently established the primary sequence and the 3-dimensional, x-ray crystallographic structure including elucidation of the metal and saccharide binding sites (4). In the last decade lectin research has increased dramatically. It has been found that besides possessing specific erythrocyte agglutination properties, lectins bind sugars specifically and specifically precipitate polysaccharides and glycoproteins (5). Some lectins, such as Con A are mitogenic; that is, they convert resting lymphocytes into actively growing and dividing

blast-like cells (6). Some lectins are also capable of specifically agglutinating malignant cells (7) and are therefore used as probes to investigate cell surface changes during malignant transformation. The increased intensity of lectin research is evidenced by four recent reviews (8,9,10,11).

Phytolectins are easily obtainable by direct extraction and chromatography techniques (12) and in most instances have been isolated from legumes. However, they have also been found in other plants such as wheat (13) and more recently in some slime molds (14).

Although their biological and chemical properties are beginning to be investigated, the exact in vivo role or roles of plant lectins is still speculative. It has been postulated that they function in a plant protection system that counteracts soil bacteria or inhibits fungal polysaccharases (15). Another suggestion is that they are involved in sugar transport and storage and cell wall extension growth (16). It has been thought that possibly they serve in the attachment of glycoprotein enzymes in organized multi-enzyme systems (17) or that perhaps they in some way control cell division and germination in the plant (18). All of these hypotheses are under study and it may be that the role of lectin in nature is not related directly to biological properties observed in laboratories.

Recently some evidence has accumulated suggesting that lectins may be involved in the localization of rhizobia bacteria on the root

hairs of legumes prior to infection and subsequent nitrogen fixation. During this infection process the rhizobia bacteria enter the plant via invagination of the cell wall at the site of binding on the root hair (25). This progressive invagination is termed an infection thread and usually contains many actively replicating bacteria. The infection thread eventually reaches a large cortical cell in the epidermis of the root where the tip of the thread ruptures. The bacteria are released into the cortical cells which then enlarge to form a nodule. Although the infected cortical cell is often tetraploid, ploidy has not been conclusively shown to be a prerequisite for infection (26). Once in the nodule the bacteria differentiate to form a bacteroid which is capable of fixing nitrogen. The ammonia produced can be combined with the products of photosynthesis to yield those compounds essential to plant growth. This symbiotic relationship between the legume host and the rhizobia is specific. In other words, only certain species of rhizobia form this association with any one legume. Obviously there is a type of recognition system operating between legume and bacteria. Some researchers feel that plant lectins may be the mediators of that recognition. Kent and Hamblin from Cambridge and the University of Alabama correlated PHA (phytohemagglutinin, a lectin from red kidney bean) binding to rhizobia and its hemagglutination activity to their observation that young plant root hairs also were capable of binding erythrocytes (19). Bohlool and

Schmidt from the University of Minnesota have shown that soybean lectin carrying a fluorescent tag binds selectively to infective strains of Rhizobium japonicum (20). Wolpert and Albersheim from the University of Colorado have demonstrated a specific interaction between the O-antigen containing lipopolysaccharides of Rhizobia and the lectin of their legume host (21). Using an affinity chromatography technique, they were able to show that the lipopolysaccharide from a rhizobium surface interacted with lectin isolated from its normal host plant but not with other non-host lectins. Working with white clover, Dazzo and Hubbel from the University of Florida showed that antibodies produced to antigen on the root surface were cross-reactive with antigen on the surface of Rhizobium trifoli and conversely anti-R. trifoli was cross-reactive with antigen on the root surface (22). They also extracted a clover lectin capable of binding to root surface and agglutinating only infective R. trifoli. On the basis of this data, they proposed a model suggesting the preferential adsorption of infective vs non-infective cells of R. trifoli on the surface of clover roots by a cross bridging of their common surface antigens with a multi-valent clover lectin. More recently they have utilized quantitative microscope techniques to examine the adsorption of rhizobial cells to clover root hairs and demonstrate rhizobial selectivity for the natural host (23). They were also able to show that the presence of host lectin greatly increased the binding of infective bacteria.

Chen and Phillips at Indiana State University have developed a reproducible, quantitative technique for studying interactions between labelled lectins and rhizobia that suggests no relationship between lectin-rhizobium interactions and the capacity to infect a plant (24). Although this data does not completely support previously published work on rhizobium-lectin interactions, it doesn't disprove the idea that the rhizobium-host specificity occurs through a recognition mechanism. It merely points out that many bacteria that are non-infective to a particular legume are still capable of binding to the root and also that lectin binding to rhizobia is minimal and may be non-specific. Clearly, more research is required to fully explain the rhizobia-plant host recognition mechanism.

