ELSEVIER

Plant Science 108 (1995) 109–117

PLANT SCIENCE

Cloning and expression of plant leghemoglobin cDNA of *Lupinus luteus* in *Escherichia coli* and purification of the recombinant protein

Michal M. Sikorski*[a], Alexey F. Topunov[a], Pawel M. Stróżycki[a], Constantin E. Vorgias[b], Keith S. Wilson[b], Andrzej B. Legocki[a]

[a] *Institute of Bioorganic Chemistry, Polish Academy of Sciences, Noskowskiego 12/14, 61-704 Poznań, Poland*
[b] *European Molecular Biology Laboratory (EMBL), c/o DESY Notkestrasse 85, 22603 Hamburg, Germany*

Received 15 December 1994; revision received 2 March 1995; accepted 21 March 1995

Abstract

The yellow lupin leghemoglobin I gene (*lbI*) was cloned in the pET-3a vector. It was overexpressed in *Escherichia coli* BL21(DE3)pLysS cells, under the control of T7 RNA polymerase promoter. The recombinant LbI protein, containing *E. coli* derived heme, was purified to homogeneity using ion exchange and gel filtration chromatography. The recombinant LbI protein has spectral and immunochemical properties identical to LbI isolated from lupin root nodules. The identity between expressed polypeptide and native LbI protein was also supported by microsequencing analysis of the N-terminus of the purified recombinant protein.

Keywords: Expression; Heme-protein; Leghemoglobin; *Lupinus luteus*; Purification

1. Introduction

Leghemoglobins are heme-containing proteins of legume plants which reversibly bind oxygen. They are the most abundant nodulins and function as oxygen carriers, facilitating its transport to nitrogen fixing bacteroids [1,2]. This process is necessary for maintaining an adequate oxygen level within the central zone of the nodule of a legume plant, where the N_2-fixation occurs. Legume plant globins are products of different members of gene families [3]. The apoprotein moieties of Lbs are encoded by plant genes expressed after the infection by symbiotic bacteria of the genus *Rhizobium*. They are induced at a defined stage of nodule formation thus suggesting a developmental control of these genes. Heme, being

Abbreviations: LbI, *Lupinus luteus* (L.) leghemoglobin I; *lbI*, gene encoding LbI; PCR, polymerase chain reaction; pET-3a/*lbI*, pET-3a expression plasmid carrying LbI encoding gene; met-Hb, methemoglobin.

The nucleotide sequence of the full-length cDNA clone for LbI was registered in EMBL GenBank under accession number X77043.

* Corresponding author, Tel.: (+48-61) 528503; Fax: (+48-61) 520532; e-mail: mmsik@pozman.edu.pl.

0168-9452/95/$09.50
SSDI 0168-9452(95)04125-E

a part of physiologically active leghemoglobins has been found to be essential for the establishment of a fully effective symbiosis. Several defined genes (e.g. *hemA*) involved in rhizobial heme biosynthesis have been identified and although plant involvement in heme biosynthesis has been disputed, it is believed that heme present in leghemoglobin is of bacterial origin [4,5].

Two major leghemoglobins (LbI and LbII) have been identified and characterized in lupin [6,7] and their primary structures are known [8,9]. The cDNA sequence of LbI has already been determined [13] as well as that of LbII (Stróżycki et al., manuscript in preparation) but their expression in an *E. coli* system has not been reported.

Globin genes also have recently been detected in nonlegume plants [11]. Their function there remains unknown. In legumes, globin genes encode for the most abundant and functionally essential group of proteins in the newly formed organ — the root nodule.

Elucidation of the oxygen binding and exchange mechanism requires detailed structural analysis. It is difficult to obtain sufficient protein material from plants for structural studies. Therefore, an efficient bacterial system for the overproduction of the recombinant LbI protein is very important.

In this paper we present the cloning and overexpression of lupin LbI cDNA (*lbI*) in *E. coli*. This is a first report on overexpression in a bacterial system of globin cDNA from higher plants. We also present here purification of the recombinant protein and its characterization.

2. Materials and methods

2.1. Cell cultures and media

E. coli strains used were HMS174 ($F^-recA^-_{K12}r^+_{K12}m^+_{K12}Rif^R$) and BL21 ($F^-ompT^-_Bm^-_B$) [12]. The cDNA coding fragment of *lbI* was introduced into *Nde*I and *Bam*HI cloning sites of pET-3a expression vector [12,13]. *E. coli* cells were grown in LB medium at 37°C supplemented when needed with 50 μg ampicillin/ml and/or 25 μg chloramphenicol/ml.

