

Molecular Cloning of the Cowpea Leghemoglobin II Gene and Expression of Its cDNA in *Escherichia coli*¹

Purification and Characterization of the Recombinant Protein

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Cowpea (*Vigna unguiculata*) nodules contain three leghemoglobins (LbI, LbII, and LbIII) that are encoded by at least two genes. We have cloned and sequenced the gene that encodes for LbII (*lbII*), the most abundant Lb in cowpea nodules, using total DNA as the template for PCR. Primers were designed using the sequence of the soybean *lbc* gene. The *lbII* gene is 679 bp in length and codes for a predicted protein of 145 amino acids. Using sequences of the cowpea *lbII* gene for the synthesis of primers and total nodule RNA as the template, we cloned a cDNA for LbII into a constitutive expression vector (pEMBL19⁺) and then expressed it in *Escherichia coli*. Recombinant LbII (rLbII) and native LbII (nLbII) from cowpea nodules were purified to homogeneity using standard techniques. Properties of rLbII were compared with nLbII by partially sequencing the proteins and by sodium dodecyl sulfate- and isoelectric focusing polyacrylamide gel electrophoresis, western-blot analysis using anti-soybean Lb_a antibodies, tryptic and chymotryptic mapping, and spectrophotometric techniques. The data showed that the structural and spectral characteristics of rLbII and nLbII were similar. The rLbII was reversibly oxygenated/deoxygenated, showing that it is a functional hemoglobin.

Roots of certain leguminous plants are infected by symbiotic soil bacteria of the genera *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium* (collectively known as rhizobia), which cause root tissues to redifferentiate into specialized organs called nodules. After a complex series of events that occur between bacteria and the plant cell (for review, see Mylona et al. [1995]), a nitrogen-fixing nodule containing bacte-

roids is formed. Bacteroids are differentiated forms of rhizobia that synthesize the nitrogenase complex.

Leguminous nodules contain a myoglobin-like heme protein, Lb, which reversibly binds molecular O₂. The role of Lb in nodules is to facilitate the diffusion of O₂ at low concentrations but rapid fluxes to the microaerophilic bacteroids (Appleby, 1992). In root nodules the O₂ concentration is maintained at low levels by a diffusion barrier in the inner cortex and by bacteroidal respiration (Hunt and Layzell, 1993). Bacteroids require O₂ for respiration for the synthesis of ATP, but high levels of O₂ not only inactivate the nitrogenase complex in the bacteroids, but also inhibit the expression of the rhizobial genes encoding for this complex (Shah and Brill, 1977; Mylona et al., 1995).

In leguminous nodules Lbs are encoded by a family of genes, with the number of detectable Lbs varying among the legume species. Eight isoLbs have been detected in soybean nodules, of which four are posttranslationally modified (Appleby et al., 1975). Cowpea nodules contain three isoLbs, I, II, and III (Dakora et al., 1991), coded by at least two genes. LbII is the major component in nodules. The physiological significance of multiple isoLbs in leguminous nodules is unknown.

Obtaining substantial quantities of functional Lbs from root nodules is difficult. Under most instances, the amount of nodule material that can be obtained is limiting, and preparation of large amounts of pure protein is cumbersome. The recombinant expression of Lbs in *Escherichia coli* obviates these hurdles. Furthermore, the availability of an expression system permits a detailed dissection of the structure and function relationships of Lbs, as has been demonstrated for other hemoglobins (Springer and Sligar, 1987). However, it is essential to establish that the recombinant Lb is identical to the native Lb that is synthesized in nodules in all biochemical characteristics, and that it is fully functional by reversible binding of O₂. The cloning and synthesis of a recombinant LbI from lupin was reported first by Sikorski et al. (1995), but they lacked the data to verify the biochemical functionality of the resulting recombinant protein. Based on the methods used by these

¹ This work was supported in part by grants from the National Science Foundation (no. OSR-92552255), the U.S. Department of Agriculture Cooperative State Research, Education, and Extension Service (no. 95-37305-2441), and the Center for Biotechnology, University of Nebraska, Lincoln. R.A.P. was partially supported by a postdoctoral fellowship from Dirección General de Asuntos del Personal Académico Universidad Nacional Autónoma de México and Consejo Nacional de Ciencia y Tecnología, México. J.F.M. is a recipient of a postdoctoral fellowship from the Ministerio de Educación y Cultura, Spain. This is published as journal series no. 11,332 of the Agricultural Research Division, University of Nebraska, Lincoln.

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Abbreviations: CFLbR, cowpea ferric leghemoglobin reductase; Hb, hemoglobin; Lb, leghemoglobin; nLbII and rLbII, native and recombinant cowpea LbII, respectively.

authors it is probable that the recombinant LbI was not functional. In this work we describe the cloning and sequencing of the cowpea *lbII* gene, the subsequent expression of the cowpea LbII cDNA in *E. coli*, and the purification and characterization of the rLbII. Results showed that the rLbII is identical to the nLbII in several of its biochemical properties, and that it is a functional Hb that reversibly binds O₂.

