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Purification of leghemoglobin from nodules of *Crotalaria* infected with *Rhizobium*

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Abstract

The leghemoglobin from nodules of *Crotalaria juncea* infected with *Rhizobium* spp. was purified to homogeneity. The protein was purified after precipitation with 40–80% $(NH_4)_2SO_4$, and chromatography by anionic exchange and gel filtration. The leghemoglobin has a single component and showed an apparent M_r of ca. 17,300 and 23,700 determined by SDS–PAGE and gel filtration, respectively. The amino acid composition showed that asparagine/aspartic acid, glutamine/glutamic acid, alanine, lysine, serine and leucine were the main amino acids. Iron was detected only in the band corresponding to the purified protein. The N-terminal amino acid sequence for the first 19 residues showed high similarities with several other leghemoglobins from other plants.

Keywords: Crotalaria juncea; Leguminosae; Crotalária; Leghemoglobin; Nodules; Purification

1. Introduction

Crotalaria juncea is a fast-growing legume originated from India, with a wide distribution in tropical areas. It has an annual life-cycle, showing a very fast initial growth. Previously, *C. juncea* was used for high quality paper production, mainly for the tobacco industry, due to the quality of its fibers (Salgado, Azzini, Feitosa, Petineli, & Veiga, 1984; Wutke, Bulisani, & Mascarenhas, 1993). Nowadays this plant has been used also to control the nematode population in infested soil and mainly as green manure in rotating culture systems (Wutke et al., 1993). This latter use is justified by its high nitrogen fixation capacity when in association with *Rhizobium* (Lombardi, 1995).

Leghemoglobin is the protein responsible for oxygen-binding in symbiotic root nodules of nitrogen-fixing plants and also in a few nonlegumes (Christensen, Dennis, Peacock, Landsmann, & Marcker, 1991). However, the detection of hemoglobin genes in barley subjected to anaerobic stress (Bogusz et al., 1988; Taylor, Nie, Macgregor, & Hill, 1994) suggests that these proteins may have functions in plants other than an exclusive control of oxygen levels in nodules. To date, little is known on rhizobia nodulation in representatives of the genus *Crotalaria*. As a part of the work developed in our laboratory concerning this aspect, we have purified the leghemoglobin (Lb) from nodules of *C. juncea* infected by rhizobia and the results are presented here.

2. Results and discussion

The chromatography of oxidized Lb in the anionic columns showed a single peak at 405 nm. Therefore, Lb of *C. juncea* has a single component. After Mono Q column apparently pure Lb was obtained. SDS–PAGE of proteins during the purification is shown in Fig. 1. The main peak resolved in the Mono Q column yielded a pink–red solution corresponding to 254 μg of protein. The proteins stained in SDS–PAGE gel from the DE-52 column (Fig. 1, lane 4) are the same as those observed for Superose (Fig. 1, lane 5), indicating that there was not a significant purification increase. However, in both cases one band was markedly more stained than others of higher M_r. This band was particularly intense in the Superose 12 fractions. Since only one peak was observed in the Superose 12 chromatography, this might be the Lb and, its M_r was estimated as 23,700 (data not shown). The apparent M_r estimated by the SDS–PAGE of the Mono Q fraction was 17,300 (Fig. 1, lane 7). Therefore, the Lb purified was a monomeric protein. This is in agreement with previous publications on Lb purification or cDNA estimation from different nodulated legumes