2.2. Chemicals and enzymes

Restriction endonucleases, T4 DNA Ligase, TaqI polymerase, deoxynucleotides, calf intestine phosphatase and antibiotics were purchased from Boehringer Mannheim; X-Gal, IPTG, Tris-Base and salts from Sigma; T7 Sequencing Kit from Pharmacia LKB; Bacto Tryptone and Yeast Extract from Difco. Synthetic oligonucleotides have been prepared at the central EMBL DNA synthesis laboratory and purified by HPLC (on Cynchropak 300 C18, 6.5 μm, MZ Analyzentechnik). *Staphylococcus aureus* Protein A (Pharmacia LKB) was labelled with Na[^{125}I] (1 mCi; Amersham) using Chloramine T (Sigma) as an oxidant.

2.3. Plant leghemoglobin I from Lupinus luteus (L.)

Lupin nodule extract was prepared according to the procedure described by Szybiak-Stróżycka et al. [14] and Lbs were purified to homogeneity using ammonium sulphate precipitation (40–80% sat.), ion-exchange chromatography on DE-52 cellulose column and size exclusion column chromatography (Superose 6 HR 10/30, Pharmacia LKB).

2.4. In vitro gene amplification of the lbI cDNA coding region

The coding region for the LbI protein was copied and amplified by PCR from a cDNA fragment containing the *lbI* gene using two oligonucleotide primers. The N primer was designed to create an *Nde*I site (CGT CGC CAT ATG GGT GTT TTA ACT GAT GTG C) at the 5′-end of the *lbI* gene. The C primer was designed to add the *Bam*HI and two stop codons at the 3′-end of the *lbI* gene (GCG GGA TCC TTA TTA AGC AGC ATC CTT CAT CTC CTT). The amplification reaction of 50 μl contained 10 mM Tris–HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 50 ng of plasmid DNA carrying *lbI* cDNA clone (pSP25) (P. Stróżycki, manuscript in preparation), 20 pmol of N primer, 20 pmol of C primer, 200 μM of each dNTP and 1.0 U of TaqI polymerase. The reaction was carried out in three steps per cycle: denaturation at 94°C for 1 min, annealing at 55°C for 1 min and polymerization at 72°C for 2 min (30 cycles) using a TwinBlock System (Ericomp Inc., USA). A 5-μl aliquot of the 50-μl reaction mixture was analysed on a 1.0% agarose gel in 1 × TBE buffer.

The amplified 462-bp *lbI* gene was further inserted into the pET-3a plasmid by direct cloning at the restriction sites *Bam*HI and *Nde*I. The construct was introduced into the *E. coli* strain HMS174. The pET-3a plasmid harbouring the *lbI* gene was verified by DNA sequencing, using the chain termination method [15]. The pET-3a/*lbI* was further introduced into *E. coli* strain BL21(DE3)pLysS for overexpression. Transformation was carried out by the $CaCl_2$ method [16]. The molecular biology procedures were performed as described in Ref. [17].

2.5. Overproduction and purification of LbI recombinant protein

The BL21(DE3)pLysS cells carrying the pET-3a/*lbI* plasmid were grown overnight in 3 ml LB medium supplemented with ampicillin and chloramphenicol. The cells were diluted 250 times and grown for the next 3 h until the $OD_{600\ nm}$ reached a value of 0.8. The overexpression of the *lbI* gene was induced by adding IPTG to final concentration of 0.5 mM. The *E. coli* cells were induced for about 3.5 h and collected by low speed centrifugation. The total bacterial proteins were analysed by 15% SDS-PAGE [18].

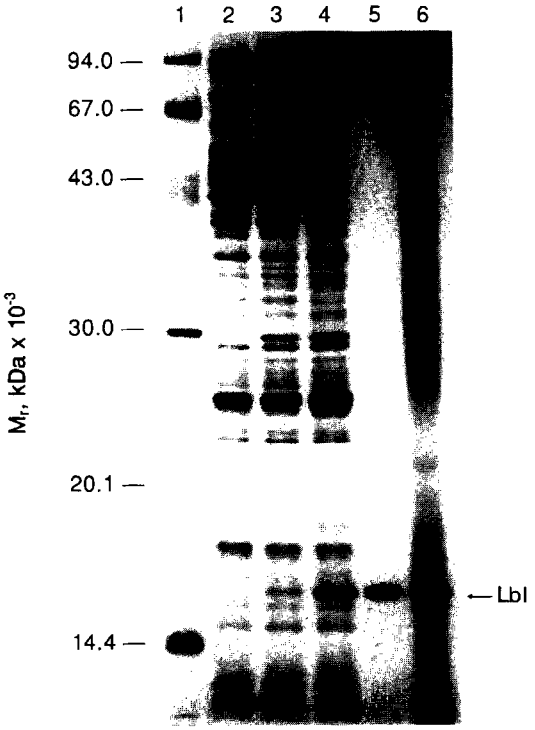


Fig. 1. Induction of *E. coli* BL21(DE3)pLysS carrying the pET-3a/*lbI* plasmid. Total proteins were analysed by 15% SDS-PAGE: Lane 1, molecular weight markers; Lane 2, non-transformed *E. coli* cells; Lane 3, transformed cells not induced; Lane 4, transformed cells IPTG-induced; Lane 5, purified LbI from lupin root nodules; Lane 6, total soluble proteins of root nodules.