MATERIALS AND METHODS

Plant Growth and Total DNA and RNA Isolation

Cowpea (*Vigna unguiculata* var. California no. 5) seeds were germinated in wet towels for 5 d. The cotyledons were removed and seedlings (3 g) were frozen in liquid nitrogen. Total DNA was isolated using a modification of the CTAB method (Doyle and Doyle, 1990).

For the isolation of total RNA from nodules, cowpea seedlings were inoculated with *Bradyrhizobium japonicum* USDA3456 and grown as described previously (Sarath et al., 1986). Nodules were harvested, immediately frozen in liquid nitrogen, and stored at -90°C until used. Total RNA was isolated from 3 g of nodules by the phenol/SDS method (Ausubel et al., 1987). RNA was quantitated spectrophotometrically assuming 1 A₂₆₀ = 40 µg RNA mL⁻¹ (Ausubel et al., 1987).

Cloning of the Cowpea *lbII* Gene and PCR Amplification

The sequences coding for helices A and H of the soybean *lbc* gene (Hyldig-Nielsen et al., 1982) were used to design primers for PCR. The sense primer was designated as SyLb/A (5' AAGCAAGAGGCTTTGGTG 3', position 193-210) and the antisense primer as SyLb/H (5' TGCCAATTCATCATAGGC 3', position 1276-1259). Both primers were synthesized by the DNA Synthesis Facility of the University of Nebraska, Lincoln.

Total cowpea DNA (approximately 1 µg) was used as the template for PCR amplification. PCR components and concentrations were as follows: 2 µM of each sense and antisense primer, 200 µM of each dNTP, 500 µM MgCl₂, and 0.5 units of *Taq* DNA polymerase (GIBCO-BRL) in a final volume of 25 µL of the PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, and 0.001% [w/v] gelatin). Before amplification tubes were incubated at 95°C for 3 min to ensure that the template DNA was completely denatured. Amplification was carried out for 45 cycles at 40°C min⁻¹ for annealing, 72°C 1.5 min⁻¹ for extension and 95°C 1.5 min⁻¹ for denaturation. An additional annealing and extension step was done at 40°C min⁻¹ and 72°C 5 min⁻¹, respectively. The total volume of the PCR samples was electrophoresed in a 1.6% (w/v) agarose gel.

Cloning of the Cowpea Nodule PolyA⁺ RNA into λ gt11

Nodule polyA⁺ RNA was isolated using a mRNA purification kit (QuickPrep, Pharmacia). Double-stranded cDNA was synthesized using a kit (Time Saver, Pharmacia) and an *EcoRI/NotI* adaptor (Pharmacia) was ligated to each end of

the cDNA. The *EcoRI*-terminated cDNAs were phosphorylated and ligated to the dephosphorylated λ gt11 vector at the *EcoRI* restriction site. The ligated products were used as templates for PCR amplification with the vector (Arredondo-Peter et al., 1995) and soybean Lb primers, as described above.

Isolating of the PCR Products and Sequencing of the DNA

The PCR products were separated by electrophoresis and isolated from the melted agarose using a kit (Geneclean, BIO 101, Vista, CA) and resuspended in 10 µL of autoclaved water. A 1-µL aliquot of the resuspended DNA was used to clone each PCR product into the linearized vector pCRII (Invitrogen, San Diego, CA) following standard procedures (Sambrook et al., 1989). Cloned fragments were partially sequenced by the dideoxy method (Sanger et al., 1977) using a nonisotopic DNA sequencing kit (United States Biochemical). These sequences were compared with sequences in the GenBank database using the BLAST program (Altschul et al., 1990). PCR fragments with sequences similar to those of *lb* genes were selected and sequenced in both directions by the DNA Sequencing Facility of the University of Nebraska, Lincoln.

Isolating of the cDNA and Cloning into pEMBL19⁺

Total RNA (1 µg) from cowpea nodules was used as the template for RNA-PCR (Wang et al., 1989). Primers for the PCR were designed using the cowpea *lbII* gene sequence. Sense (5' CCATGGTGCCTTCTCTGAC 3') and antisense (5' GAATTCCTAATATGCTTTTAAATAGCTGC 3') primers started at the START and the STOP codons, respectively, and were degenerated with restriction sequences for *NcoI* and *EcoRI* (underlined). An RNA-PCR kit (Perkin-Elmer Cetus) was used for the reverse transcription and amplification of the transcripts following the manufacturer's protocol. PCR conditions were as follows: 35 cycles at 55°C min⁻¹ for annealing, 72°C min⁻¹ for extension, and 95°C min⁻¹ for denaturation. An additional annealing and extension step was carried out as above.

