

PAWEŁ STRÓŻYCKI and ANDRZEJ B. LEGOCKI

ISOLATION AND CHARACTERIZATION OF ROOT NODULE PROTEINS FROM LUPIN *

*Department of Biochemistry, University of Agriculture,
Wołyńska 35, 60-637 Poznań*

*Institute of Bioorganic Chemistry, Polish Academy of Sciences,
Noskowskiego 12, 61-704 Poznań, Poland*

Received 22 June, 1987

A group of root nodule-specific plant proteins (nodulins) has been isolated from yellow lupin (*Lupinus luteus*) by immunoaffinity chromatography. The cytoplasmic nodule protein extract was initially enriched in nodulins on a column with immobilized IgG fraction. It was then purified by chromatography on Sepharose 4B - bound IgG against uninfected root proteins and finally on Sepharose 4B - bound IgG against *Rhizobium lupini* proteins. Rocket immunoelectrophoresis showed that the nodulin preparation did not react with antibodies against root or bacterial proteins. SDS gel electrophoresis of lupin nodulins revealed at least 23 polypeptides ranging in M_r from 7 000 to 70 000, probably representing protein subunits.

The root nodule is a structurally complex which develops after infection of legume plants with *Rhizobium*, a Gram negative soil bacteria. Due to this intracellular symbiotic association with the host plant, *Rhizobium* bacteroids are able to fix atmospheric nitrogen [1]. Root-nodule symbiosis depends on a number of genetic [2] and biochemical [3] events that involve both symbionts, even during the initial recognition process.

Electrophoretic analysis of host proteins from uninfected roots and root nodules of soybean has revealed that the majority of proteins are common to both tissues [4]. However, immunological analysis showed the presence of several plant polypeptides which are restricted to root nodules. These proteins called "nodulins" have been detected in soybean [5], pea [6], lupin [7]

* This work was supported by the Polish Academy of Sciences within the Project 04.12.2.

and alfalfa [8] root nodules. Lack of some nodulins in ineffective root nodules [6] suggested that nodulins may play a direct role in the nitrogen fixation process. Biological functions of the following nodulins are known: leg-hemoglobins [9], nodule-specific form of uricase [10], sucrose synthase [11], glutamine synthetase [12], choline kinase II [13] and xanthine dehydrogenase [14]. Specification of the function of other nodulins present at low concentration in root nodules requires their isolation. The present paper describes a general, immunological, convenient method for isolation of legume plant nodule on a preparative scale.

MATERIALS AND METHODS

Plant culture. Lupin seeds (*Lupinus luteus* var. Ventus) were surface sterilized and germinated at 27°C for three days in the dark. To obtain mature uninfected roots, seedlings were grown at 27°C for 21 days on perlite with complete Dilworth solution [9] containing 5 mM Ca(NO₃)₂ and without CaCl₂. For root nodule isolation, three days-old seedlings were inoculated with *Rhizobium lupini* strain 3045 (USDA) and repotted on sterile perlite. They were grown at 27°C in a controlled environment, watered daily and given nitrogen-free Dilworth solution twice a week. Root nodules were harvested usually five weeks after infection. For some experiments root nodules were collected from field cultures 4-5 weeks after germination; for developmental studies they were collected earlier.

Plant protein preparation. Uninfected roots or root nodules were pulverized with liquid nitrogen and extracted with 2 vol. of extraction buffer containing 50 mM Tris/HCl, pH 8.7, 20 mM KCl and 10 mM MgCl₂ [4]. Nodule extracts were centrifuged at 23 000 ×g for 20 min and the supernatant was recentrifuged at 106 000 ×g for 2.5 h. The soluble cytoplasmic proteins were precipitated with solid ammonium sulphate to 0.9 saturation, dissolved in 20 mM Tris/HCl, pH 7.5 buffer, and dialysed against the same buffer.

Bacterial proteins. *Rhizobium lupini* strain 3045 (USDA) was grown (4 days at 4°C) in a solution containing: 0.5 g KH₂PO₄; 0.2 g MgSO₄ · 7 H₂O; 0.1 g NaCl; 10 g mannitol; 0.5 g yeast extract per 1 liter (pH ranging from 6.8 to 7.0). Bacteria were then centrifuged at 3000 ×g for 10 min, washed four times with extraction buffer (the same as for plant protein preparation), suspended in a small volume of extraction buffer and sonicated for 20 min. The cell extract was centrifuged at 23 000 ×g for 20 min and proteins from the supernatant were precipitated with ammonium sulphate to 0.99 saturation, dissolved in 20 mM Tris/HCl, pH 7.5 buffer, and dialyzed against the same buffer.