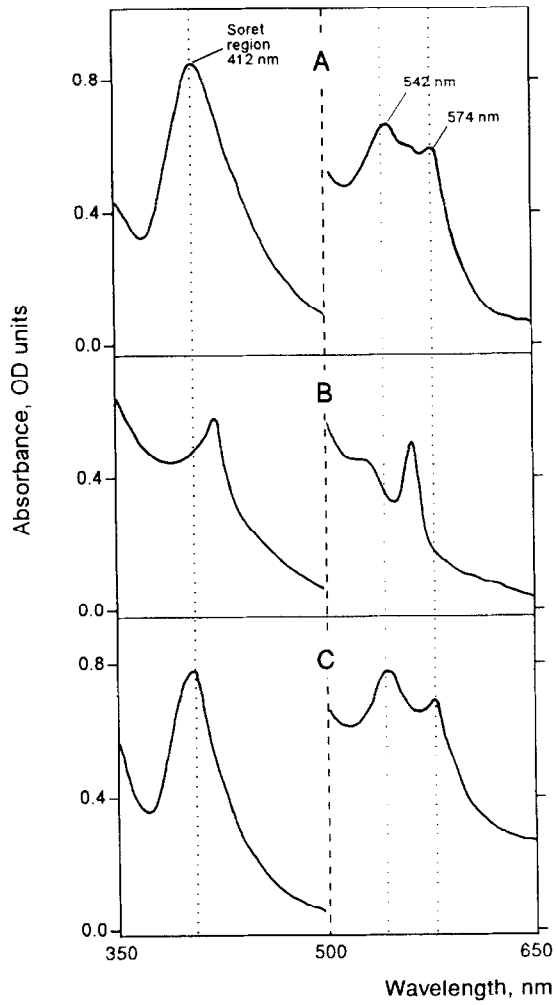


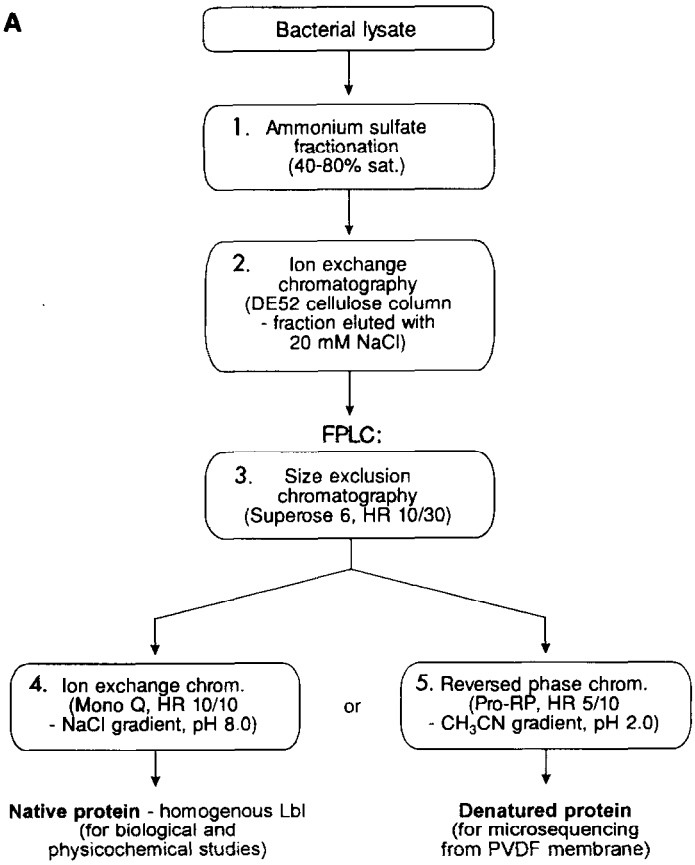
Fig. 2. Spectrophotometric analysis of total protein extract of *E. coli* cells harbouring the pET-3a/*lbI* plasmid after IPTG induction (A); total protein extract of *E. coli* cells without the plasmid (B) and total soluble protein from lupin root nodules (C).

For preparative purposes, a 1-l culture of transformed *E. coli* cells was grown and induced as described above. Then cells were collected by low speed centrifugation and resuspended in 50 mM Tris–HCl buffer pH 8.0 containing 10 mM EDTA. Cell lysis was achieved by repeated freezing and thawing of the cell suspension to break cell walls and membranes in order to release the T7 Lysozyme coded by the coexisting plasmid pLysS, as described in Ref. [12].