DNA manipulation and transformation in host cells were done by standard procedures (Sambrook et al., 1989). The PCR products were separated by electrophoresis and cloned into the pCRII vector. The cloned fragments were sequenced in both directions.

The construct pCRII::LbII was digested with the restriction enzymes *NcoI* (15 units) and *EcoRI* (10 units) to generate cohesive ends. The clone LbII was directionally subcloned into the *NcoI* and *EcoRI* cloning sites of the expression vector pEMBL19⁺ (Springer and Sliagar, 1987). *Escherichia coli* TB-1 cells were transformed with the construct pEMBL19⁺::LbII, and colonies were selected in Luria broth plates containing ampicillin (50 µg mL⁻¹).

Bacterial Growth and Purification of the Recombinant LbII

Host cells with the pEMBL19⁺::LbII construct were grown in a 100-L fermentor using Luria broth medium containing ampicillin (50 µg mL⁻¹). Cultures were vigorously aerated at 37°C and grown until the absorbance reached 2 to 3. Cells

were harvested by centrifugation and the cell paste was stored at -90°C until used. An aliquot of the cell paste (25 g) was resuspended in 100 mL of buffer (50 mM sodium phosphate, pH 7, 1 mM EDTA, 1 mM PMSF) and sonicated (model 450 sonifier, Branson, Danbury, CT) at maximum power (three cycles of 1 min each). After sonication, lysozyme (2 mg mL^{-1}), DNase (40 units mL^{-1}), and RNase (3 units mL^{-1}) were added and the suspension was incubated overnight at 4°C with gentle stirring.

The suspension was centrifuged at $48,000g$ for 20 min to remove undisrupted cells and cell debris, and the resulting supernatant was saved. Proteins precipitating between 55 and 90% (w/v) saturation with ammonium sulfate at 4°C were collected and resuspended in 20 mM Tris-HCl (pH 8) containing 1.8 M ammonium sulfate. This solution containing soluble proteins was chromatographed on a Phenyl-Sepharose column ($2.5 \times 20\text{ cm}$, Pharmacia) equilibrated with 20 mM Tris-HCl (pH 8) containing 1.8 M ammonium sulfate. The fraction containing the red rLbII was eluted from the column with 20 mM Tris-HCl (pH 8) containing 0.8 M ammonium sulfate, concentrated to 5 mL, and dialyzed against 20 mM Tris-HCl (pH 8). The concentrated solution containing the rLbII was chromatographed on a DEAE-cellulose column ($2.5 \times 30\text{ cm}$; Whatman DE-52) previously equilibrated with 20 mM Tris-HCl (pH 8), and the rLbII was eluted with a NaCl gradient (0–60 mM) in the same equilibrating buffer. Fractions containing the rLbII were collected and concentrated to 2 mL by ultrafiltration on membranes (YM10, Amicon, Beverly, MA). The final purification of the rLbII was done by preparative PAGE (Prep Cell, Bio-Rad) using 9% (w/v) native gels. Protein purification steps were evaluated using SDS-PAGE (Laemmli, 1970). Protein concentrations were determined by a dye-binding assay (Bio-Rad) using BSA as a standard. Heme concentration was measured by the pyridine hemochromogen assay, as described by Appleby and Bergersen (1980). Cowpea nLbII and soybean Lb were purified from nodules as described by Jun et al. (1994a).

Characterization of the Cowpea rLbII

Immunoblot Analysis of rLbII

The rLbII was electrophoresed in a 12.5% (w/v) SDS-PAGE gel and then western-blotted using anti-soybean Lb_a antibodies, as described by Sarath and Wagner (1989). Pure soybean Lb_a and cowpea nLbII were included as controls.

Peptide Sequence of rLbII

The partial amino acid sequences of the N terminus were determined for the pure rLbII eluted from IEF gels (Robertson et al., 1987) and for the proteins transferred onto membranes (Immobilon-P, Millipore) (Jun et al., 1994b). Automated Edman degradation (Procise 494, ABI-Perkin Elmer) was performed using protocols recommended by the manufacturer.

Tryptic and Chymotryptic Mapping of rLbII and nLbII

Recombinant and native LbII were digested with trypsin and chymotrypsin essentially as described by Jun et al.

(1994a). An aliquot containing 400 μg of the cowpea rLbII or nLbII was digested with 8 μg each of trypsin or chymotrypsin in 20 mM Tris-HCl (pH 8) containing 10 mM CaCl_2 and incubated for 24 h at 37°C . An additional 8 μg each of trypsin or chymotrypsin was added and incubated for an additional 3 h. Enzymatic reactions were terminated by the addition of 10% (v/v) trifluoroacetic acid to obtain a final concentration of 0.1% (v/v) trifluoroacetic acid. Peptides in the digested samples were separated by reverse-phase HPLC on a C₁₈ column (218TP42, Vydac Group, Hesperia, CA) using gradients with acetonitrile. The eluate was monitored at 215 nm.

