Leghemoglobin in Lupin Plants  
*(Lupinus albus cv Multolupa)*

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**ABSTRACT**

Leghemoglobin was localized by immunogold techniques in nodules of *Lupinus albus* cv Multolupa inoculated with *Bradyrhizobium* sp. (*Lupinus*) strain ISLU 16. The protein localization was performed in nodules embedded in Spurr's and Araldite epoxy resins and Lowrycril K4M. A very good preservation of both the ultrastructure and antigenicity was obtained with the tissues embedded in Araldite following glutaraldehyde fixation and un-postfixed in osmium tetroxide. Lupin leghemoglobin is a stable and abundant protein which allows a conventional method to be safely used for localization of leghemoglobin. Labeling of leghemoglobin was specifically confined to the cytosol matrix and nuclei. Gold particles were never observed in the peribacteroid spaces nor in the cytoplasmic organelles of the infected cells. Decrease of leghemoglobin was observed when the plants were grown with 10.7 micromolar and 21.4 micromolar of nitrate.

Gas diffusion in nodules. Witty et al. (30) also concluded that rates of nitrogen fixation in nodules from various legumes are limited by the resistance to the diffusion of oxygen.

For our study, we used lupin plants (*Lupinus albus* L. cv Multolupa), a crop of importance in Spain as an alternative crop to soybean. Nitrogen compounds formed in the lupin nodules are exported as amides. The central region of the nodule consists entirely of bacteroid-infected cells. The nitrogen-fixing bacteroids are encircled (one or several bacteroids) by the peribacteroid membrane and the peribacteroid spaces are relatively large.

Our main objective was to localize LHb in nodule cells of lupin. We also studied the effect of combined nitrogen on the immunocytochemical localization and amount of LHb.

**MATERIALS AND METHODS**

**Plant Material**

Seedlings of *Lupinus albus* L. cv Multolupa were inoculated with the *Bradyrhizobium* sp. (*Lupinus*) strain ISLU 16 (10⁸ bacterial/mL) and cultivated on vermiculite watered with a Bond nutrient solution. Potassium nitrate at 0, 10.7, and 21.4 mm was added to nodulated plants 5 d after nodulation. The growth chamber conditions were 70% RH, 26°C temperature, with a 16 h photoperiod and 190 µE m⁻² s⁻¹ irradiance. Plants were harvested 30 d after inoculation. Nodules to be used for leghemoglobin purification were kept at −70°C until employed. Nodules used to prepare specimens for electron microscopy were always fresh.

**Preparation of Soluble Nodule Protein**

Lupin root nodules were homogenized in a Sorvall Omni-mixer in cold 0.1 M sodium phosphate buffer (pH 6.8), 4 mL/g, containing 1 mM phenyl methyl sulfonylfluoride. Bacteroids were removed by centrifugation for 4 min at 10,000g at 4°C. The resulting supernatant contained the soluble cytoplasmic proteins and the protein enclosed by the peribacteroid membrane. Total protein concentrations were estimated by the Bradford (7) assay before electrophoresis and western blotting.

**Purification of Lupin Leghemoglobin and Preparation of Antisera**

Leghemoglobin was prepared as described by Dilworth (11) with modifications. Nodules (10 g) were homogenized in

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0.1 M sodium phosphate buffer (pH 6.8) (40 mL). The homogenate was centrifuged at 10,000g for 20 min and the supernatant was fractionated between 50 and 80% saturation of ammonium sulfate. The precipitate was collected by centrifugation at 10,000g 15 min, redissolved in 3 mL of 0.1 M Tris-HCl with 0.1 mM EDTA (pH 7.7) and dialyzed overnight against the same buffer. The concentration of heme-groups in this solution was measured by the hemochromogen method (23). The solution was taken to pH 6, oxidized with four equivalents of K3Fe(CN)6 at room temperature, and was then applied to a 3 x 25 cm Sephadex G 25 column equilibrated and eluted with 0.1 M Tris-HCl (pH 9.2). The absorbance at 403 nm was measured and the fractions having an absorbance larger than 0.25 were pooled, concentrated over an Amicon YM 10 membrane, and dialyzed overnight against 10 mM phosphate buffer (pH 7.0). The dialyzed solution was then loaded onto a DEAE Sephaloc column (1.5 x 10 cm) which was equilibrated with 10 mM phosphate buffer (pH 7.0), and was eluted with 50 mM phosphate buffer (pH 7.0). Fractions having A403 > 0.25 were collected, concentrated over a YM 10 membrane, and applied to 1.6 x 40 cm Sephadex G 100 column equilibrated and eluted with 10 mM phosphate buffer (pH 7.0). Fractions with A403 > 0.25 were concentrated over a YM 10 membrane, protein and heme groups were measured, and the purified leghemoglobin was stored in liquid nitrogen.