2.6. Purification of the overproduced LbI protein

The chromatin of bacterial lysate was separated from the total lysate by ultracentrifugation at 100 000 × *g* for 60 min at 4°C and the soluble proteins were further fractionated with ammonium sulphate. The LbI protein was quantitatively precipitated between 40 and 80% saturation of ammonium sulphate. This protein fraction was pelleted by centrifugation and desalted on a NAP-column (Pharmacia LKB). The enriched LbI protein fraction was directly applied to a DE-52 cellulose column (1.5 × 15 cm) equilibrated with buffer A (20 mM Tris–HCl pH 8.0, 5% glycerol, 10 mM β-mercaptoethanol and 6 M urea) and bound LbI protein was eluted in one step with 0.02 M NaCl in buffer A. The LbI fractions were applied to a Superose 6 HR 10/30 column (FPLC) in buffer A containing 50 mM NaCl, where a substantial amount of impurities was removed. The final purification step was the chromatography on Mono Q column HR 10/10, where bound LbI was eluted with 80 ml of NaCl linear gradient (from 0.01 to 0.30 M in buffer A).

Fig. 3



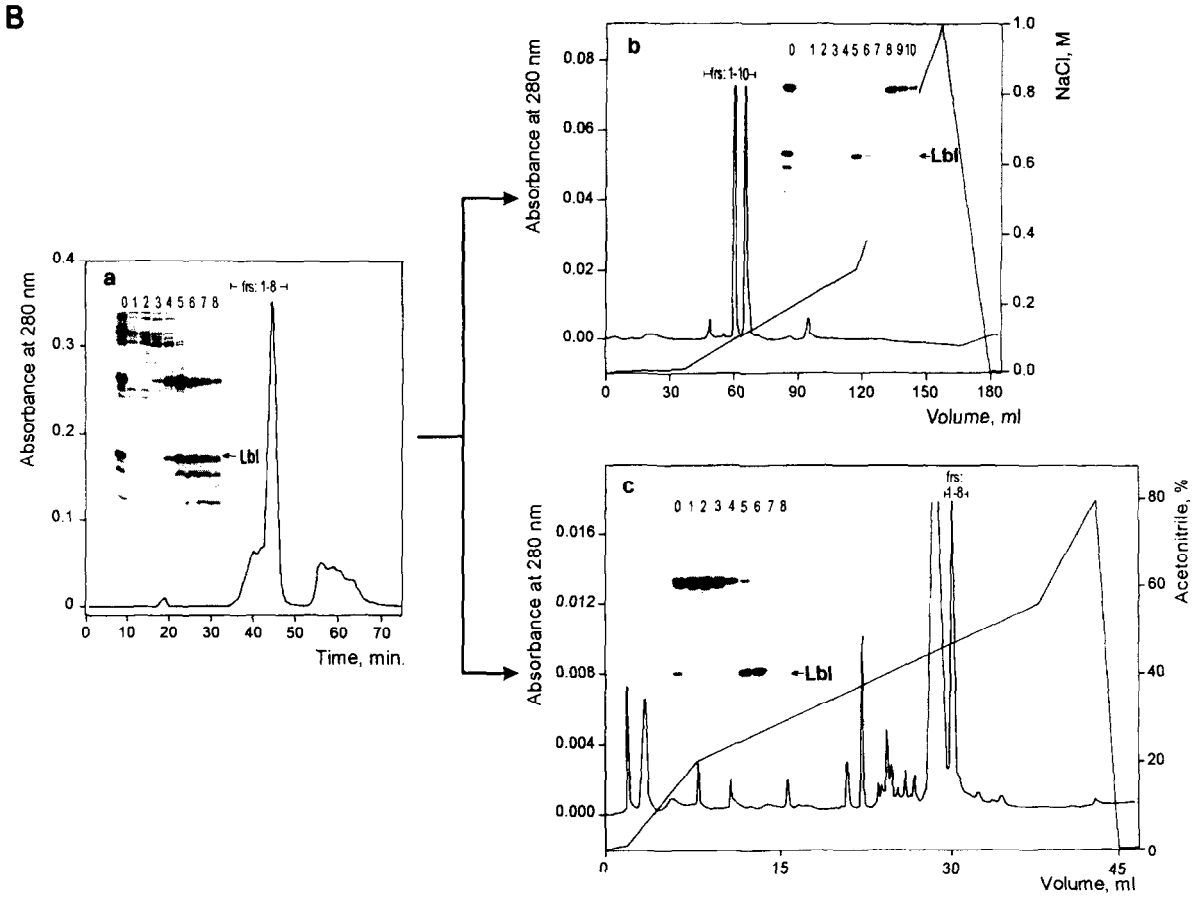


Fig. 3. (A) Flow diagram for the purification of leghemoglobin I (LbI) protein overproduced in *E. coli* BL21(DE3)pLysS cells. (B) FPLC chromatographic steps for LbI purification. Size exclusion chromatography on Superose 6 HR 10/30 column in buffer A containing 50 mM NaCl (a); ion-exchange chromatography on Mono Q HR 10/10 column of LbI-containing fractions after size exclusion chromatography on Superose (factions 5–8), performed with NaCl linear gradient from 0.01 to 0.30 M in buffer A (b); reversed phase chromatography on Pro-RP HR 5/10 column of LbI-containing fractions after size exclusion chromatography on Superose (fractions 5–8). The proteins were eluted from the column with a CH_3CN linear gradient from 0 to 80% in the presence of 0.1% TFA, 0.01% 2-mercapto-ethanol, pH 2.0 (c). Inserts on a, b and c show 15% SDS-PAGE of LbI-containing peaks.

2.7. *Analysis of recombinant protein*

The electrophoretic analysis of protein samples was carried out by 15% sodium dodecylsulphate/polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [18]. Gels were stained with Coomassie brilliant blue R-250. The Pharmacia LKB low molecular weight markers were used.

The optical absorption spectra of LbI samples were determined in the wavelength region of 350–650 nm, at room temperature on a Beckman spectrophotometer, model DU-65, using a 1-cm pathlength quartz optical cuvettes. The protein concentration was determined according to the method of Bradford [19]. The microsequencing was performed in the Protein Laboratory at EMBL Heidelberg by Dr R. Kellner.

2.8. *Immunochemical assay*