This current study is part of an interdisciplinary research program directed toward improvement of the legume, sainfoin (Onobrychis viciifolia), a forage and pasture crop which is unfortunately a poor nitrogen fixer. A lectin isolated from sainfoin was studied with the objective of determining its possible role in the rhizobia-host symbiosis. Localization of sainfoin lectin in the plant tissue was undertaken to help elucidate such a role. Because of the high degree of sensitivity and specificity of the antigen-antibody reaction, immunofluorescent localization was the technique chosen. If lectin does participate in rhizobia-host recognition, microscopic evaluation of root tissue sections treated with lectin specific immunofluorescent

stain should locate it on the outer surface of root tissue. The lectin's availability to interact with rhizobia, a critical prerequisite, was defined in this study by successful tissue localization.

In a larger sense, it is probable that phytolectins are multifunctional. The immunofluorescent technique was used to examine several tissues from sainfoin in addition to root in order to suggest other functions. Seed, nodule, and hypocotyl were among the other tissues examined and the location of lectin determined. Although localization can never completely define a function, it can serve as a valuable signpost to guide the investigator in further research. In this particular study it has answered certain specific questions as well as suggesting several new and interesting problems for investigation.

RESEARCH OBJECTIVES

The general objective of this study is to localize sainfoin lectin, via immunofluorescent techniques, in the seed, root, and nodule tissue of the plant. Specific objectives are:

a. Development of appropriate histological methodology.

b. Development of appropriate immunofluorescent staining procedures and instrumentation.

c. Localization of the lectin in plant tissue as evidenced by photomicroscopy.

MATERIALS AND METHODS

Isolation and Purification of Sainfoin Lectin Antigen

Extraction. A simple phosphate buffered saline (PBS) extraction was used in the initial isolation process (36). Dehulled, finely ground sainfoin seeds were extracted at 4°C with constant stirring in PBS buffer for four hours. The extraction buffer was 0.01 M in phosphate, 0.15 N in NaCl, 0.01 M in ascorbate and 0.1 M in glucose. The buffer was titrated to a pH of 7.0. Sodium Azide, 0.025% w/v, was added to the buffer as a preservative. Following the initial extraction period, the extract was centrifuged at 10,000 rpm's for 20 minutes. The supernatant solution was collected and brought to 40% saturation with ammonium sulfate. This mixture was stirred for four hours at 4°C and then centrifuged. The supernatant solution was again collected and additional ammonium sulphate was added to bring the solution to 80% saturation. The mixture was stirred for four hours at 4°C and then centrifuged again. After this centrifugation, the precipitate was redissolved in 100 ml of PBS buffer. This buffer was the same as the extraction buffer except that the ascorbate was deleted and the glucose concentration was 0.25 M. The dissolved precipitate was dialyzed in the cold for 24 hours against frequent changes of buffer. The dialysis buffer was the same as the extraction buffer except that it contained no ascorbate or glucose.

Affinity chromatography. Since sainfoin lectin's saccharide specificity includes mannose (36), the lectin was isolated on an affinity chromatography column of mannose covalently attached to sepharose 6B via the divinyl sulphone technique of Porath and Fornstedt (27). A column of this matrix 1.5 cm in diameter and 2.0 to 4.0 cm in height was prepared and equilibrated with PBS buffer which was the same as the dialysis buffer. The dialyzed sample was centrifuged and the supernatant solution was applied to the column. The column was then washed with the PBS buffer used in equilibration until the $O.D._{280}$ of the wash was 0.02 or below. Elution of the lectin from the column was performed with PBS buffer 0.25 M in glucose. The effluent was monitored spectrophotometrically. Fractions containing protein were pooled and dialyzed against PBS to remove the glucose prior to repurification.

Repurification. The lectin was repurified by repeating the affinity chromatography process. A small column 0.9 cm by 1.5 cm was prepared using virgin mannose-sepharose as the matrix. Fractions containing the protein were pooled and stored frozen in the PBS-glucose elution buffer at -20°.

