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Nodule Specific Proteins in Alfalfa (*Medicago sativa* L.)[1]

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Abstract

Legume root nodule formation, a complex developmental process that is dependent upon both the host plant and *Rhizobium*, ultimately results in fixation of nitrogen and assimilation of that fixed nitrogen. Accompanying this process, we report the presence of at least nineteen nodule specific proteins detected with an enzyme-linked immune assay. Most of these nodule specific proteins appear to be plant proteins. However, one appears to originate from bacteria and three may originate from bacteroids. Ineffective nodules controlled by the plant gene in_1 had reduced concentrations of most nodule specific polypeptides. The nodule specific protein compliment of ineffective nodules controlled by the plant gene in_1 was similar to that of ineffective nodules controlled by the not$^+$ fix$^-$ *Rhizobium meliloti* strain 102F26. This similarity suggests that common mechanisms may regulate ineffectiveness induced by certain plant and rhizobial genes. By contrast, tumor-like ineffective nodules controlled by the plant gene in_3 had little crossreactivity with nodule specific antiserum. The protein in tumor-like nodules thus appears to be more related to the protein of root tissue than to that of effective nodules. Antibodies prepared against effective alfalfa nodules and made nodule-specific crossreact with nodule-polypeptides from both amide and ureide forming legumes. Proteins specific to alfalfa nodules may be conserved across a diverse range of legumes.

Key words: N_2-fixation, nodulins, ineffective nodules, amide, ureides.

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1. Introduction

Rhizobium-legume induced root nodules are highly organized hyper-plastic tissue masses derived from root cortical cells (Sutton, 1983; Vance, 1983). Root nodule formation and function require numerous specific events which are controlled by both the host plant and *Rhizobium* genomes (Jordan, 1974; Vance, 1983). While substantial progress has been made in identifying rhizobial genes and gene products that regulate root nodule symbiosis, relatively little progress has been made in understanding the contribution of plant genes to symbiosis (Bisseling et al., 1983; Verma et al., 1984). Several symbiotic genes have been documented through classical genetic studies of pea (Holl, 1975), clover (Nutman, 1969), soybean (Caldwell, 1966), and alfalfa (Peterson and Barnes, 1981), yet the physiological and biochemical manifestation of these genes is poorly understood.

Studies with soybean showed that host plant genes code for some twenty nodule specific proteins (Legocki and Verma, 1980; Auger and Verma, 1981). These are plant proteins found only in root nodules and not in roots or other host organs. Using immunological techniques dependent on ^{35}S and ^{125}I approximately thirty nodulins were detected in pea nodules (Bisseling et al., 1983). In contrast, using similar techniques only nine nodulins were detectable in alfalfa (Lang-Unnasch and Ausubel, 1985). Although the explanation for the reduced number of nodulins in alfalfa is not readily evident, it may be due to the immunization procedure. In pea (Bisseling et al., 1983) and soybeans (Legocki and Verma, 1980) rabbits were immunized with mg quantities of nodule protein while in the alfalfa study (Lang-Unnasch and Ausubel, 1985) rabbits were immunized with 50 to 150 μg of nodule protein. Thus proteins that either exhibit low antigenicity, or that were in low concentrations in nodules may not have elicited a satisfactory immune response.

Nodulin expression has been shown to be a function of the stage of nodule development and dependent upon the effectivenss of the symbiosis (Verma et al., 1984). Fuller and Verma (1984) showed that leghemoglobin and nodulin genes in soybean are activated prior to visible nodule morphogenesis and appeared to be independent of N_2-fixation, while in pea the majority were detected concomitantly with leghemoglobin and active N_2-fixation (Bisseling et al., 1983). Lang-Unnasch and Ausubel (1985) detected two alfalfa nodulins prior to the appearance of visible nodules, the majority during development of visible nodules, and one nodulin appeared to be associated with the late stages of nodule development. Ineffective *Rhizobium* strains (nod$^+$ fix$^-$) had pleiotropic affects on nodulin expression in soybean (Fuller and Verma, 1984), pea (Bisseling et al., 1983) and alfalfa (Lang-Unnasch and Ausubel, 1985). In most instances nodulin synthesis appeared to be reduced. To date, nodulins have not been evaluated in root nodules that are ineffective due to the host plant genome.