Preparation of antibodies. Female New Zealand rabbits were injected subcutaneously each week with 20 mg of crude protein extracts, or with 0.4 mg of purified lupin nodulins in Freund's complete adjuvant (1:1 v/v). Rabbits were bled ten days after the fourth injection, and the specificity of the antiserum was checked by the Ouchterlony immunodiffusion technique [15]. The IgG fractions were obtained by ammonium sulphate fractionation (0 to 0.33 saturation), followed by DEAE-Sephadex A-50 chromatography [16].

Preparation of nodule-specific immunoglobulins. Nodule-specific immunoglobulins were prepared using immunoaffinity chromatography. To 5 ml Sepharose gel modified with proteins from uninfected roots (see below) 6 mg of IgG against nodule proteins was added in 10 ml 10 mM Tris/HCl buffer, pH 8.6. The suspension was stirred gently overnight at 4°C, then the unbound fraction was collected and rechromatographed on the same gel. This step was repeated twice. The gel was regenerated each time with 0.1 M potassium-phosphate; 0.5 M NaCl buffer, pH 8.0, containing 8 M urea.

Preparation of affinity adsorbents. CNBr-activated Sepharose 4B was prepared according to manufacturer's instructions (Pharmacia). After washing with carbonate buffer (0.2 M NaHCO₃, pH 8.6 for immunoglobulins or 0.1 M NaHCO₃, pH 8.3 for plant proteins) the suitable proteins were added at concentration of 10 mg per 1 ml of the Sepharose gel, and stirred gently overnight at 4°C. Under these conditions 95% of proteins was bound to the adsorbent. The coupled gel was subsequently filtered, washed with 0.1 M bicarbonate buffer, pH 8.6 and the remaining active groups of the gel were blocked with 0.2 M glycine buffer, pH 8.0 at room temperature. The excess of unbound immunoglobulin was washed with 0.1 M sodium acetate buffer, pH 4.0, containing 0.5 M NaCl, followed by 0.1 M NaHCO₃, pH 8.6, containing 0.5 M NaCl.

Purification of lupin nodulins by immunoaffinity chromatography. Total cytoplasmic proteins from root nodules were initially fractionated on a gel containing the IgG fraction for nodule-specific proteins coupled to Sepharose 4B (see above). Usually 20 mg of protein in 10 ml of 0.1 M potassium-phosphate buffer, pH 8.0, containing 0.5 M NaCl were added to 3.5 ml of the sedimented gel; the suspension was mixed gently for 16 h at 4°C and loaded into a small column. The unbound proteins were washed with the same buffer until the adsorption of the eluate dropped to zero. Fractions enriched in nodule-specific proteins were eluted with phosphate-NaCl buffer, pH 8.0, containing 8 M urea, pooled and dialyzed against 20 mM Tris/HCl, pH 8.6. They were subsequently concentrated by lyophilization and submitted to a second affinity chromatography using Sepharose 4B coupled with the IgG fraction against root proteins. Two milligrams of

proteins in 0.1 M potassium-phosphate-0.5 M NaCl buffer were mixed gently for 16 h with 5 ml of the gel, previously equilibrated with the same buffer. Proteins did not bind to the gel and were eluted from the column. The column was then regenerated with phosphate-NaCl buffer containing 8 M urea and the unbound fraction was rechromatographed on the same column. To remove residual contaminations with bacterial proteins the nodule were finally purified on a Sepharose 4B column (5 ml) coupled with IgG against *R. lupini* proteins. The chromatographic conditions were as for the previous column. The presence of the nodulins in the eluate was tested by rocket immunoelectrophoresis. All chromatographic steps were performed at 4°C. All three columns were used 3-5 times without significant evidence of deterioration.

Immunoelectrophoresis. Rocket, tandem-crossed and line electrophoresis were performed in 20 mM Tris/HCl buffer, pH 8.6 using 1% agarose gels according to Axelsen *et al.* [16]. Samples containing 50-80 micrograms of proteins were applied into wells located in the lower gel section (rocket and tandem-crossed), while approximately 400 micrograms of protein were mixed with the gel for line immunoelectrophoresis. The upper gel section contained 15% (w/v) of the respective IgG fraction. The electrophoresis was carried out at 4°C at 3-5 V/cm for 17-24 h and at 16 V/cm for 1.5 h in case of tandem-crossed immunoelectrophoresis. Gels were washed for 2 days in 0.1 M NaCl to remove unprecipitated proteins, and stained with Kenacid blue R.