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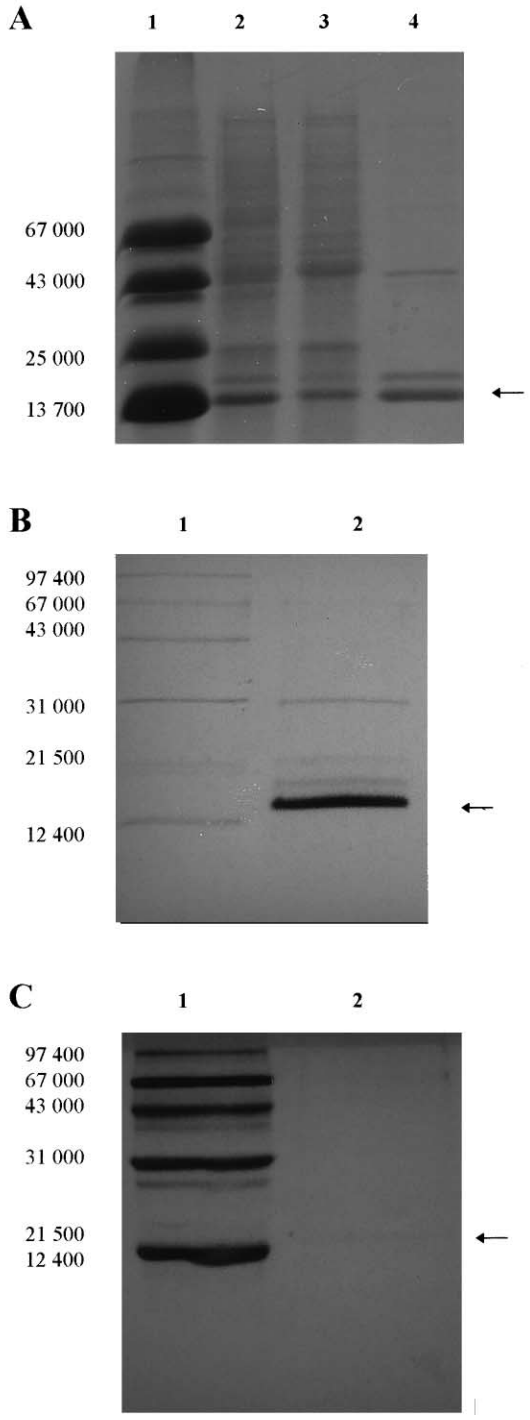

Fig. 1. SDS–PAGE of crude protein extract (A, lane 2); 40–80% $(NH_4)_2SO_4$ precipitation (A, lane 3); DE-52 (A, lane 4); Sepharose (B, lane 2) and Mono Q (C, lane 2) columns. Proteins in lanes 1 are M_r protein markers. Arrows indicate Lb position in the gel. Proteins stained with Coomassie Blue.

(Barbosa & Meirelles, 1994; Bogusz, Kortt, & Appleby, 1987; Lehtovaara, Lappalainen, & Ellfolk, 1980) where values ranged from 14,000 to 18,000.

During the Mono Q chromatography the main peak detected at 280 nm was coincident with the red color eluting from the column. However, in order to be sure that the purified protein was Lb and that other bands observed in the SDS–PAGE of Superose 12 fraction were not subunits not seen in the Mono Q SDS–PAGE lane; they were analyzed for the presence of Fe. This metal was detected only in the more stained band.

It has been shown that several Lbs from different plant sources are composed of different molecules called components, which present a high degree of homology. After $(NH_4)_2SO_4$ precipitation, usually they are separated by ion-exchange chromatography using a gradient with acetate buffer at pH ~ 5 (Bogusz et al., 1987; Lehtovaara & Ellfolk, 1975; Thulborn, Minasian, & Leach, 1979). However, some components may be separated by SDS–PAGE (Thulborn et al., 1979). Bogusz et al. (1987) observed that the five among seven components of Lb of *Sesbania rostrata*, separated by ionic-exchange chromatography, showed M_r ranging from 16,000 to 16,200. When native PAGE was used, four components ranged from 14,800 to 15,500 and three from 16,200 to 16,600. On the other hand, for some legumes, such as *Phaseolus vulgaris*, only one Lb band was observed in PAGE (Becana & Sprent, 1989). As in *P. vulgaris*, we determined here that Lb of *C. juncea* has only one component. The amino acid N-terminal sequence of this component is shown below.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
–	G	G	L	T	E	S	Q	V	G	L	V	K	S	S	W	E	A	L

The highest sequence homologies found in the Entrez databank of the National Center for Biotechnology were with Lb II (81%) and Lb I (68%) of *Lupinus luteus*, Lb of *Medicago truncatula* (66%), Lb K of *Vicia faba* (66%), Lb of *Canavalia lineata* (64%), Lb of *P. vulgaris* (64%), Lb of *Psophocarpus tetragonolobus* (64%), class 2 non-symbiotic hemoglobin of *Arabidopsis thaliana* (65%) and Lb I of *Casuarina glauca* (62%).