Since the leghemoglobin contained some contaminants, it was re-purified by SDS-polyacrylamide gel electrophoresis. Samples contained 4.5 mg leghemoglobin were boiled for 3 min in electrophoresis sample buffer (19) and applied to a single slot, 12% acrylamide, 1.5-mm thick gel. Protein bands were visualized by immersing the gel onto 10 volumes of 4 M sodium acetate during 30 min (17). The major band was excised, macerated, and protein eluted from the gel by shaking into 10 mM NH4HCO3, 0.05% SDS for 12 h (twice). Protein concentration was measured by the Lowry method (21). The yield was approximately 30% of the first purification. Electrophoretically purified leghemoglobin was concentrated over a YM 10 membrane and stored frozen.

Preparation and Assay of Antibodies to Lupin Leghemoglobin

Approximately 0.4 mg of SDS-gel electrophoresis-purified leghemoglobin in Freund's complete adjuvant were injected intradermically at several sites in the back of male white New Zealand rabbits. The injections were repeated 2 and 4 weeks later employing the same antigen in Freund's incomplete adjuvant. Rabbits were then boosted at 1 month intervals, blood being obtained from the ear vein and finally by cardiac puncture.

Sera were characterized by ELISA (14) and western blotting (16, 27) as described by Andreu et al. (1), employing preimmune sera as controls. Antileghemoglobin IgG2 was prepared from immune sera by precipitation with 45% saturated ammonium sulfate and chromatographed on DEAE Sephadex (8).

Abbreviation: IgG, immunoglobulin G.

Figure 1. Electrophoresis by SDS-PAGE and Western blots of lupin hemoglobin. 1, SDS-PAGE electrophoresis of the total proteins of the lupin nodule; 2, purified lupin LHb, containing some contaminants; 3, electrophoretically repurified lupin LHb (lanes 1-3 were stained with Coomassie blue); 4, autoradiogram of an immunoblot showing the specificity antilupin LHb antiserum (the total proteins of the lupin nodule were blotted); 5, autoradiogram of an immunoblot showing the specificity antilupin IgG LHb for LHb among the total proteins of the lupin nodule.

Immunogold Labeling of Thin Sections

Samples (2–3 mm) of nodules from plants harvested 30 d after inoculation, were fixed in 2.5% (v/v) glutaraldehyde in a 0.05 M cacodylate buffer (pH 7.4), under vacuum for 2 h at room temperature. Some of the samples were postfixed for 2 h in 1% (w/v) osmium tetroxide in the same buffer at 2°C. Specimens were cut with a Reichert OM U2 ultramicrotome and collected on uncoated nickel grids.

Prior to labeling, some sections of osmium postfixed tissue were treated with aqueous 0.56 M sodium metaperiodate for 1 h (12) in order to overcome the masking of antigenic sites by osmium (4). After periodate treatment, grids were rinsed with distilled water.

For immunolabeling, sections placed on grids were submerged for 1 h at 37°C in a Tris-buffered saline solution (TBST) (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.3% Tween 20) containing 0.2 mg mL−1 Na azide and 20 mg mL−1 BSA. They were later immersed for another hour at 37°C in a fresh solution as above to which the antilupin leghemoglobin antibody had been added at a concentration of 50 μg IgG mL−1. The grids were washed by gentle agitation in three changes of 2 mg mL−1 BSA in TBST at room temperature and later immersed in goat anti-rabbit IgG-Au (15 nm diameter colloidal gold, Janssen, Life Sci.) diluted 1:10 in the same solution for 1 h at 37°C. The grids were then washed by gentle agitation in three changes of BSA, 2 mg mL−1 in TBST, followed by another washing in the same solution but containing 0.1% Triton X-100, and finally with distilled water.
Parallel controls were performed with preimmune rabbit IgG or without the antileghemoglobin IgG.

Sections were post-stained in 2% aqueous uranyl acetate for 10 min and for another 2 min in lead citrate (24) before observation with a Philips 300 electron microscope at 80 kV.