The recombinant LbI was also immunologically characterized. Western blot analysis was carried out as described in [20]. Purified LbI from 32-day-old root nodules was used as a positive control, and protein extract from non-transformed *E. coli*

cells as a negative control. The protein samples were transferred onto Immobilon P membrane in the MilliBlot-SDE transfer system and Western blot analysis was carried out according to the Millipore protocol. The membrane was incubated with IgGs against lupin nodulins (50 μg/ml) in 10 mM Tris–HCl pH 7.5 containing 0.9% NaCl and 1% BSA. The antigen–antibody complexes were detected with iodinated Protein A (350 000 cpm/ml). Rabbit antibodies against native LbI protein and against nodule-specific from yellow lupin were prepared according to the procedure previously described [14,21].

The IgGs against lupin nodulins were kindly provided by Dr. C.J. Mądrzak (University of Agriculture, Poznań).

3. Results

3.1. Clone selection and probes

A full length cDNA clone for *Lupinus luteus* LbI (846 bp) was selected from a lupin cDNA library [22]. The 754-bp *Hin*dIII fragment consisting of the coding region of the *lbI* cDNA clone was used as a probe [10]. The LbI cDNA clone was further subcloned into the *Pst*I site of the pK18 plasmid (P. Stróżycki, manuscript in preparation).

3.2. In vitro amplification, cloning and expression of lbI coding DNA sequence

The pK18 plasmid carrying the 846-bp *lbI* cDNA inserted into *Pst*I site (pSP25) was used as a template to copy and amplify by PCR the open reading frame for the LbI protein. Two oligonucleotide primers (N and C) containing the two unique restriction sites *Nde*I and *Bam*HI were designed to copy and amplify the DNA fragment in order to allow direct cloning of the *lbI* gene into the pET-3a expression vector in the correct orientation without shifting the reading frame. The amplified DNA fragment was extensively digested with *Nde*I and *Bam*HI restriction endonucleases and subcloned into the pET-3a expression vector. This plasmid was introduced into bacterial cells (strain HMS174) for storage and preparative purification. The pET-3a/*lbI* construct was verified by nucleotide sequence determination according to the Sanger method [15]. A clone without any misreading was used for transformation of BL21-(DE3)pLysS.

The pET-3a/*lbI* construct allows for the expression of the *lbI* gene under the control of T7 RNA polymerase promoter after IPTG is added to the growing cells [12]. The induction of *lbI* gene in BL21(DE3)pLysS under different conditions (described in the legend to Fig. 1) was analysed by 15% SDS-PAGE. The recombinant LbI protein migrates in the gel at the same position as the LbI protein from lupin root nodules and represents a substantial amount of the total bacterial proteins.