Spectral Analysis

Purified rLbII and nLbII were characterized spectrophotometrically using a spectrophotometer (Cary 1-Bio, Varian, San Fernando, CA) interfaced to a microcomputer. Ferrous Lb was oxidized to ferric Lb by the addition of potassium ferricyanide (final concentration of 5 mM) in 50 mM sodium phosphate buffer (pH 6), and then chromatographed on a PD-10 column (Pharmacia) equilibrated with 50 mM phosphate buffer at pH 7. Ferrous Lb was formed by the addition of a few crystals of sodium dithionite (Fluka). CO or air was bubbled carefully through the Lb solutions to generate the CO- or O₂-ligated forms of Lb, respectively. Nicotinate at a final concentration of 5 mM was used to form the nicotinate complex of the ferrous or ferric Lb.

Reduction of the Ferric rLbII by the CFLbR

CFLbR was purified from cowpea root nodules as previously described by Ji et al. (1991). Ferric rLbII or nLbII (approximately 50 μM) in 50 mM sodium phosphate buffer (pH 7) was mixed with CFLbR (approximately 35 nM), and NADH (10 mM) was added to start the reaction. Spectra (350–650 nm) were determined at 5-min intervals at room temperature.

RESULTS AND DISCUSSION

Isolation and Sequence of the Cowpea *lbII* Gene

When total DNA from cowpea was used as the template with primers designed for the soybean *lb_c* gene, two PCR products of approximately 660 and 470 bp were obtained. These PCR products were purified, cloned, and sequenced, and the sequences were compared with sequences deposited in the GenBank database. The sequence of the smaller fragment (470 bp) showed no similarity with Lbs and was a nonspecific amplification. The sequence of the larger fragment (660 bp) was similar (>70%) to several Lbs and corresponded to an authentic *lb* gene. The clone, named cowpea *lbII*, coded for a predicted protein with a sequence that was identical to an authentic LbII that had been isolated, purified, and partially sequenced from cowpea nodules (Sarath et al., 1990).

The clone cowpea *lbII* is 599 bp in length, which corresponds to approximately 90% of the total gene. The missing 5' and 3' flanking sequences for the gene were obtained by

cloning the nodule polyA⁺ RNA into the λgt11 vector and using inverse soybean *lb*c and λ primers for PCR amplification (Fig. 1). The full *lbII* gene is 679 bp in length and codes for a predicted protein that is 145 amino acids in length (Fig. 2). The sequence of the predicted cowpea LbII is most similar to many of the plant Hbs, particularly to Lbs in the bean group (>80%).

The coding (exon) and noncoding (intron) sequences of the cowpea *lbII* gene was determined using RNA-PCR on the total (or polyA⁺) nodule RNA, and then sequencing the amplified Lb transcript. Cowpea *lbII* gene has four exons and three introns (Fig. 2), which is consistent with all of the known plant *hb* genes.

Cloning and Expression of the Cowpea rLbII cDNA in *E. coli* TB-1

When total RNA from cowpea nodules was used as the template with the specific primers for cowpea Lb, a PCR product approximately 450 bp in length was obtained. To determine if this was a LbII cDNA, it was cloned into the pCRII vector and sequenced in both directions. The sequence of this product was identical to the cowpea *lbII* gene, confirming that it was a LbII cDNA. The clone LbII was subcloned into the constitutive expression vector pEMBL19⁺, which contains a strong promoter of the *Pseudomonas putida* Cyt P-450 gene (Springer and Sligar, 1987), and then transformed in *E. coli* TB-1. The resulting transformed cells were reddish-brown in color and, after cell lysis, the red protein was found mostly in the supernatant. Spectra of the red supernatant exhibited absorption maxima at approximately 535 and 570 nm, suggesting that rLbII was synthesized by the *E. coli* cells and the resulting Lb was in the oxygenated form.

Purification of the Cowpea rLbII

Cowpea rLbII was purified by a multiple-step procedure, including ammonium sulfate precipitation, followed by hydrophobic and ionic-exchange chromatography, and then preparative electrophoresis (Fig. 3). Phenyl-Sepharose chromatography was a critical step because it eliminated many contaminating proteins (Fig. 3, lane 5). The rLbII that was eluted from the DEAE-cellulose column was highly

Figure 1. General strategy for sequencing the total cowpea *lbII* gene. An approximately 600-bp fragment was amplified from cowpea total DNA using the primers SyLb/A and SyLb/H. After cloning the polyA⁺ cowpea nodule RNA into λ vector, the inverse primers invSyLb/A and invSyLb/H were used in combination with the λ primers (Arredondo-Peter et al., 1995) to amplify the missing flanking sequences (coding and noncoding). Specific cowpea CPLb/ATG and CPLb/TAG primers were used to amplify the total *lbII* gene (679 bp). Arrows show the position and orientation of the PCR primers.