Root lectin. Lectin from the root was isolated and purified in the same manner as seed lectin with the exception that fresh washed root tissue was homogenized in PBS containing glucose in a Waring

blender. The resulting supernatant was subjected to affinity chromatography as previously described.

Production of Specific Antisera to Sainfoin Lectin

Injection schedule. Antisera was prepared in the rabbit system. Primary immunization was carried out using 13.0 mg of purified seed lectin in 2.0 ml of PBS mixed with 2.0 ml of Freund's adjuvant and injected in 1.0 ml portions near the four axial lymph nodes. At week one the animal was immunized using 4.0 mg of purified lectin and Freund's incomplete adjuvant. At week two the animal was immunized again with 5.0 mg of purified lectin and Freund's incomplete. The animal was bled by cardiac puncture 10 days after the last injection. The blood was allowed to clot and then the serum fraction was separated from the cells by centrifugation. The serum was divided into 1.0 ml aliquots and stored in a Revco deepfreeze at -80°C.

Gel double diffusion. To establish antisera specificity and antigen purity gel double diffusion (29) was run with antisera vs purified lectin and antisera vs crude extract after affinity chromatography. The antigenic identity of seed lectin and root lectin was also compared by gel double diffusion with antisera to seed lectin. The gel double diffusion plates were set up using small petri dishes containing 1% agar. The wells held a sample volume of 10 microliters.

Diffusion was allowed to proceed 24 hours in a moist chamber, then the gels were washed with distilled water and photographed.

Titer. An Ouchterlony gel double diffusion (37) was carried out using serial dilutions of the antisera in the six outer wells of the plate and lectin antigen in the center well. Diffusion was allowed to proceed 24 hours at which time pattern development was complete.

Immunoelectrophoresis of seed and root lectin. Scheidigger's microtechnique for immunoelectrophoresis was used (29). A microscope slide of standard size was used for a gel support and 2.0 ml of buffered agar, pH 8.5 were poured on top giving a gel layer of about 1 mm thickness. Seed and root lectin (1 microliter) were applied to small circular wells 1 mm in diameter that were punched out of the gel. A longitudinal trench 40 mm x 2 mm was cut central and parallel to the long side of the slide at a suitable distance from the circular wells. An E-C Apparatus Corporation electrophoresis cell was filled with barbital buffer, pH 8.5, and the gels were electrophoresed two hours at a potential drop of 6 v/cm in the gel. At the end of this time 0.05 ml antisera was added to the central trough and double diffusion was allowed to proceed for 24 hours at which time pattern development was complete. The gels were stained with Coomassie blue for 15-30 minutes, destained for 5-10 minutes with acid alcohol and soaked for two 24

hour periods in distilled water. The stained gels were then photographed.

Preparation and Characterization of Rhodamine Anti-rabbit IgG Conjugate

Preparation. Rhodamine-B isothiocyanate (mixed isomers) was purchased from Sigma Chemical Corporation, St. Louis, Missouri. Purified IgG fraction of anti-rabbit IgG produced in the goat was purchased from Miles Laboratories, Incorporated, Elkhart, Indiana. The purified anti-rabbit IgG was dialyzed into the reaction buffer, 0.2 M sodium carbonate, pH 9.6, prior to carrying out the reaction. Rhodamine-B isothiocyanate (10 micrograms per milligram of protein) was dissolved in 0.5 ml of DMSO and was added to the stirring, buffered protein solution in 3 aliquots at hour intervals at room temperature. The volume of DMSO used to solubilize the rhodamine did not exceed 10% v/v final concentration in the reaction mixture. The reaction was allowed to continue for 24 hours at 4°C. To stop the reaction, the conjugate solution was slowly titrated to pH 7.0 and was applied to a sephadex G-50 column 1.8 by 50.0 cm which had been equilibrated with 0.01 M PBS buffer 0.1 M in glucose. Unreacted dye was separated from conjugate on this column by molecular sieve filtration. The conjugate fraction was collected, dialyzed and stored frozen in small aliquots at -80°C.

Characterization. Absorption spectra of a standard solution (10 micrograms per milligram) of rhodamine and also of a sample of the conjugate were obtained on a Varian Techtron UV-Vis spectrophotometer model 635. Using the Lambert-Beer Law, a molar extinction coefficient for rhodamine was calculated from the absorbance maxima and known concentration of the standard solution of the free dye. The standard solution of free dye was prepared using DMSO and PBS as in the reaction procedure. Fluorescence excitation and emission spectra of the conjugate were obtained using a 500 mm Bausch and Lomb grating monochromator, a EMI 9558 QC photomultiplier tube, an Osram XBO 150 W xenon lamp and a Hewlett Packard 7030A x-y recorder. The molar rhodamine to protein ratio was calculated using a modification of the method of Wells et al. (30).