Pelleted *E. coli* cells after expression of LbI had a red-brown colour and the bacterial lysate was also red. Such colour is characteristic for heme-containing proteins. Total lysates were spectroscopically analysed. Spectrum A in Fig. 2, which represents the bacterial lysate of induced *E. coli* cells clearly shows three characteristic peaks at 412, 542 and 574 nm. The 412-nm peak is the maximum in the Soret region (region corresponding to heme-containing proteins) and the two other peaks at 542 and 574 nm are due to oxygenated Lb. In the negative control where bacterial lysate of non-transformed cells was used, as shown in spectrum B of Fig. 2, the absence of these characteristic peaks is obvious. These data are in agreement with

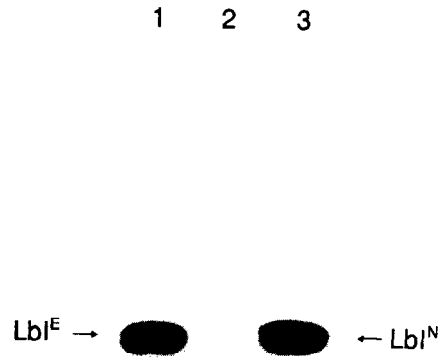


Fig. 4. Western blot analysis of expressed LbI. The *Staphylococcus aureus* Protein A was ^{125}I-labeled and the immunoreaction was visualized by autoradiography: Lane 1, LbI expressed in *E. coli* cells; Lane 2, protein extract of non-transformed *E. coli* cells; Lane 3, LbI purified from lupin root nodules.

Table 1
Purification steps of leghemoglobin I overproduced in *E. coli* BL21(DE3)pLysS cells carrying the pET-3a/*lbI* plasmid

Purification step		Volume (ml)	Concentration (mg/ml)	Total amount (mg)	Yield (%)
1.	Crude bacterial lysate*	120.0	2.2	264.0	100.0
2.	Ammonium sulphate precipitation, 40–80% sat.	11.5	16.0	184.0	70.0
3.	DE-52 Cellulose chromatography	13.6	2.0	6.8	2.5
4.	FPLC-Superose 6 column	4.5	0.7	3.2	1.2
5.	FPLC-Mono Q column	2.0	0.9	1.8	0.7

*Crude bacterial lysate was prepared from a 2-l culture of bacterial cells.

spectral characteristics of lupin Lbs [23]. Spectrum A is also identical with that of protein extract of lupin nodules (spectrum C in Fig. 2).

3.3. *Purification of the recombinant LbI protein*

The bacterial protein extract of induced cells was subjected to further purification. The procedure is outlined in Fig. 3A and includes initial fractionation of the total bacterial lysate by ammonium sulphate followed by conventional chromatography steps.

During the initial step, a substantial enrichment of LbI protein was achieved. The second step was an ion exchange chromatography on DE-52 cellulose. The LbI protein was weakly bound on this column at pH 8.0 in the presence of the strong denaturing agent urea and it was eluted from the column in one step, using 20 mM NaCl (data not shown). The third step was gel filtration on a high performance Superose 6 column in the absence of urea and in the presence of 50 mM NaCl. A part of the protein was eluted in the void volume of the column, possibly due to aggregation of LbI with other high molecular weight impurities as shown in Fig. 3B (insert, lane 0). This step was nevertheless essential because it separated the soluble part of LbI protein from the aggregated molecules. The next and final purification step had two alternatives. The first was application of high performance liquid chromatography using a ProRP HR column. The LbI obtained by this method was still contaminated with the 30-kDa protein however, it was well separated from other polypeptides. After electroblotting of the LbI fraction into PVDF membrane, N-terminal amino acid sequence was performed (20 residues determined were identical to the native LbI from yellow lupin — results not shown). The alternative and very effective chromatography is the Mono Q high resolution column which is able to quantitatively separate the two major proteins as two distinct peaks. The purified recombinant LbI protein was immunologically compared to the native LbI as shown in Fig. 4 and both were found to be crossreactive, proving once more that the overproduced and purified polypeptide is the correct protein.

The yield of the purification of the LbI protein overproduced in BL21(DE3) pLysS cells carrying the pET-3a plasmid is shown in Table 1.

4. Discussion

Leghemoglobin is a heme-containing protein present in all nitrogen fixing legume plants. This hemeprotein reversibly binds oxygen and is expressed in root nodule tissue containing bacteroids. Monospecific antibodies to Lb have subcellularly localized this protein in the cytoplasm [24]. Leghemoglobin comprises a heme prosthetic group (synthesized from the bacterial precursor-protoporphyrin IX) [4,5,25] and its protein structure is similar to that of monomeric myoglobin. Leghemoglobins represent a group of highly related proteins different in their primary structures. There is usually more than one protein type in every legume species. Yellow lupin has two major leghemoglobins: LbI and LbII, as in pea, whereas soybean has four leghemoglobins. Such a hetero-

geneity may suggest different functionality and expression of Lbs at various stages of the symbiotic relationship. Leghemoglobins are thought to be products of symbiotically coordinated synthesis by *Rhizobium* (heme) and by the host plant (apoleghemoglobin). They exhibit high oxygen affinity in low pressure areas and maintain low oxygen pressure in the central tissue of the nodule during vigorous oxygen consumption by the rhizobial bacteroids. Leghemoglobin releases oxygen for rhizobial respiration at low pressure, maintaining it throughout the life of a nodule and protecting the oxygen-sensitive nitrogenase enzyme complex [25].