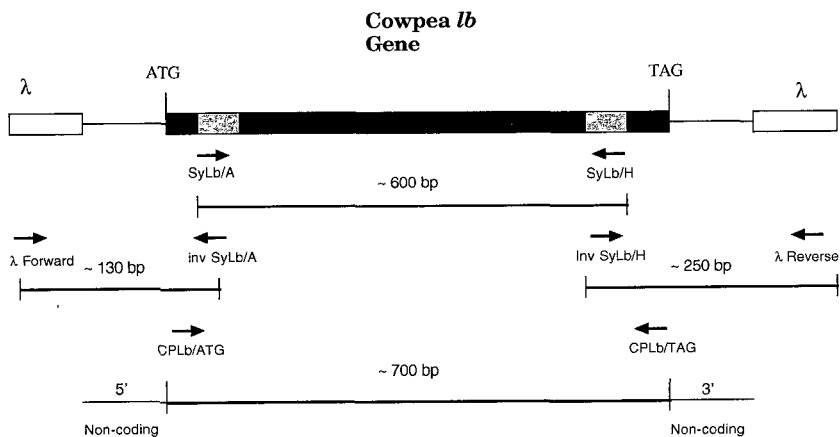
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60
ATGGTTGCCTTCTCTGACAAGCAAGAGGGTTTGGTGAATGGCGCATATGAAGCATTC AAG
M V A F S D K Q E G L V N G A Y E A F K
120
GCAGACATTCTAAATACAGTGTGTGCTTCTACACCACGcttagtatttgtatcttcctta
A D I P K Y S V V F Y T T
180
aacatctgtttttcatgcatctctatgtgtttgtgtttgtttgtttgacaaaacaaatgaa
240
cagaAATACTGGAGAAGCACCAGCAGCAAAGAACCTGTCTCGTTTCTAGCTAACGGAGT
I L E K A P A A K N L F S F L A N G V
300
TGATGCCACTAATCCTAAGCTCAGGGTCTATGCTGAAAAGCTTTTGGATTGGTaaagtgt
D A T N P K L T G H A E K L F G L V
360
gaaacaaatgaaattgaaattttgtttgttatgtttgtatgaaagattgaaatgaaaa
420
cgtaggtGCGTgATTcAGTcGCACAACtTAGAGCAAGtGGAGGAGTGGTGGCTGATGCTG
R D S A A Q L R A S G G V V A D A
480
CACTTGGTGTGTGCACTCTCAGAAAGCAGTGAACGATGCTCAGTTTGTGgtatgattag
A L G A V H S Q K A V N D A Q F V
540
tatgagtaagaataaagaatgaatatagaagattgatgtgaaataaagaagagaatgt
600
aaatgaaatgcagTGGTAAAAGAAGCTTGTGTCAAAACATtGAAAAGAAGCAGTtGGAGA
V V K E A L V K T L K E A V G D
660
CAAATGGAGTgATGAACtGGGCACTGCTGTGGAACtAGCCTACGATGAATtGGCAGCAGC
K W S D E L G T A V E L A Y D E L A A A
140
679
TATTAATAAAAGCATATTAG
I K K A Y - 145
    
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Figure 2. Total nucleotide sequence of the cowpea *lbII* gene and predicted protein sequence. Coding sequences (exons) are shown in uppercase and noncoding (introns) are shown in lowercase. Nucleotide sequences used for the synthesis of oligonucleotides for PCR amplification are underlined.

pure, but a few contaminants of approximately 40-kD were still detected on SDS-PAGE gels (Fig. 3, lane 6). Preparative electrophoresis on a 9% (w/v) native polyacrylamide gel removed all of the contaminants, resulting in a pure rLbII, as determined by SDS-PAGE (Fig. 3, lane 7) and on IEF gels (not shown). On a heme-to-protein basis, the rLbII was purified 63-fold relative to the original bacterial extracts, with a final yield of 0.23 mg pure rLbII per gram bacterial cell paste (Table I).

Cowpea rLbII (Fig. 3, lane 7) and nLbII (Fig. 3, lane 8) have identical mobilities on SDS-PAGE and a molecular