Verma et al. (1984) suggested that there may be two distinct groups of nodulins: 1) C-nodulins that are conserved between species and that are common to all legumes; and 2) S-nodulins that are species specific. The conservation of nodule specific leghemoglobin between legume species has been documented

(Jing et al., 1982). Similarly Cullimore and Miflin (1984) recently demonstrated that immune serum prepared against *Phaseolus vulgaris* root nodule specific glutamine synthetase (EC 6.3.1.2) cross reacted with pea and soybean glutamine synthetase. Alfalfa nodule specific immune serum (Lang-Unnasch and Ausubel, 1985) appeared to cross react with two polypeptides from pea nodules. The cross reactivity of nodule specific antiserum between a number of diverse legume species has not been documented. Such experiments may assist in resolving the presence of C- and S-type nodulins within a species.

The objectives of our studies were: 1) to assess nodule specific proteins in alfalfa using antiserum induced in rabbits in response to injection of mg quantities of nodule soluble protein; 2) to determine the occurrence of nodule specific proteins in nodules that are ineffective due to the host plant genome; 3) to evaluate if alfalfa nodule specific antiserum crossreacts with nodule proteins from a number of diverse species. In contrast to previous studies requiring ^{35}S and ^{125}I, we used an extremely sensitive non-radioactive enzyme-linked immunosorbent assay. A preliminary report of these studies has been presented (Vance et al., 1984).

2. Materials and Methods

2.1 *Plant material*

Alfalfa (*Medicago sativa* L., cv. Saranac) and birdsfoot trefoil (*Lotus corniculatus* L. cv. Norcen), were grown as previously described (Vance et al., 1979), Adzuki bean (*Vigna angularis* Willd, Ohwi and Ohasmi, cv. Takara), soybean (*Glycine max* L. Merr. cv. Hodson), pea (*Pisum sativum* L. cv. Alaska), Texas bluebonnet (*Lupinus subcarnosus* L. cv. Hook) and clover (*Trifolium pratense* L. cv. Lakeland) were grown in a glasshouse, in sand, in 15 cm clay pots with supplemental fluorescent light at a quantum flux density of 400 μE m^{-2} sec^{-1} during a 16/8-hr light/dark cycle at 24/19°C. Plants ineffective due to the host plant genome in_1ag (Agate background), in_1sa (Saranac background) and in_3 (tumor-like) were grown as described by Peterson and Barnes (1981). At the time of seeding the sand was inoculated with the appropriate preparation of commercially available *Rhizobium* species (Nitragin Co., Milwaukee, WI)[2]. Macro- and micronutrients except for N were incorporated into the sand at planting. All plants were maintained in sand throughout the experiments with no supplemental N added. Effective nodules were collected from 6-week old plants, frozen and stored at -20°C until used.

2.2 *Antiserum production*

Nodule soluble protein for production of antiserum was prepared from frozen nodules of 6-week old greenhouse grown alfalfa plants. Nodules were homogenized in phosphate buffered saline (PBS, 10 mM K_2HPO_4, 150 mM NaCl, pH 7.2) and cell debris removed by centrifugation at 20,000 × g for 20 min. The supernatant

was treated with solid $(NH_4)_2SO_4$ to 90% saturation and the precipitated protein collected by centrifugation. The pellet was solubilized in a minimum volume of PBS. All protein determinations were performed on 10% TCA precipitates following the method of Lowry et al. (1951).

Rabbits (NZW) were initially immunized by subcutaneous injections of 25 mg of total nodule soluble protein in Freund's complete adjuvant (Difco, Detroit, MI)[2]. At least 4 booster injections of 25 mg protein each in incomplete adjuvant were given at bimonthly intervals. Rabbits were bled initially at 1 month and at 10 days after each booster immunization. Blood serum was concentrated with 80% saturated $(NH_4)_2SO_4$ according to Herbert et al. (1973). The serum was precipitated twice and solubilized in PBS at one-half the original volume. Serum titer was determined by double diffusion (Ouchterlony, 1968).

Antiserum prepared to total soluble protein from effective nodules was made "nodule specific" by absorption with uninfected root protein (Legocki and Verma, 1980; Bisseling et al., 1983). Root protein (30, 60, 120, 240, and 480 μg) was sequentially added to a 200 μl aliquot of anti-nodule serum. After each addition of root protein the nodule antiserum was incubated at 4°C for 6 to 8 hr and the resulting immunoprecipitate was removed by centrifugation.