Yellow lupin leghemoglobin I (LbI) was overexpressed in *E. coli* BL21(DE3)pLysS strain under the control of T7 RNA polymerase promoter. The LbI coding sequence (462 bp) was copied and amplified by polymerase chain reaction from a pK18 plasmid carrying the entire cDNA clone of 836 bp. The *lbI* coding DNA fragment was inserted into the pET-3a expression vector with the correct open reading frame. The LbI protein produced in *E. coli* harbouring the pET-3a/*lbI* appeared to be heme-containing protein (plant leghemoglobin I). A cell pellet as well as the lysate containing proteins from transformants were bright-red in colour. It was shown by SDS-PAGE analysis of bacterial lysates (Fig. 1) that a 17.2-kDa protein appearing in IPTG-induced *E. coli* cells was identical to native leghemoglobin purified from lupin root nodules.

Spectral analysis of bacterial cell lysates of IPTG-induced cells (Fig. 2A) was consistent with that of ferric-hemoprotein with maximum absorption in the Soret region at 412 nm (Fig. 2C, spectrum of total soluble proteins of lupin root nodules). Two peaks with maximum absorption at 542 and 574 nm correspond to the oxygenated form of leghemoglobin (spectra A and C, Fig. 2). The spectrum of expressed LbI is in agreement with that presented earlier [23].

Physiologically active oxygen-binding leghemoglobin is in a reduced form due to the presence of met-Hb or met-Lb-reductase in *E. coli* cells [26]. It has already been speculated that *Vitreoscilla* hemoglobin is enzymatically reduced by met-Hb-reductase which may be present in bacterial cells [27,28]. In our case, the absorption in the Soret region in non-transformed cells (Fig. 2B) was due to heme-containing proteins (e.g. cytochromes) present in *E. coli* cells. However, IPTG-induced *E. coli* cells revealed typical spectral properties of myoglobins or hemoglobins from other sources, including protozoans. The fact that LbI is synthesized in *E. coli* cells as a heme-containing protein is in agreement with expression of bacterial *Vitreoscilla* hemoglobin [27,29] and hemoglobin of the cyanobacterium *Nostoc* [30]. However, it should be indicated at this point that this paper presents the first report of overexpression of globin cDNA from higher plants.

Spectral analysis confirms that bacterial protoporphyrine IX (the immediate precursor of heme) is used for synthesis of native hemoglobin-like proteins in bacterial expression systems. The bacterial protein lysate containing the expressed LbI was purified according to the scheme shown in Fig. 3A, including ammonium sulphate fractionation, ion exchange chromatography on DE-52 cellulose column and two FPLC steps — size exclusion and anion exchange chromatography on Mono Q column or reversed phase chromatography on Pro-RP column. The resulting homogeneous LbI was then used for microsequencing and Western blot analysis.

N-terminal sequence analysis of polypeptide confirmed that the expressed protein was identical to native LbI. The expressed LbI is immunochemically active with either monospecific antibodies against native LbI (data not shown or antibodies against total lupin nodulins).

We conclude that the bacterial expression system applied in this work can be successfully used for synthesis of plant leghemoglobins and preparation of large quantities of those proteins for structural studies.

Acknowledgements

This work was partially supported by grant from State Committee for Scientific Research No. 6-6282-92-03. We thank Miss Alina Akuszewska and Dr Zbigniew Michalski for their excellent technical and editorial assistance.

References

[1] C.A. Appleby, Leghemoglobin and *Rhizobium* respiration. Annu. Rev. Plant Physiol., 35 (1984) 443–478.

[2] J.B. Wittenberg and B.A. Wittenberg, Mechanism of cytoplasmic hemoglobin and myoglobin function. Annu. Rev. Biophys. Biophys. Chem., 19 (1990) 217–241.

[3] J.J. Hyldig-Nielsen, E.O. Jensen, K. Paludan, O. Wiborg, R. Garrett, P. Jørgensen and K.A. Marcker, The primary structures of two leghemoglobin genes from soybean. Nucleic Acid Res., 10 (1992) 689–701.

[4] M.L. Guerinot and B.K. Chelm, Bacterial δ-aminolevulinic acid synthase activity is not essential for leghemoglobin formation in the soybean/*Bradyrhizobium japonicum* symbiosis. Proc. Natl. Acad. Sci. USA, 83 (1986) 1837–1841.

[5] S.A. Leong, G.S. Ditta and D.R. Helinski, Heme biosynthesis in *Rhizobium*. Identification of a cloned gene coding for δ-aminolevulinic acid synthetase from *Rhizobium meliloti*. J. Biol. Chem., 257 (1982) 8724–8730.

[6] S.S. Melik-Sarkissyan, V.V. Yarovenko and W.L. Kretovich, Isolation and fractionation of leghemoglobin from nodules of yellow lupin. Doklady Akad. Nauk SSSR, 188 (1969) 1390–1393.