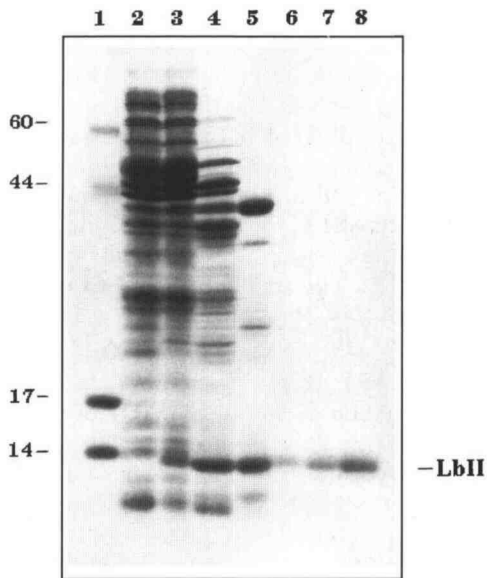


Figure 3. Purification of the cowpea rLbII synthesized by *E. coli* TB-1. Aliquots (10–50 μ g of total protein) were separated by SDS-PAGE in a 12.5% (w/v) gel. The gel was stained with Coomassie blue. Lane 1, Molecular mass markers; lane 2, supernatant of the untransformed *E. coli* TB-1; lane 3, supernatant of the *E. coli* TB-1 transformed with the pEMBL19⁺::LbII construct; lane 4, 55 to 90% (w/v) ammonium sulfate precipitation; lane 5, Phenyl-Sepharose; lane 6, DEAE-cellulose; lane 7, preparative electrophoresis on 9% (w/v) native-PAGE; and lane 8, pure cowpea nLbII. Markers are shown in kD.

mass of approximately 14 kD. Analysis in IEF gels showed that the rLbII and nLbII have an identical pI of 4.4. These data indicate that the rLbII synthesized by *E. coli* is similar to nLbII.

Characterization of the Cowpea rLbII

Immunoblot Analysis

Western blots were performed to evaluate if major changes were evident in rLbII. Pure cowpea rLbII, nLbII, and soybean Lb_a were electrophoresed in a SDS-PAGE gel, transferred to a nitrocellulose membrane, and probed us-

Table 1. Purification steps of the cowpea rLbII synthesized by *E. coli* TB-1

Step	Total Protein	Heme	R Heme: Protein ^a	Purification
	mg	mg	$\times 10^{-3}$	
Bacterial crude extract ^b	2799	1.0	0.36	1
Ammonium sulfate precipitation ^c	1185	0.48	0.40	1.1
Phenyl-Sepharose	243	0.24	0.99	2.75
DEAE-cellulose	12.6	0.18	14.3	39.7
Preparative electrophoresis	5.7	0.13	22.8	63.3

^a Relative purity expressed as the ratio heme:protein. ^b Soluble fraction obtained after lysing 25 g of cell paste and centrifugation. ^c Fraction corresponding to the protein precipitated between 55 and 90% (w/v) saturation with ammonium sulfate.

ing anti-soybean Lb_a antibodies. Bands of equal intensity were observed for rLbII and nLbII, indicating that antibodies to soybean Lb_a cross-reacted with the cowpea Lbs (Fig. 4). The observation that the two bands had comparable intensities suggests that rLbII and nLbII have similar antigenic epitopes.

Partial Sequence of the N Terminus of rLbII

The N terminus of the rLbII was sequenced to determine if it was identical to the predicted LbII from the gene sequence. Pure rLbII was electrophoresed on an IEF gel and then transferred to a membrane (Immobilon P, Millipore) for microsequencing of the first 10 amino acid residues at the N terminus of the rLbII. The sequence was determined to be VAFSDKQEGL, which is identical to the LbII sequence predicted from the *lbII* gene (Fig. 2).

Tryptic and Chymotryptic Maps of the rLbII and nLbII

Pure rLbII and nLbII were subjected to extensive tryptic and chymotryptic digestions, and the resulting peptides were separated on HPLC (Fig. 5). Chromatograms of the digested rLbII and nLbII are nearly identical, with no detectable differences substantiating that the rLbII and nLbII are structurally similar.

Spectral Analysis

Ferrous Lbs are known to bind with many ligands, including O₂, NO, CO, and nicotinate, and these ligated Lbs exhibit characteristic absorption maxima (Appleby et al., 1983; Appleby, 1984; Fleming et al., 1987). Ferric Lbs also bind nicotinate, which is a natural component of legume nodules (Appleby et al., 1973; Klucas and Appleby, 1991).

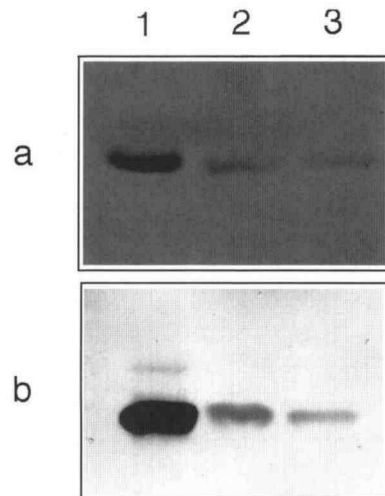


Figure 4. Western-blot analysis of soybean Lb_a and cowpea Lbs challenged with the anti-soybean Lb_a antibody. Soybean Lb_a (1) and cowpea nLbII (2) and rLbII (3) were electrophoresed in 12.5% (w/v) SDS-PAGE (a) and transferred to a nitrocellulose membrane for western blotting (b).