Leghemoglobin purified according to Jing et al. (1982) was used to elicit antibody production in rabbits (NZW). Initially 5 mg of leghemoglobin in Freund's complete adjuvant was injected subcutaneously. Three booster injections of 2.5, 5.0, and 5.0 mg in incomplete adjuvant were given at 10 day intervals. Two more injections of 10 mg of leghemoglobin conjugated with glutaraldehyde (10:1 vol/vol, followed by dialysis overnight in PBS) were then given at two week intervals. Serum was collected six weeks after the initial immunization and at one week after the final immunization. Serum titer was assessed by double diffusion (Ouchterlony, 1968).

2.3 *Electrophoresis*

All samples for electrophoresis were homogenized in PBS containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 μM antipain. Extracts were centrifuged at 20,000 $\times$ g to remove cell debris and bacteroids. The supernatant was centrifuged again at 105,000 $\times$ g for 2 hr (Legocki and Verma, 1980). The supernatant containing soluble cytoplasmic proteins was dialyzed overnight at 4°C in 10 mM Tris pH 7.8 containing 1 mM PMSF. Extracts diluted to a protein concentration of $5 mg \cdot ml^{-1}$ and antipain was added to a final concentration of 10 μM. Protein samples of 5 to 10 μg were diluted prior to electrophoresis with sample buffer 1:1 (vol/vol) containing 5% B-mercaptoethanol and 4% SDS. The samples of SDS protein were treated by boiling. Samples were routinely not boiled. However, as a check, some samples were boiled and we found no changes in band number and in resolution. Proteins were separated in 10% SDS-polyacrylamide gels using the discontinuous buffer system of Maizel (1971). Gels were run at a constant

current of 15 mAmps for 3 hrs with constant cooling. Molecular weight markers consisted of phosphorylase B (94,000), bovine serum albumin (68,000), creatine phosphokinase (40,000), soybean trypsin inhibitor (21,000), RNAase (14,000), and cytochrome C (12,400). Proteins were visualized by silver staining (Merrill et al., 1981).

2.4 *Visualization of nodulins*

Prior to protein transfer to nitrocellulose, the SDS-polyacrylamide gels were incubated in 3 changes of transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol (vol/vol)). Protein was transferred electrophoretically to nitrocellulose (Towbin et al., 1979). Gels were then silver stained to insure complete protein transfer. Proteins transferred to nitrocellulose were visualized with an enzyme-linked immunosorbent assay (Towbin et al., 1979). Nonspecific binding sites on the nitrocellulose filter were blocked for 8 hr with 1% BSA (wt/vol) in PBS. Nitrocellulose blots were then incubated overnight at 4°C with primary antiserum (preimmune, nodule specific, or leghemoglobin) in PBS containing 0.5% BSA (wt/vol) and 0.05% Tween-20 (vol/vol). Nitrocellulose blots were then extensively washed in PBS containing 0.05% Tween-20 and incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad, Richmond, CA)[2] in PBS containing 1% gelatin (wt/vol). Blots were rinsed in 3 washes of PBS-Tween-20, 2 washes of PBS and 1 wash of 50 mM K_2HPO_4. Purple bands were developed by reacting blots in 120 ml of peroxidase substrate solution containing 60 mg 4-chloro-napthol and 0.015% H_2O_2 (vol/vol) (Hawkes et al., 1982).

3. Results

At least nineteen nodule specific proteins were detected from effective alfalfa nodule soluble proteins separated by SDS polyacrylamide gel electrophoresis, then transferred to nitrocellulose blots and visualized with an enzyme-linked immunosorbent assay (Figs. 1, 2, 3, and 4). A lane containing root protein was usually included as a control to assess the specificity of our nodule specific antiserum (Fig. 1). Typically no distinct protein bands were visualized in roots that corresponded to the polypeptides from effective nodules, thus indicating that our antiserum was nodule specific. Nodule specific proteins ranged in molecular weight from about 12.5 kd up to 140 d. Although there was some inconsistency in detection of certain proteins (i.e. 75 and 140 kd), those labelled in Figs. 1-4 were observed at a frequency of 75% or greater. With effective nodule proteins, 5 $\mu g \cdot well^{-1}$ was easily detectable on nitrocellulose blots with the enzyme-linked immunosorbent assay. Above 7.5 μg protein$\cdot$well^{-1} problems occurred with overstaining and smearing. However, occasionally 7.5 μg of protein from ineffective nodules was loaded into wells to assess if our technique was below the limits of detection. Preimmune serum did not resolve any protein bands in nodules and roots.