Tissue Preparation

Non-infected tissue. Sainfoin seedlings which had not been exposed to infective rhizobia bacteria were germinated and grown in 1 inch dialysis tubing planted in sterile vermiculite. The seeds were surface sterilized with Chlorox for 15 minutes prior to planting and were placed singly in the tubing at a depth of 1/2 inch below the surface of the vermiculite. One end of the tubing was left exposed approximately 1 inch above the vermiculite and left open to allow growth of the cotyledons. The seedlings were fed sterile Thornton's nitrogen

free liquid media (31), initially on the surface of the vermiculite and then from the bottom of the pot after the appearance of cotyledons. Samples of the root tissue from these seedlings were taken at 96 hours, 10 days, 20 days, and 30 days after planting.

Infected tissue. Samples of root and nodule were taken from 30 day old seedlings that had been grown in sterile vermiculite, watered with nitrogen free water and infected with a mixture of several strains of rhizobia known to infect sainfoin. The seedlings were inoculated 4-5 days after planting.

Seed. Seeds were dehulled and soaked in distilled water for 24 hours prior to sectioning.

Sectioning. Serial cross sections of all tissue examined except the seed were cut at 10 microns on a standard Universal cryostat-microtome (32). Seed sections of 10 microns were cut longitudinally on the same instrument. Tissue was frozen fresh, sectioned and affixed to standard microscope slides without the use of adhesive.

Fixation. All tissue sections were fixed 15 minutes in 95% ethanol after cutting and prior to staining.

Photomicroscopy

A Leitz Ortholux microscope equipped with a Ploempak vertical illuminator was used to evaluate and photograph the immunofluorescent stains. An HB 200 mercury vapor lamp was the energy source. All tissue sections were observed with transmitted darkfield illumination. The condenser was adjusted for maximum illumination of field. Spectra of various primary and secondary filters were compared. The filters chosen were a 546 wideband interference primary filter and a K580 secondary filter. Photographs were taken with Kodak High Speed Ektachrome film. The exposure time varied from 1 to 2 minutes depending upon the intensity of the stained sections. Exposure time for the controls was always the same as that used for the corresponding stained section.

Staining Procedures

Hematoxylin-eosin. Delafield's hematoxylin was used and the standard procedure for routine H&E histological staining was followed (33). Histological stains for each type of tissue examined were prepared. The tissue sections were stained in the filtered hematoxylin for 6 minutes, washed briefly in tap water, dipped in acid alcohol and washed in running tap water for 15 minutes. They were then placed in 70% ethanol for 5 minutes prior to counterstaining for 30 seconds with eosin. The tissue was then passed briefly through baths of 95%

ethanol, absolute ethanol and 3 successive baths of xylene. The sections were coverslipped with Permount mounting media immediately after the final xylene wash.

Immunofluorescent. A dilution series of both antisera and rhodamine conjugate was used in the initial staining to establish optimal concentrations. Dilutions of 1:100 v/v for the antisera and 1:50 v/v for the conjugate were found to offer the best staining and were then used routinely. Both antisera and conjugate were diluted with PBS, pH 7.0, 0.1 M in glucose and 0.3% in Triton X-100 (34). Staining was carried out in a moist chamber. The diluted antisera was applied directly to the tissue sections; approximately two drops per section. The sections were stained with antisera for one hour and then were washed five minutes in a solution of PBS, pH 7.0, 2% in Triton X-100. After washing, the sections were allowed to drain and then the conjugate was applied in the same manner as the antisera and allowed to stain for one hour. The sections were then washed again for five minutes in PBS, pH 7.0, 2% in Triton X-100. Following this wash they were rinsed in distilled water, allowed to drain, mounted and coverslipped. The mounting media was glycerol diluted 1:1 with PBS, pH 6.0 (35).