Polyacrylamide gel electrophoresis. SDS slab gel electrophoresis was carried out as described by Laemmli [17], with two modifications: 8-15% polyacrylamide gradient and 1-5 M urea were employed.

RESULTS AND DISCUSSION

The nodule-specific proteins (nodulins) have been identified in soybean [4, 5], pea [6] and alfalfa [8]. We have recently reported the presence of nodulins in root nodules of yellow lupin (*Lupinus luteus*) [7]. Using immunochemical techniques, about 15-25 plant polypeptides were detected in lupin nodules. According to our estimation lupin nodulins account for 6-11% of the total nodule cytoplasmic proteins.

With the exception of certain nodulins from soybean, the biological function of nodulins has not been elucidated. Their identification has heretofore been based on immunochemical tests [4, 5, 6, 7, 10]. Nodule-specific polypeptides were visualized by tandem-crossed and line immunoelectrophoreses. Plate 1 shows tandem-crossed immunoelectrophoresis of soluble cytoplasmic proteins from lupin nodules and from uninfected roots. Although this technique provides only tentative estimate, proteins visualized as precipitation areas

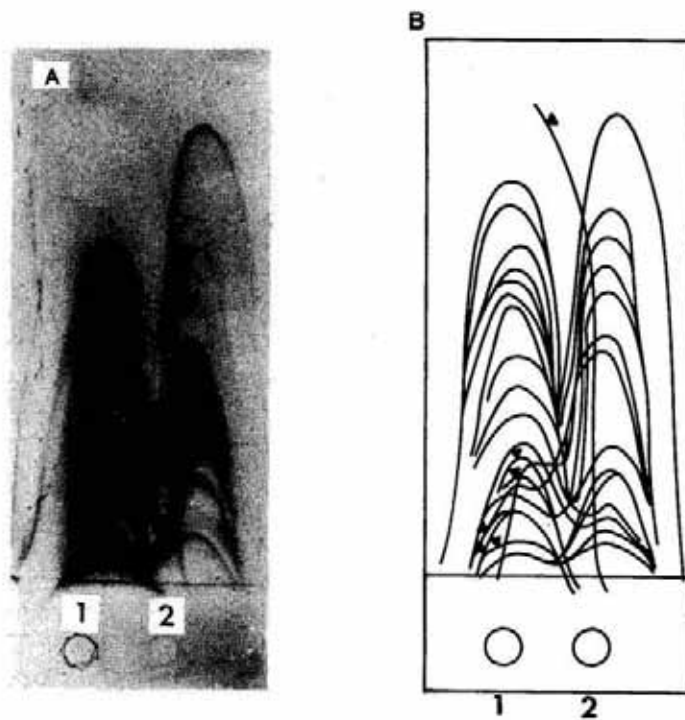


Plate 1. Tandem-crossed immunoelectrophoresis of soluble cytoplasmic proteins from lupin root nodule (1) and uninfected roots (2). Panel A shows gels stained with Kenacid blue R, panel B indicates the presence of nodule-specific proteins (▼)

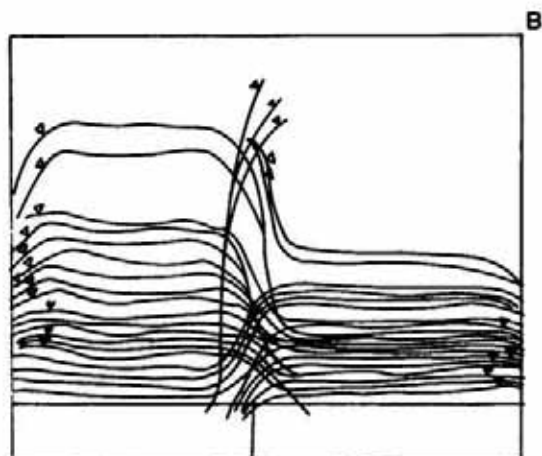
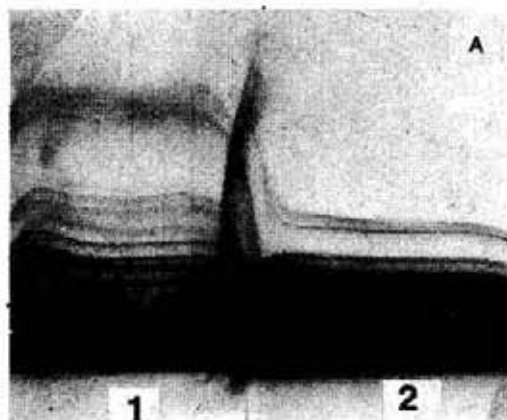