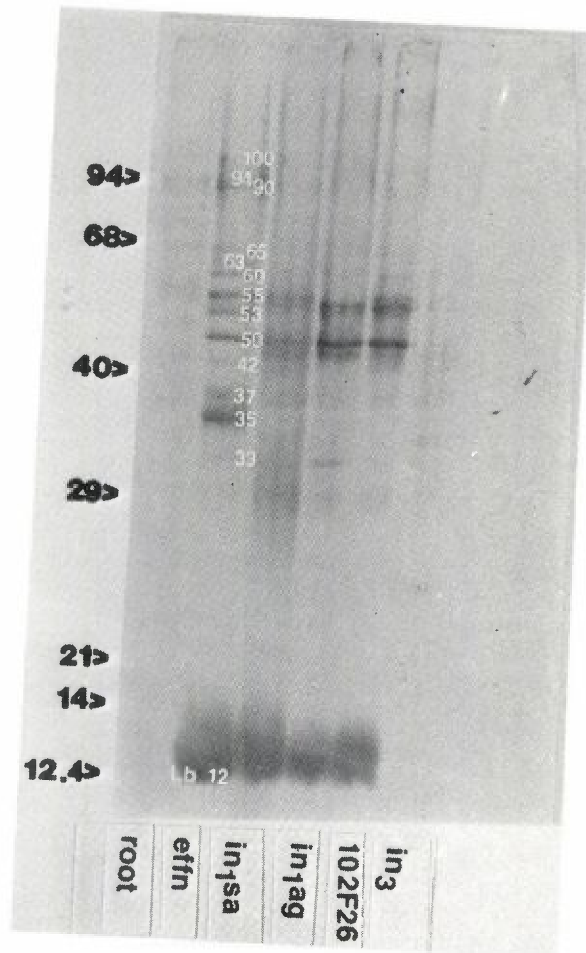

Figure 1. Nodule specific proteins of effective (effn), plant gene controlled ineffective (in_1sa, in_1ag, in_3), and *Rhizobium* strain controlled ineffective (102F26) nodules and roots of alfalfa. Protein separated by SDS-polyacrylamide gel electrophoresis was transferred to nitrocellulose and visualized using nodule specific antiserum coupled to an enzyme linked immunoassay. Each lane contains 5 μg protein except in_3 which contains 7.5 μg. The molecular weights (kd) of protein standards are indicated by the bold numbers. The smaller white numbers indicate the molecular weights of nodule specific proteins. Leghemoglobin is indicated by Lb. Nodule specific proteins were numbered according to nomenclature suggested by van Kammen (1984).

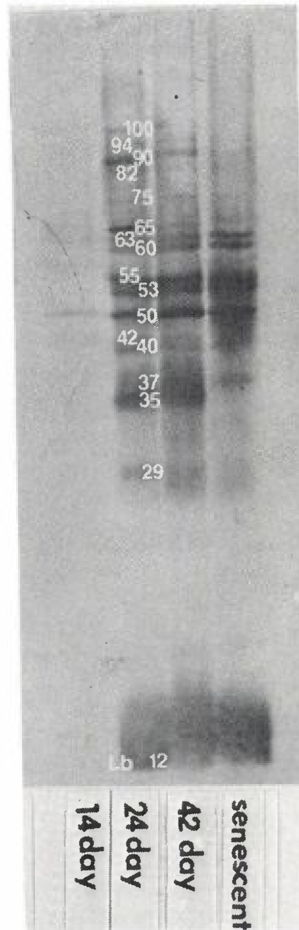

Figure 2. Developmental profile of nodule specific proteins found in effective nodules taken 14, 24, and 42 days planting and in senescent alfalfa nodules (taken 7 days after detopping of shoots). Proteins were separated by SDS-polyacrylamide gel electrophoresis Western blotted and probed with effective alfalfa nodule specific antiserum. Each lane contains 5 μg of protein. The white numbers indicate molecular weight (kd) and location of nodule specific proteins. Leghemoglobin is indicated by Lb.