[7] W.J. Broughton, M.J. Dilworth and C.A. Goodfrey, Molecular properties of lupin and seradella leghemoglobins. Biochem. J., 127 (1972) 309–314.

[8] Ts.A. Egorov, M.Yu. Feigina, V.K. Kazakov, M.I. Shakhparonov, S.I. Mitaleva and Yu.A. Ovchinnikov, The complete amino acid sequence of leghemoglobin I from yellow lupin. Bioorgan. Khimiya, 2 (1976) 125–128.

[9] Ts.A. Egorov, V.K. Kazakov, M.I. Shakhparonov, M.Yu. Feigina and P.V. Kostetsky, The complete amino acid sequence of leghemoglobin II from yellow lupin (*Lupinus luteus* L.). Bioorgan. Khimiya, 4 (1978) 476–480.

[10] A. Konieczny, Nucleotide sequence of lupin leghemoglobin I cDNA. Nucleic Acid Res., 15 (1987) 6742.

[11] D. Bogusz, C.A. Appleby, J. Landsmann, E.S. Dennis, M.J. Trinick and W.J. Pecock, Functioning hemoglobin genes in non-nodulating plant. Nature, 331 (1988) 178–180.

[12] F.W. Studier, A.H. Rosenberg, J.J. Dunn and J.W. Dubendorff, Use of T7 RNA Polymerase to direct expression of cloned genes. Methods Enzymol., 185 (1990) 60–89.

[13] F.W. Studier and B.A. Moffatt, Use of bacterial T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol., 189 (1986) 113–130.

[14] U. Szybiak-Stróżycka, P. Stróżycki, M. Sikorski, B. Golińska, C.J. Mądrzak and A.B. Legocki, Lupin leghemoglobin during root nodule development. Acta Biochim. Polon., 34 (1987) 79–85.

[15] F. Sanger, S. Nicklen and A.R. Coulson, DNA sequencing with chain-terminal inhibitors. Proc. Natl. Acad. Sci. USA, 74 (1977) 5463–5467.

[16] M. Mandel and A. Higa, Calcium-dependent bacteriophage DNA infection. J. Mol. Biol., 53 (1970) 159–162.

[17] J. Sambrook, E.F. Fritsch and T. Maniatis, Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989).

[18] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227 (1970) 680–685.

[19] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein due binding. Anal. Biochem., 72 (1976) 248–254.

[20] H.W. Burnette, Western blotting: electrophoretic transfer of protein from SDS-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem., 112 (1981) 195–203.

[21] P. Stróżycki and A.B. Legocki, Isolation and characterization of root nodule proteins from lupin. Acta Biochim. Polon., 35 (1988) 39–50.

[22] A. Konieczny, E.O. Jensen, K.A. Marcker and A.B. Legocki, Molecular cloning of lupin leghemoglobin cDNA. Mol. Biol. Rep., 12 (1987) 61–66.

[23] S.S. Melik-Sarkissyan, N.F. Bashirova, L.P. Vladzievskaya, G.P. Karpilenko and W.L. Kretovich, Legoglobin investigation of lupin nodules by means of isoelectric focusing. Izv. Akad. Nauk SSSR Ser. Biol., 6 (1975) 837–845.

[24] J.G. Robertson, B. Wells, T. Bisseling, K.J.F. Farden and A.W.B. Johnston, Immuno-gold localization of leghaemoglobin in cytoplasm in nitrogen-fixing root nodules of pea. Nature, 331 (1984) 254–256.

[25] R. Powell and F. Gannon, The leghaemoglobins. BioEssays, 9 (1988) 117–121.

[26] V.V. Talyzin, A.F. Topunov, N.F. Bashirova and W.L. Kretovich, Bacterial reductases that reduce oxygen-transporting hemoproteins. Dokl. Akad. Nauk SSSR (English translation), 303 (1988) 403–404.

[27] K.L. Dikshit and D.A. Webster, Cloning, characterization and expression of the bacterial globin gene from *Vitreoscilla* in *Escherichia coli*. Gene, 70 (1988) 377–386.

[28] R.P. Dikshit, K.L. Dikshit, Y. Liu and D.A. Webster, The bacterial hemoglobin from *Vitreoscilla* can support the aerobic growth of *Escherichia coli* lacking terminal oxidases. Arch. Biochem. Biophys., 293 (1992) 241–245.

[29] C. Khosla and J.E. Bailey, The *Vitreoscilla* hemoglobin gene: molecular cloning, nucleotide sequence and genetic expression in *Escherichia coli*. Mol. Gen. Genet., 214 (1988) 158–161.

[30] M. Potts, S.V. Angeloni, R.E. Ebel and D. Bassam, Myoglobin in a cyanobacterium. Science, 256 (1990) 1690–1692.