The amino acid composition of the purified Lb is shown in Table 1. The amino acid composition of other Lbs showed that asparagine/aspartic acid, glutamine/glutamic acid, alanine, lysine and leucine are the main amino acids (Bogusz et al., 1987; Lehtovaara et al., 1980; Kortt, Inglis, Fleming, & Appleby, 1988). This is in agreement with the our results, although we have also observed significant amounts of serine (Table 1).

3. Experimental

3.1. Plant material and nodule production

Rhizobium strain isolated from *Crotalaria retusa* (Mendoça, 1997) was used in this study. Seeds of *C. juncea* were germinated in 1.5 l plastic pots containing vermiculite and immediately inoculated with 2 ml of rhizobia suspension produced with medium "79" (Fred & Waksman, 1928).

Table 1
Amino acid composition of Lb from *Crotalaria juncea* obtained by acid hydrolysis

Amino Acids	mol(%)
Asp/Asn	12.72
Glu/Gln	14.36
Ser	12.08
Gly	7.84
His	4.76
Arg	4.16
Thr	3.88
Ala	9.16
Tyr	1.04
Val	6.48
Met	0.32
Ile	4.48
Leu	10.04
Phe	3.72
Lys	8.88

A new 2 ml inoculation was repeated one week later. Nodules were collected 90 days after sowing. During this period, the plants were maintained in greenhouse conditions with daily irrigation with nutrient solution deprived of N.

3.2. Purification of Lb

Nodules from several plants (32 g) were extracted with cold Drabkin's solution, centrifuged (27,000*g* at 4°) and partially purified by 40–80% $(NH_4)_2SO_4$ precipitation (Dilworth, 1980). The satd pellet was solubilized and dialyzed against 3 mM K–Pi buffer, pH 7.0. The protein concentration was determined with a ready-to-use reagent from BioRad. To oxidize the Lb, ferricyanide–nicotinate solution was added (Becana & Sprent, 1989) and the extract treated on Sephadex G25 PD-10 minicolumns (Pharmacia). The Lb from *C. juncea* was purified using a 2.6×10 cm DEAE–cellulose column (DE-52 Beckman), a 3.1×30 cm Superose 12 column (Pharmacia), and a Mono Q HR 5/5 column (Pharmacia). A linear gradient with NaOAc buffer pH 5.4 was used with the anion exchange columns (Thulborn et al., 1979). The elution of the proteins from the columns was monitored, setting the UV monitor of the HPLC system at 280/405 nm. The protein purification was repeated twice, with very similar results. Here, we present the data of one purification.

3.3. Electrophoresis

Samples from each purification step were reserved to be analyzed in SDS–PAGE using discontinuous gel, with 6% in the stacking gel and 17% of acrylamide in the main gel (Laemmli, 1970). Proteins were stained with Coomassie Blue R250.

3.4. Amino acid composition of Lb

The band in a SDS–PAGE gel corresponding to Lb was cut out, washed several times with deionized H_2O and finely ground with a glass rod in an Eppendorf tube containing 250 µl of deionized H_2O. The gel debris was removed by centrifugation in 0.22 µm Eppendorf filters (Amicon) and proteins precipitated three times by addition of TCA to 5% final concentration. The recovered pellet was solubilized with 6 M HCl and transferred to cap sealed glass tubes for digestion at 110° for 24 h. The digest was dried under vacuum at 40° and the residue solubilized in 200 µl of deionized H_2O. Amino acids were analyzed in HPLC with fluorescence detection after derivatization with *o*-phthaldialdehyde (Jarret, Coosky, Ellis, & Anderson, 1986).

3.5. N-terminal amino acid sequencing

Purified Lb separated in a SDS–PAGE gel was electrotransferred to immobilon-p membrane (Millipore) for amino acid N-terminal sequencing by the Edman degradation method in a Applied Biosystems Procise Sequencer. The Entrez databank of the National Center for Biotechnology (USA) was consulted for sequence homologies.

3.6. Iron determination

Fe concentration was determined in four bands separated in SDS–PAGE gels of frs collected from the Superose 12 column. These bands were cut out from the gel, rinsed several times with deionized H_2O for 24 h and ground in a glass tube with a glass rod. HNO_3 (1 ml) and deionized H_2O (4 ml) were added and left at 150° until the gel had dissolved. Nonstained gel pieces were used as blanks. Fe in the cooled digests was determined in a spectrometer of argon plasm atomic emission (Mod. 50P, Jobin Yvon). Three replicates were analyzed for each stained band.

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