Plate 2. Line immunoelectrophoresis of soluble cytoplasmic proteins from uninfected roots (1) and lupin root nodules (2). Panel A shows the stained gel, panel B the presence of both repressed (∇) and induced root proteins (▼)

may be classified into three groups: polypeptides common to root nodules and uninfected roots, nodule-specific proteins (nodulins), and root proteins that disappear in the nodule tissue as a result of infection of the host plant with *Rhizobium*. This clearly indicates that despite a specific induction of defined plant genes leading to the synthesis of nodulins, a significant repression of root-specific sequences occurs during the symbiosis events. The root proteins are better resolved by line immunoelectrophoresis than by the tandem-crossed one. Plate 2 shows that relative concentration of root polypeptides decreases significantly in the nodule tissue as a result of both induction of nodulin and suppression of several root proteins during nodule formation and development.

At the early stages of this work we attempted to purify lupin nodulins by ion exchange and hydrophobic chromatography. These methods, however, did not lead to satisfactory results. We therefore applied an immunochemical approach for purification of a group of lupin nodulins (Fig. 1). Initially protein extracts from lupin nodules were applied to a Sepharose 4B column coupled with IgG fraction against nodulins (see Materials and Methods). The fraction enriched in nodulins was usually eluted in a single symmetric peak. To remove residual contaminations with root and bacterial proteins, the nodulin-enriched fraction was further chromatographed using: a column with IgG against root proteins and subsequently a second one with IgG fraction against *R. lupini* proteins. The purity of nodule-enriched proteins was tested by rocket immunoelectrophoresis. The final preparation was free of root and bacterial contaminations as shown on Plate 3A and B. The yield of nodulins was found to be approximately about 5% of the total nodule proteins. This value confirms earlier estimations of nodulin contents in lupin root nodules [7].

It should be pointed out that immunoaffinity chromatography presumably does not resolve large polypeptides since several proteins with an M_r above 40 000 are present in the final preparation. It is likely that some small lupin nodulins represent protein subunits since immunoelectrophoresis of the purified nodulin preparation under non-denaturing conditions reveals a lower number of precipitation arcs than in the presence of denaturing agents (results not shown).

We found the foregoing procedure very convenient for preparative scale isolation of a group of nodulins. It should greatly facilitate the purification and characterization of the biological function of individual nodulins in the legume plant-*Rhizobium* symbiotic interactions.