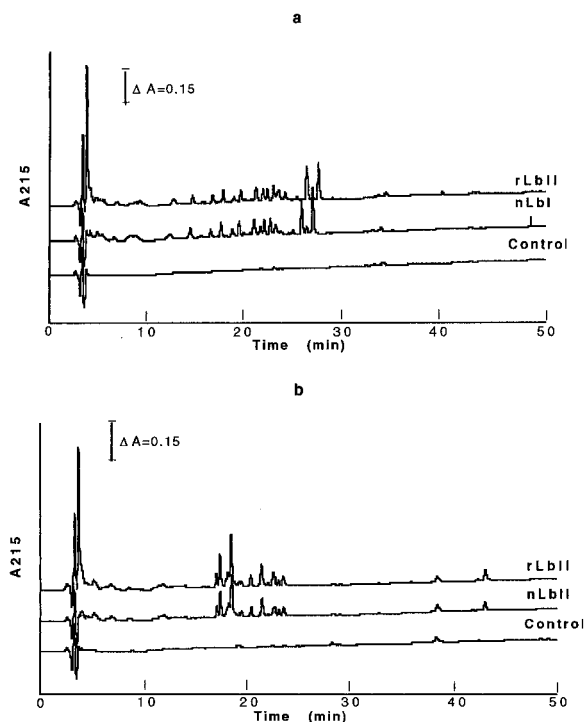


Figure 5. Tryptic (a) and chymotryptic (b) maps of the cowpea rLbII and nLbII. Peptides were separated on a Vydac-C₁₈ (218TP42) column at a flow rate of 1 mL min⁻¹.

Functional recombinant Lbs should possess comparable ligand-binding and spectral characteristics. Spectra of the various oxidation states and ligand complexes of the rLbII were similar to those of the nLbII. Values for the absorption maxima in the Soret region and for the α and β peaks were similar for rLbII and nLbII, as well as the maxima for the ferric forms and the ferric and ferrous rLbII-nicotinate complex (Table II).

Additional characteristics of functional Hbs are that they bind O₂ reversibly and that CO displaces O₂. The absorption maxima of the oxygenated and deoxygenated forms of rLbII were similar to those of the nLbII (Table II) and, qualitatively, the spectra of the rLbII and nLbII (Fig. 6) were similar to the spectra of the soybean Lbs (Appleby,

1974). When a solution of LbIIO₂ was bubbled with CO, rLbIICO was formed, a property that is unique for Hbs but not other heme-proteins (Fleming et al., 1987). The heme that was incorporated into the rLbII was identical to that found in nLbII. The differential (ferric versus ferrous) spectra of the dipyridine complexes of the rLbII and nLbII had a peak at 556 nm and a trough at 539 nm (not shown), which is characteristic of the heme B-dipyridine complex (Appleby and Bergersen, 1980). Our results indicated that the rLbII was a functional Hb and possessed all of the characteristics of nLbII.

Enzymatic Reduction of the Ferric rLbII

Another evaluation of the functionality of rLbII was to determine if it was a substrate for ferric Lb reductase, an enzyme postulated to be important for maintaining Lb in the functional ferrous state in nodules. In leguminous nodules ferric Lbs are thought to be formed by auto-oxidation of LbO₂ and one-electron oxidation of ferrous Lb; therefore, enzymatic and nonenzymatic mechanisms should exist in nodules to reduce the ferric Lb to functional ferrous Lbs (Becana and Klucas, 1990). An enzyme (ferric Lb reductase) with this activity has been isolated and purified from soybeans and shown to have a high affinity for Lbs (Ji et al., 1991, 1994). This reductase is also a very useful and non-destructive method for generating LbO₂ from ferric Lb (Saari and Klucas, 1984). We have isolated and purified a corresponding reductase from cowpea nodules (P. Luan and R.V. Klucas, unpublished data) and evaluated its effectiveness at reducing the ferric rLbII to ferrous rLbII. The rLbII was reduced by the ferric Lb reductase from cowpea nodules (Fig. 6) at rates comparable to those observed for the soybean ferric Lb reductase-soybean Lb system (Saari and Klucas, 1984; Ji et al., 1991). The isobestic (cross-over) points that occurred at 522 and 588 nm provided strong evidence that two main forms of rLbII (ferric rLbII and rLbIIO₂) were present throughout the reaction, and that there was no denatured Lb or other contaminant compounds.