RESULTS AND DISCUSSION

Figure 1 shows the results of the purification of the lupin leghemoglobin. As seen in lane 2, six minor bands with different mol wt were obtained besides the leghemoglobin on the SDS-polyacrylamide gel. The absorption spectrum confirmed the two components of the leghemoglobin, the heme one at 403 nm and the protein component at 280 nm in approximately a 0.76:1 ratio (2). Preparative electrophoresis was performed to further purify the leghemoglobin protein (Figure 1, lane 3), which was employed to raise specific antibodies. Lane 1 in Figure 1 shows the soluble proteins of the lupin nodule. Only the leghemoglobin reacted with the antiserum (lane 4). Antileghemoglobin IgG was purified and its specificity verified (lane 5).

The antigenicity of lupin leghemoglobin was checked by ELISA. The titer of the antiserum was higher than 1:3000. To further characterize the specificity of our antibodies, it was shown that there was no major cross-reaction between the LHbs of soybean and lupin plants and their antisera by either ELISA (Fig. 2A) or western blot (Fig. 2B). A similar result was already reported by Hurrel et al. (18) employing radioimmunoassay.

Localization of Leghemoglobin

Satisfactory results were obtained by the conventional technique of fixing in glutaraldehyde and embedding in Araldite with the omission of OsO₄-postfixation. Antigenicity did not decrease when the tissues were postfixed and the osmium was removed with sodium metaperiodate (not illustrated). The Lowcryl technique preserved the antigenicity of leghemoglobin but not the ultrastructure. On the other hand, the lupin leghemoglobin has antigenic determinants that are different from those of soybean leghemoglobin (18) and seem to be stable in plastic sections. Since we obtained a high titer antiserum and lupin leghemoglobin is a stable and an abundant protein, the conventional method was safely used for its localization.

Immunogold staining proved to be very specific for the cytoplasm of the host cells (Fig. 3A). This is in agreement with results obtained for pea nodules (25). The location was homogeneous throughout the cytoplasm matrix. Immunolocalization was also observed on the peribacteroid membrane. No association of gold particles with subcellular organelles of cell was found.

Specific staining of LHb was also detected in nuclei (Fig. 3B). The determination of the density of gold staining gave a ratio for the plant cytoplasm and nuclei of 1:0.85 gold particles per unit area. Robertson et al. (25) also obtained nuclear localization in 15-d infected pea nodule cells. The significance of this nuclear labeling remains unclear, and additional work is required to clarify the physiological significance of this result, and whether LHb has any functional role in nuclei.

Perhaps leghemoglobin, a small mol wt protein, penetrates the nuclear membrane via nuclear pores. Verma and Bal (28), applying antibodies conjugated with ferritin, observed localization of leghemoglobin around the nuclear membrane.

The control test (Fig. 3C) confirmed the specificity of the immunogold staining of LHb.
Figure 3. Thin sections of lupin nodule infected cells. A, Section embedded in Araldite resin immunogold labeled with antilupin leghemoglobin. The cytosol of the host cells is intensively labeled ×32,000; B, section immunogold labeled with antilupin leghemoglobin showing gold particles in the nucleus, ×65,000; C, section incubated in preimmune serum, followed by immunogold labeling ×78,000. B, Bacteroid; CW, cell wall; CY, cytosol; M, mitochondria; N, nucleus; P, peroxisome; PBM, peribacteroid membrane; PBS, peribacteroid space.
Figure 4. Thin sections of lupin nodule cells from plants grown in the presence of combined nitrogen. A, Gold localization in the cytosol of infected cells of nodule treated with 10.7 mM nitrate, ×32,000; B, gold localization in nodule cells from 21.4 mM nitrate grown plants, ×32,000. B, bacteroid; CY, cytosol; PBS, peribacteroid space.
The nitrogenase activity in nodules from plants grown in 10.7 and 21.4 mM nitrate decreases considerably (20). The gold density measured in nodules from plants grown at different concentrations (0, 10.7, 21.4 mM nitrate) gave a ratio of 9:2:1 (Fig. 4, A and B). The decrease in nitrogenase activity produced by nitrate was probably caused by the decrease of leghemoglobin content of the nodules. Bisseling et al. (6) observed a decrease in the heme content of nodules by the influence of nitrate, observing a correlation between nitrogenase activity and heme concentration in the nodule.

Inhibition of legume nitrogen fixation by nitrate has been intensively studied for many years without any conclusive evidence being obtained as to the mechanism of inhibition. The hypothesis that nitrate inhibits nitrogenase activity as a result of nitrite production in bacteroid-containing cells was recently shown by Sprent et al. (26) to be invalid. A new hypothesis based on experiments with white clover (22) and soybean (10) is that nitrate increases the resistance to oxygen diffusion within the nodule.

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LITERATURE CITED