Nodule specific proteins in effective and ineffective nodules were compared simultaneously on protein blots prepared from whole nodule protein separated by SDS polyacrylamide gel electrophoresis. Blots were probed with effective alfalfa nodule specific antiserum. The nodule specific protein compliment of ineffective nodules was strikingly different than that of effective nodules (Fig. 1). Several proteins were either reduced in quantity or occurred at concentrations too low to

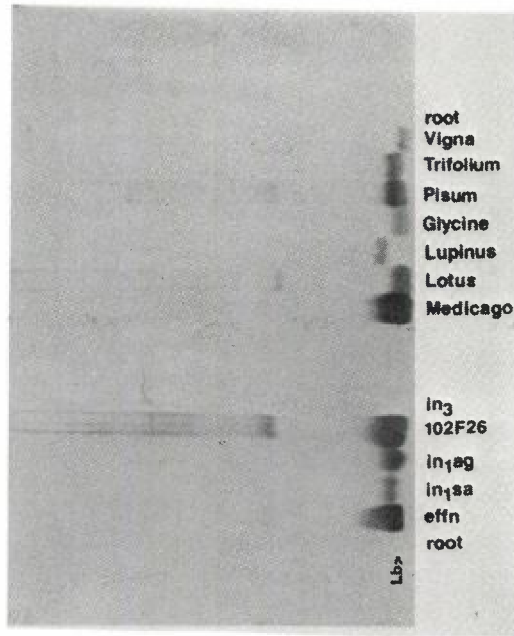

Figure 3. Nitrocellulose blot of leghemoglobin showing the presence of leghemoglobin in effective and ineffective alfalfa nodules and the crossreactivity of alfalfa leghemoglobin antibodies with leghemoglobin polypeptides from effective nodules of other legume species. Proteins, Western blotted from an SDS-polyacrylamide electrophoresis gel, were probed with antibodies prepared against purified alfalfa nodule leghemoglobin. Lane designations are: effective alfalfa, effn and *Medicago*; plant controlled ineffective in_1sa, in_1ag, in_3; *Rhizobium* controlled ineffective, 102F26; birdsfoot trefoil, *Lotus*; Texas bluebonnet, *Lupinus*; soybean, *Glycine*; pea, *Pisum*; clover, *Trifolium*; adzuki bean, *Vigna*; root. Leghemoglobin is indicated by Lb. Each lane contains 5 μg protein.

detect. Conspicuously absent or reduced were proteins of 35, 53, 63, 90, 94, and 100 kd molecular weight. However, two protein regions at 50 and 55 kd molecular weight appeared to be enhanced in ineffective nodules induced by the plant in_1 gene in in_1ag and in nodules induced by the ineffective *R. meliloti* strain 102F26.

Ineffective nodules induced by the plant in_1 gene in the cultivar Saranac (in_1sa) and Agate (in_1ag) were compared to ascertain if expression of this gene was similar in different cultivars (Fig. 1). Similar nodule proteins were detected in the cultivars, however, the bands detectable at 50 and 55 kd were substantially reduced in in_1sa. The nodule specific protein pattern of in_1ag appeared more similar to that of *R. meliloti* 102F26 induced nodules than to that of nodules induced by in_1sa.

Little crossreactivity was observed when protein prepared from the tumor-like ineffective nodules of in_3 was probed with effective nodule specific antibodies (Fig.