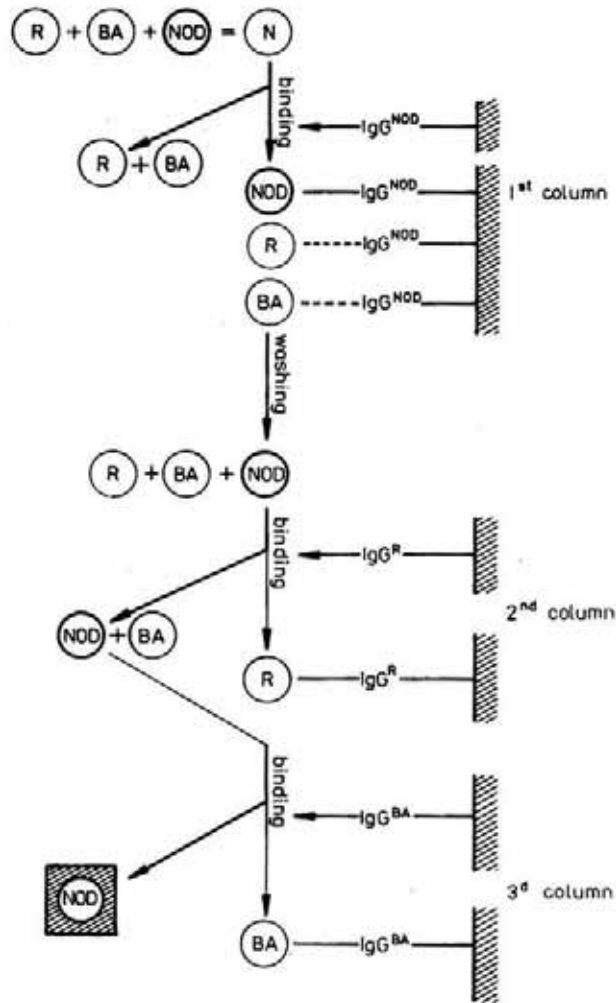


Fig. 1. Schematic representation of immunochromatographic purification of lupin nodulins. R, root proteins; NOD, nodulins; N, nodule proteins; BA, bacterial proteins; IgG^{NOD}, IgG against nodulins; IgG^R, IgG against root proteins; IgG^{BA}, IgG against *R. lupini* proteins

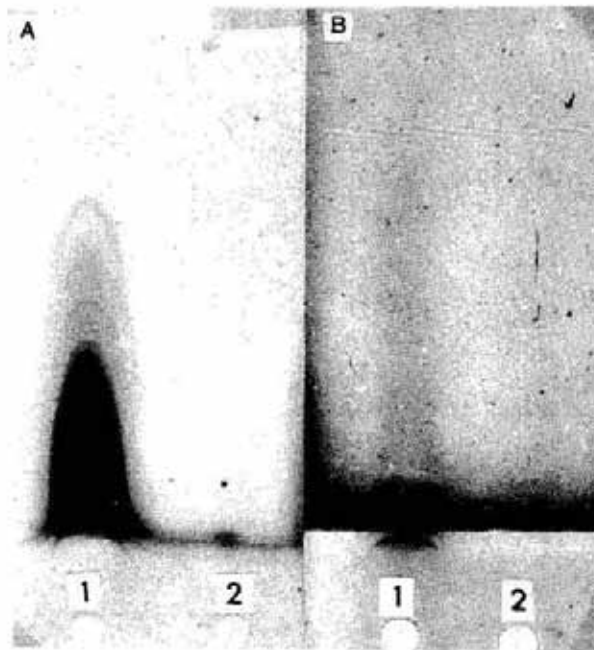


Plate 3. Rocket immunoelectrophoresis of soluble cytoplasmic proteins from root nodule (1) and purified lupin nodulins (see Materials and Methods) (2). Electrophoresis of 60 μ g of proteins was carried out in 1% agarose gel containing 4 mg/ml of either IgG against root proteins (A) or IgG against *R. lupini* proteins (B)

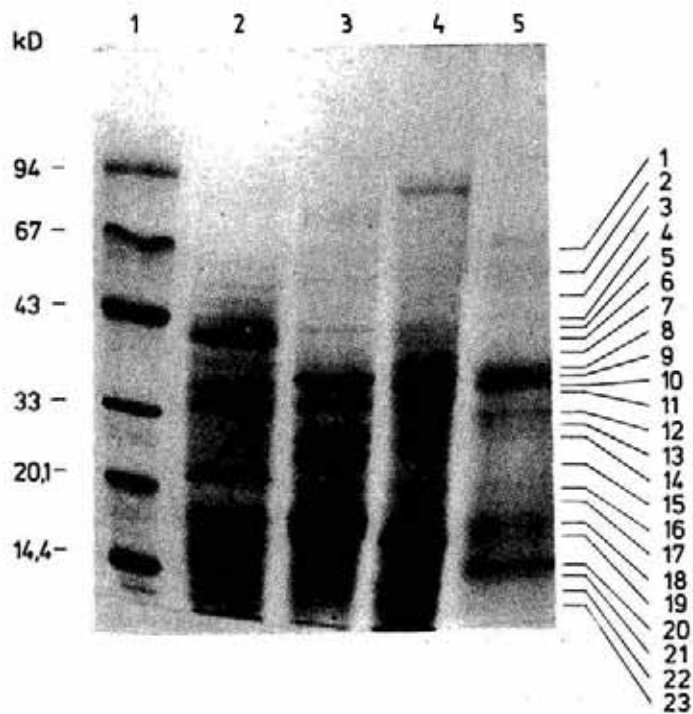


Plate 4. SDS-polyacrylamide gel electrophoresis of cytoplasmic proteins from *R. lupini* (lane 2), cytoplasmic proteins from uninfected lupin roots (lane 3), proteins from root nodule (lane 4) and purified lupin nodulins (lane 5). 40 μ g of proteins were electrophoresed. Lane 1 represents marker proteins