Controls

General staining. Sainfoin lectin was covalently attached to sepharose 6B by the cyanogen bromide activation procedure of Cuatrecasas (44). A micro-chromatography column, 0.5 cm by 1.0 cm, was poured using that matrix. Two similar columns were also prepared; one of methylated chymotrypsin covalently attached to sepharose 6B and one of sepharose 6B without a protein ligand attached. All three columns were equilibrated with PBS, pH 7.0. One ml of the antisera, diluted as in the immunofluorescent staining procedure, was applied to each column. After the sample had been absorbed, the columns were washed with several column volumes of PBS. One ml of conjugate, also diluted as in the staining procedure, was then applied to each column and allowed to penetrate the matrix. Again, the columns were washed with PBS. After extensive washing a sample of beads was taken from the top of each column, mounted in mounting media on microscope slides, and observed microscopically for fluorescence.

Tissue staining. Autofluorescence controls were run for each tissue examined by substituting PBS for antisera and conjugate in the staining process. Non-specific binding controls were run for each tissue examined by substituting normal rabbit serum (NRS) for specific antisera in the staining procedure. The control sections were

microscopically evaluated and photographed under identical conditions as the sections treated with the complete stain.

RESULTS AND DISCUSSION

Antigen-Antibody Characterization

Antigen preparation. Pure Sainfoin lectin was prepared by affinity chromatography using sepharose columns containing covalently linked mannose. Figure 1 shows that treatment of the column with glucose resulted in displacement of the lectin which emerged from the column as a single sharp peak. The yield of repurified lectin from 200 g of ground seeds was typically near 50 mg. Time required for the entire lectin preparation was 2-3 days. Unpublished characterization studies (36) have shown the lectin to be free of contaminating proteins. It is a glycoprotein containing 6 per cent carbohydrate and the molecular weight as established by gel filtration is 57,000 daltons. The molecular weight measured by sodium dodecyl sulfate polyacrylamide electrophoresis is 28,000. This antigen was used in the rabbit immunization program for elicitation of specific antibody. The purity of antigen and specificity of antibody obtained was demonstrated as described below.

Ouchterlony double diffusion. Figure 2 gives the result of a double diffusion experiment employing antisera in the center well. The outer wells contained samples of the crude extract before and after affinity chromatography and a sample of purified lectin. The purified lectin and the initial sainfoin extract formed a confluent precipitin

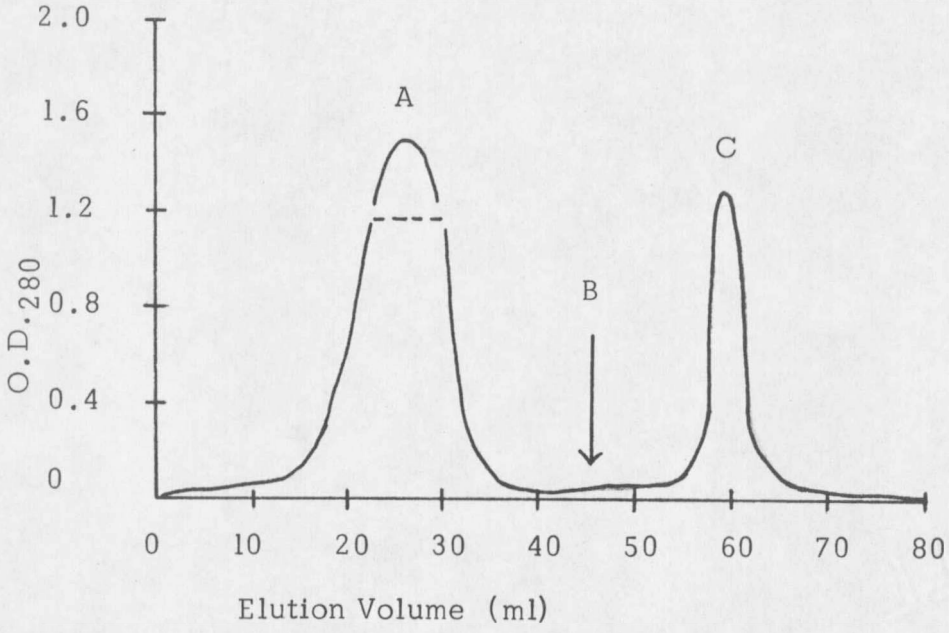

Figure 1. Elution of sainfoin lectin from mannose-sepharose affinity chromatography column, 1.5 cm by 4.0 cm. A - peak resulting from application of crude protein extract, B - application of elution buffer, PBS - Glu (0.5 M), C - peak resulting from elution of pure sainfoin lectin antigen.