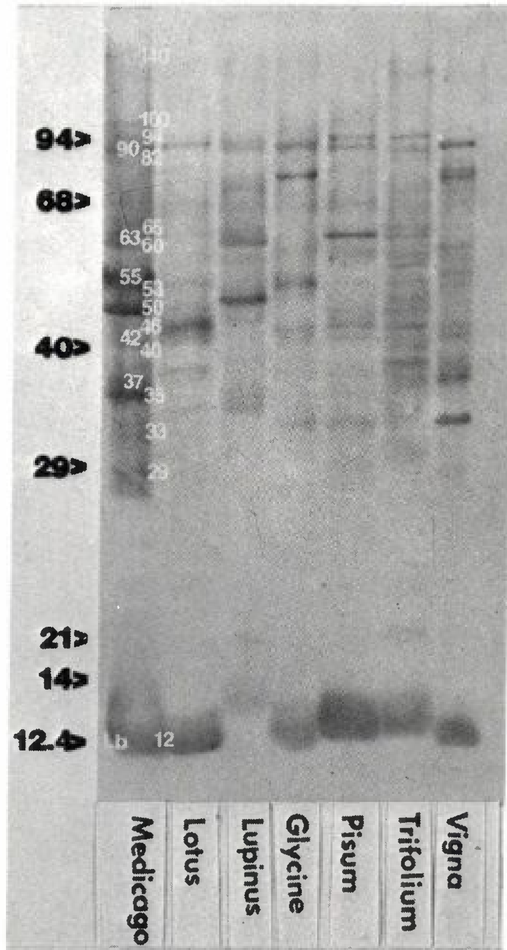

Figure 4. Crossreactivity of alfalfa nodule specific antiserum with effective nodule proteins of various legume species. Nodule soluble protein (5 μg) from each species was separated by SDS-polyacrylamide gel electrophoresis, transferred to nitro-cellulose, and probed with effective alfalfa nodule specific antiserum. Molecular weights (kd) of protein standards are indicated by bold numbers. The white numbers indicate the molecular weights of effective alfalfa specific proteins. Leghemoglobin is indicated by Lb. Band designations are: alfalfa, *Medicago*; birdsfoot trefoil, *Lotus*; Texas bluebonnet, *Lupinus*; soybean, *Glycine*; clover, *Trifolium*; aszuki bean, *Vigna*.

1). Other than the slight crossreactivity at 50 and 55 kd, the in_3 lane appeared similar to the root control lane. Protein prepared from tumor-like nodules in_2 and in_4in_5 also showed very little crossreactivity with nodule specific antiserum (data not shown).

To assess the developmental profile of nodule specific proteins, extracts prepared from nodules 14, 24, and 42 days after planting were electrophoresed, blotted

and probed with nodule specific antiserum (Fig. 2). Senescent nodules were also collected from 42 day-old plants that had been detopped 7 days prior to nodule collection. Nodules collected at 14 days after planting were 0.5 to 1.0 mm in diameter, white, and contained few bacteroids and do not fix nitrogen (Vance et al., 1980). At this time only the 50, 63 and 65 kd proteins were conspicuous. By 24 days after planting nodules were small (2 to 3 mm), uniformly pink, and vigorously fixed nitrogen. The majority of nodule specific proteins were easily detectable. At 42 days after planting flower buds are beginning to form and the nodules were large (4 to 6 mm), and uniformly pink. The nodule specific protein compliment is similar to that at 24 days. However, proteins at 35, 60, and 63 kd appear to have increased in intensity while those at 40 and 65 kd appear to be reduced. Seven days after detopping 42 day old plants, nodules begin to senesce and nitrogenase activity is reduced by 80% (Vance et al., 1979). These visual changes are reflected in a dramatic reduction in the nodule specific proteins at 35, 50, 65, 90, 94, and 100 kd. We did not assess nodule specific proteins as plant tops began to regrow and nodules recover.

Antiserum prepared against purified alfalfa nodule leghemoglobin was used to assess the presence of leghemoglobin in ineffective nodules and the crossreactivity of alfalfa leghemoglobin with other legume species (Fig. 3). Leghemoglobin was detectable in ineffective nodules controlled by the plant gene in_1 and ineffective nodules controlled by *R. meliloti* strain 102F26. However, leghemoglobin polypeptides were not detectable in either roots or tumor-like ineffective nodules controlled by the plant gene in_3. Leghemoglobin polypeptides were also not detectable in tumor-like ineffective nodules controlled by the plant genes in_2 and in_4in_5 (data not shown). Antiserum to alfalfa leghemoglobin crossreacted strongly with comparable low molecular weight polypeptides from *Lotus*, *Pisum* and *Trifolium*. In contrast, antiserum to alfalfa leghemoglobin was much less crossreactive with *Lupinus*, *Glycine* and *Vigna*. Results similar to these were also observed with nodule specific antiserum such as in Figs. 1 and 4.