CONCLUSIONS

We have cloned and expressed in *E. coli* a cDNA that encodes for the cowpea LbII. The expression vector used in

Table II. Spectral characteristics of the cowpea rLbII and nLbII

State/ligand		Absorption Maxima		
		nm		
rLbII				
Ferric	404.8	535	565 (Shoulder)	622 (Shoulder)
Ferrous/deoxygenated	425		556	
Ferrous/oxygenated	411	540		574
Ferrous/CO	416.5	538.5		562
Ferrous/nicotinate	419	525.5	554.5	
nLbII				
Ferric	404	534	560 (Shoulder)	620 (Shoulder)
Ferrous/deoxygenated	428		556	
Ferrous/oxygenated	411	540		574
Ferrous/CO	416	538		562
Ferrous/nicotinate	419	525	555	

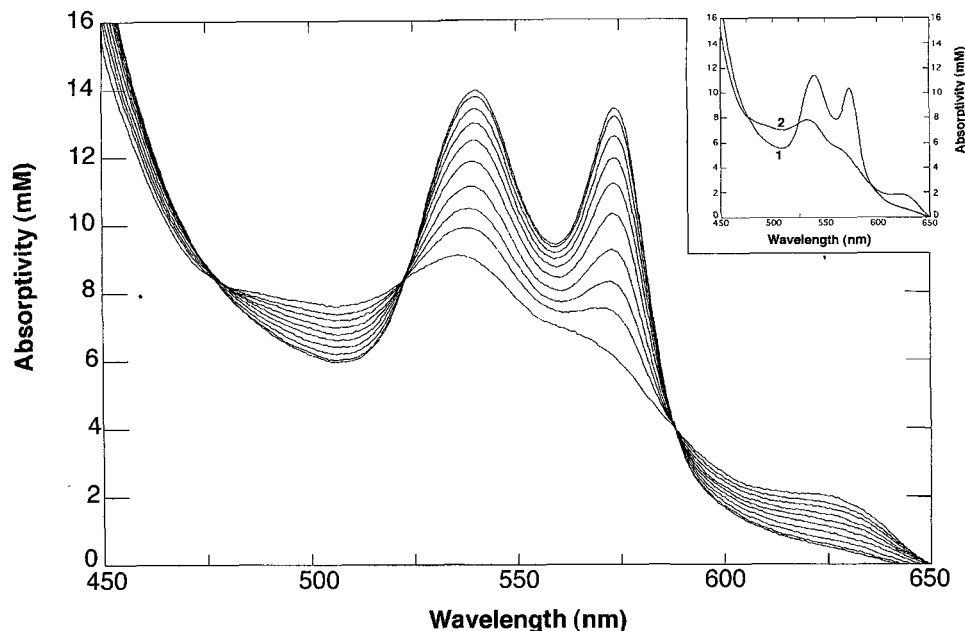


Figure 6. Enzymatic reduction of the rLbII by the CFLbR. The pure rLbII was oxidized by the addition of potassium ferricyanide, and the spectrum of the ferric form was obtained after the excess of cyanide was removed by filtration through a PD-10 column (see "Materials and Methods"). The ferric rLbII was then incubated with the CFLbR for reduction, and the spectra were recorded at 5-min intervals. Inset, Spectra of the nLbII isolated from nodules: curve 1, oxygenated; curve 2, ferric. The ferric spectrum of the nLbII was obtained as described above for rLbII, a few crystals of sodium dithionite were added and air was carefully bubbled to the nLbII solution to obtain oxygenated nLbII.

our work, pEMBL19⁺, does not incorporate additional amino acid residues onto the recombinant protein, so, as expected, a rLbII was expressed with structural and functional properties similar to nLbII. An additional feature was that most of the rLbII synthesized in *E. coli* was soluble, with very little recombinant protein in inclusion bodies. This eliminated problems usually associated with the use of detergents to solubilize and reconstitute the proteins in inclusion bodies.

The purified rLbII had structural and spectral properties similar to the nLbII isolated from cowpea nodules. Data from sequencing of the LbII cDNA cloned into pEMBL19⁺ and the partial sequence of the rLbII amino terminus, as well as the same tryptic and chymotryptic maps of the rLbII and nLbII, proved that the rLbII synthesized in *E. coli* had the same sequence as the nLbII synthesized in nodules. Modifications of the rLbII were also not detected by SDS-PAGE and western-blot analysis. Spectra of rLbII and nLbII had similar absorption maxima, and the rLbII was reversibly oxygenated and deoxygenated. Moreover, ferric rLbII was enzymatically reduced by the CFLbR, and the isobestic points showed that only two forms of Lb were present in the reaction. These data indicate that the rLbII is fully functional, with similar structural and functional properties as the nLbII synthesized in nodules. Cowpea LbII cloned into the expression vector pEMBL19⁺ is therefore a good system for generating large quantities of unmodified or mutated rLbII.

ACKNOWLEDGMENTS

The authors wish to express their gratitude to Drs. Mark Hargrove and John Olson (Rice University, Houston, TX) for the kind gift of the expression vector pEMBL19⁺, and to Eileen Singleton (Rice University) for the growth and harvest of *E. coli*.

Received December 30, 1996; accepted February 7, 1997.

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Sequences reported in this work are deposited in the GenBank data base under the accession nos. U33207 for the cowpea *lbII* gene and U33205 for the LbII RNA sequences.

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