Table 1

M_r of lupin nodulins

	<i>M_r</i>		<i>M_r</i>
1.	70 600	13.	33 600
2.	63 100	14.	31 700
3.	54 400	15.	26 700
4.	47 500	16.	22 500
5.	45 300	17.	21 300
6.	42 200	18.	18 900
7.	41 100	19.	17 800
8.	40 000	20.	15 800
9.	38 100	21.	15 300
10.	36 900	22.	14 200
11.	36 400	23.	7 200
12.	34 400		

REFERENCES

1. Verma, D. P. S. (1982) Host-*Rhizobium* interactions during symbiotic nitrogen-fixation; in *The molecular biology of plant development* (Smith, H. & Gierson, D., eds.) pp. 437 - 466. Univ. Calif. Press, Berkeley.
2. Van den Bos, R. C., Schetgens, T. M. P., Bisseling, T., Hontelez, J. G. J. & van Kammen, A. (1983) Analysis of nodule-specific plant and bacteroid proteins in pea plant inoculated by transposon mutagenized *Rhizobium leguminosarum*; in *Molecular genetics of the bacteria-plant interaction* (Puhler, A., ed.) pp. 121 - 129. Springer-Verlag, Berlin.
3. Long, S. R. (1984) Genetics of *Rhizobium* nodulation; in *Plant microbe interactions* (Kosuge, T. & Nester, N. E., eds.) vol. 1, pp. 265 - 306. Macmillan Publ. Comp., New York.
4. Legocki, R. P. & Verma, D. P. S. (1980) Identification of "nodule specific" host proteins (nodulins) involved in the development of *Rhizobium*-legume symbiosis. *Cell*, **20**, 153 - 163.
5. Legocki, R. P. & Verma, D. P. S. (1979) A nodule-specific plant protein (nodulin-35) from soybean. *Science*, **205**, 190 - 193.
6. Bisseling, T., Been, C., Klugkist, J., van Kammen, A. & Nadler, K. (1983) Nodule-specific host proteins in effective and ineffective root nodules of *Pisum sativum*. *EMBO J.*, **2**, 961 - 966.
7. Stróżycki, P., Konieczny, A. & Legocki, A. B. (1985) Identification and synthesis *in vitro* of plant-specific proteins in yellow lupin root nodules. *Acta Biochim. Polon.*, **32**, 27 - 34.
8. Lang-Unnasch, N. & Ausubel, F. M. (1985) Nodule-specific polypeptides from effective Alfalfa root nodules and from ineffective nodules lacking nitrogenase. *Plant Physiol.*, **77**, 833 - 839.
9. Dilworth, M. (1980) Leghemoglobins; in *Meth. Enzymology* (San Pietro, A., ed.) vol. 69C, pp. 812 - 823.
10. Bergmann, H., Preddie, E. & Verma, D. P. S. (1983) Nodulin 35: a subunit of specific uricase (uricase II) induced and localized in the uninfected cells of soybean nodules. *EMBO J.*, **2**, 2333 - 2339.

11. Thummler, F. & Verma, D. P. S. (1987) Nodulin-100 of soybean is the subunit of sucrose synthase regulated by the availability of free heme in nodules. *J. Biol. Chem.*, **262**, 14730 - 14736.
12. Gebhardt, Ch., Oliver, J. E., Forde, B. G., Saarelainen, R. & Mifflin, J. (1986) Primary structure and differential expression of glutamine synthetase genes in nodules, roots leaves of *Phaseolus vulgaris*. *EMBO J.*, **5**, 1429 - 1435.
13. Mellor, R. B., Christensen, T. M. I. E. & Werner, D. (1986) Choline kinase II is present only in nodules that synthesize stable peribacteroid membranes. *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 659 - 663.
14. Triplett, E. (1985) Intracellular nodule localization and nodule specificity of xanthine dehydrogenase in soybean. *Plant Physiol.*, **77**, 1004 - 1009.
15. Ouchterlony, O. (1953) Antigen-antibody reactions in gels. *Acta Path. Microbiol. Scand.*, **32**, 231 - 240.
16. Axelsen, N. H., Kröll, J. & Wecke, B. (1973) A manual of quantitative immunoelectrophoresis. Methods and applications. *Scand. J. Immunol.*, **2**, suppl., 1, 37 - 46.
17. Lammli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)*, **227**, 680 - 685.