We investigated common (conserved) type C-nodulins in diverse species by comparing alfalfa (*Medicago*), *Lotus*, *Lupinus*, *Glycine*, *Pisum*, *Trifolium*, and *Vigna* whole nodule soluble protein extracts subjected to SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose blots and probed with alfalfa nodule specific antiserum (Fig. 4). In addition to leghemoglobin at 12 kd at least 8 other nodule specific proteins may be candidates as type C-nodulins. Consistent crossreactivity was observed between alfalfa nodule specific antiserum and protein of other legume species with molecular weights of 33, 40, 42, 55, 63, 65, 90, and 94 kd. The 33, 40, and 42 kd bands were in all species except *Lupinus*. The 55, 65, and 94 kd band occurred in all species. The protein bands at 63 and 90 kd may occur in all species, however, we need further documentation for those bands. There are intense alfalfa nodule specific bands at 29, 35, and 50 kd that do not appear to crossreact in other species. These protein subunits may be specific for alfalfa.

4. Discussion

Including leghemoglobin, we resolved at least nineteen nodule specific proteins from effective alfalfa nodules using an enzyme-linked immunosorbent assay. In addition to the nine proteins resolved by Lang-Unnasch and Ausubel (1985) we resolved nodule specific proteins of 33, 35, 40, 42, 46, 53, 55, 63, and 94 kd. We also obtained a protein band at 65 kd that corresponds to the B-66 band reported by Lang-Unnasch and Ausubel (1985). The discrepancies in their resolution versus ours may result from differences in antibody production as well as from procedures involved in obtaining nodule specific antiserum.

We induced antibodies by injecting rabbits with 100-fold more nodule protein than did Lang-Unnasch and Ausubel (1985). Thus we may have obtained antibodies to a wider range of proteins particularly those occurring in nodules at low concentrations. The total amount of protein that we used for immunization is similar to that used in soybean (Legocki and Verma, 1980) and pea (Bisseling et al., 1983) nodulin studies. The total of nineteen nodule specific proteins in alfalfa is similar to that reported for soybean (Legocki and Verma, 1980) and somewhat less than that reported for pea (Bisseling et al., 1983). Our numbering of bands is conservative since two dimensional gels were not run to resolve polypeptides with similar subunit molecular weights but unique isoelectric points.

Similar to pea and soybean studies, we obtained nodule specific antiserum by absorbing whole nodule antiserum with root antigens. In contrast, Lang-Unnasch and Ausubel (1985) produced nodule specific antiserum by preadsorption of whole nodule antiserum with root proteins bound to cyanogen-bromide activated sepharose. Our procedure enabled us to resolve *de novo* synthesized nodule specific proteins as well as some root proteins that may have been highly enriched in nodules. Although we did not absorb whole nodule antiserum with free living *R. meliloti* protein, we did analyze blots of *R. meliloti* bacterial and bacteroid protein with nodule specific antiserum. Similar to Lang-Unnasch and Ausubel (1985) the only bacterial protein to crossreact was the 65 kd band. However, bacteroid protein bands at 35, 60, and 63 kd as well as 65 kd band crossreacted indicating that those bands may be bacteroid derived. Therefore in effective alfalfa nodules at least potentially fifteen plant-derived nodule specific proteins were detected in our study.

The reduction and loss of nodule specific proteins in ineffective nodules induced by the plant gene in_1 are similar to those reported for pea (Bisseling et al., 1983), soybean (Auger and Verma, 1980), and alfalfa (Lang-Unnasch and Ausubel, 1985) that were ineffective as a result of nod$^+$ fix$^-$ strains of *Rhizobium*. The nodule specific protein profile of plant gene in_1 ineffective nodules was also similar to the profile of ineffective nodules induced by the nod$^+$ fix$^-$ 102F26 *R. meliloti* strain. This similarity between plant and bacterial controlled ineffective nodules suggests that the ultimate expression of some ineffectiveness may be similar whether controlled by plant or *Rhizobium*. Although the overall profile of in_1 was similar in Agate and Saranac backgrounds there were differences in intensity

of protein bands at 50 and 55 kd. These differences and previously documented differences in development (Vance and Johnson, 1983) indicate that the biochemical and physiological expression of the in_1 gene is cultivar dependent.

The nodule specific protein compliments of tumor-like in_3 ineffective nodules appear substantially different than any previously reported ineffective nodule. Protein from in_3 nodules had little crossreactivity with effective nodule specific antiserum. These data provide support for the concept that the physiology and biochemistry of tumor-like nodules may be more similar to root cortical tissue than to effective root nodules. Readily visible nodule specific protein bands occur at 50 to 55 kd in in_1ag, in_1sa and 102F26 ineffective nodules. Yet these bands are reduced substantially or absent in tumor-like in_3 nodules. This difference may relate to the presence of bacteria in the nodule. Structural studies of these nodules (Vance et al., 1980; Vance and Johnson, 1983) showed that in_1ag, in_1sa and 102F26 contained numerous bacteroids while bacteria were absent from in_3 nodules. Studies of in_2 and in_4in_5 tumor-like nodules have also shown an absence of bacteroids (Vance et al., 1980; Vance and Johnson, 1983). The protein from in_2 and in_4in_5 nodules also has little crossreactivity with effective nodule antiserum. This data provide additional support for the concept that some nodule specific proteins are involved in nodule development while others are involved in their effectiveness (Legocki and Verma, 1979).

The decline in nodule specific proteins in ineffective nodules can be directly related to the reduction in activity of certain enzymes. We previously documented (Vance and Johnson, 1983; Vance et al., 1983) that plant controlled in_1, in_2, in_3 and in_4in_5 ineffective nodules and *Rhizobium* controlled (nod$^+$ fix$^-$) nodules are reduced in total soluble protein, glutamate synthase (GOGAT), glutamine synthetase (GS) and phosphoenolpyruvate carboxylase (PEPC). Some of the protein bands that are either reduced or absent in ineffective nodules probably correspond to polypeptides of these enzymes.

Previous studies (Bisseling et al., 1983; Legocki and Verma, 1979; Lang-Unnasch and Ausubel, 1985) have shown that some nodule specific proteins appear prior to visible nodule formation. In small white nodules lacking visible leghemoglobin and bacteroids we did detect three nodule specific proteins. Similar to other developmental studies, the development of bacteroids and nitrogenase activity was accompanied by the formation of the majority of nodule specific proteins.

Although our developmental study of nodule specific proteins is less extensive, in terms of sampling dates, than previous studies, we are the first to artificially induce nodule senescence and then evaluate the expression of nodule specific proteins. We induced nodule senescence by removing the plant shoot, a treatment similar to that an alfalfa plant would receive in natural conditions. We previously demonstrated that shoot removal induced nodule senescence (Vance et al., 1979). This results in increased proteolysis, reduced nitrogenase, GS, GOGAT, PEPC activities and a decreased bacteroid content. Studies reported herein indicate that senescence is accompanied by either reduced amounts or complete loss of certain

proteins. Similar to ineffective nodules, some of these proteins that are reduced in senescence probably correspond to GS, GOGAT, and PEPC.

Our nodule specific immune serum crossreacts with a number of polypeptides from many diverse legume species. This observation gives support to the Verma et al. (1984) suggestion that nodules contain specific polypeptides that are common (C-nodulins) to all legumes and that these are related to nodule function. Likely candidates for these common nodulins are key enzymes of ammonia and carbon assimilation. Root nodule GS from *Phaseolus* (Cullimore and Miflin, 1984) has a molecular weight of 42 kd and antigens to the enzyme crossreact with pea and soybean GS. Alfalfa nodule GS is also 42 kd in molecular weight (Groat and Schrader, 1982). We consistently observe crossreactive bands at 42 kd. Inclusively these observations suggest that our crossreactive bands at 42 kd may be GS. Bergmann et al. (1983) indicated that nodulin-35 uricase may be conserved between ureide producing species. This particular nodule specific protein would probably be absent from amide producing legumes. We detected a nodule specific protein at 35 kd. However, alfalfa nodule specific antiserum did not crossreact with other species at 35 kd. Another possible C-nodulin is PEPC. All effective nodules tested to date fix CO_2 (Vance et al., 1983). Circumstantial evidence suggests that the 94 kd protein band is the C-nodulin PEPC. Alfalfa nodule PEPC comprises about 2% of the total soluble protein of effective nodules and has a subunit molecular weight of 94 to 100 kd (Vance and Stade, 1984). Alfalfa nodule specific antiserum strongly crossreacts with all species at 94 kd.

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