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Purification and Properties of *Phaseolus vulgaris* Leghemoglobin (PhLb)

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Leghemoglobin components *a* and *b* (PhLb*a* and PhLb*b*) were purified from root nodules of *Phaseolus vulgaris*. Homogeneity of PhLb*a* was demonstrated by polyacrylamide gel disc electrophoresis. Calibrated gel filtration gave a molecular weight of 16 900 for PhLb*a*. The total amino acid compositions of PhLb*a* and PhLb*b* suggest that PhLb*b* is a deamidation product of PhLb*a*. The amino acid composition of PhLb*a* is: Lys_{12}, His_2, Arg_2, Asx_{14}, Thr_7, Ser_{11}, Glx_{15}, Pro_4, Gly_8, Ala_{25}, Val_{11}, Ile_4, Leu_{17}, Tyr_4, Phe_7, Trp_2. These 145 amino acid residues correspond to a molecular weight of 16 115 for the hemoprotein. The *N*-terminal sequence of PhLb*a* is *Gly-Ala-Phe-Thr-Glx-* and the *C*-terminal residue is alanine. The isoelectric point of PhLb*a* is 4.70 and that of PhLb*b* 4.55.

Leguminous root nodule leghemoglobin may make an important contribution to the knowledge of the structure—function relationship of heme proteins. Leghemoglobin is structurally and functionally related to animal hemoglobins and myoglobins. So far only soybean leghemoglobin component *a* has been extensively studied.[1] The present work describes the purification and some molecular properties of kidney bean (*Phaseolus vulgaris*) leghemoglobin.

MATERIALS AND METHODS

Planting of kidney beans. Seeds of kidney bean *Phaseolus vulgaris*, var. Kaiser Wilhelm, were surface sterilized, inoculated with *Rhizobium phaseoli* (Lantbrukshögskolan, Ultuna, Sweden) and grown in sea sand in a greenhouse essentially as described previously.[2] EDTA-Fe(III)-Na salt was used instead of ferric citrate. The harvested root nodules were stored at −16 °C.

Purification of leghemoglobin. The nodules were extracted in a mortar with ice-cold 20 mM phosphate buffer, pH 6.8. All other operations were performed at 4 °C. Leghemoglobin was precipitated from the centrifuged nodule extract by adding crystalline ammonium sulfate to 55 − 80 % saturation. The pH was maintained at 6.5 − 7.0 with ammonium hydroxide. The centrifuged precipitate was dissolved in the extraction buffer, dialyzed against distilled water and concentrated by pressure filtration on an Amicon UM-10 membrane. Fractionation of PhLb was performed on DEAE-Sephadex A-25 (Pharmacia) equilibrated with 15 mM acetate buffer pH 5.6. Convex gradient elution with respect to ionic strength was employed. A closed mixing chamber containing 250 ml of the equilibration buffer was connected to a 52 mM buffer reservoir, and, after PhLb*a* had emerged from the column, to a 100 mM buffer of the same pH.

Disc electrophoresis. Disc electrophoresis in 7.5 % polyacrylamide gels at pH 7.5 was conducted as described by Maurer,[3] except that the samples were laid directly onto the separation gels. The protein bands were visualized by staining with Coomassie blue.[4]

Analytical gel filtration. A column (1.5 × 92 cm) of Sephadex G-50 superfine (Pharmacia) equilibrated with pH 6.0 phosphate buffer of ionic strength 0.05 was used. 1.5 ml fractions were collected at a flow rate of 6 ml/h. The blue dextran void volume was detected by following A_{625}, and the elution volumes of the marker hemoproteins by A_{409}. The molecular weight of PhLb*a* was determined essentially as described by Andrews.[5]

Isoelectric focusing. The leghemoglobin fraction precipitating at 55 − 80 % ammonium sulfate saturation from the root nodule extract was dialyzed, and a sample of 6 − 10 mg was used for the electrofocusing performed according to Vesterberg and Svensson[6] in a 110 ml LKB 8101 column. A 1 % concentration of carrier ampholytes in the pH range 4.5 − 5.0 (Ampholine, LKB) was used. The column was cooled with 2 °C water. After focusing for 24 h

at 700 V, fractions of 2 or 1 ml were collected and the pH values were measured immediately at 2 °C (Radiometer PHM 4c). The pH meter was calibrated with 0.05 M potassium diphthalate and pH 7 standard buffer (Beckman No. 3581). Leghemoglobin was detected by following the absorbance at 404 nm.

Dry weight determination. Dialyzed leghemoglobin samples were dried to constant weight at 105 °C and weighed on a Cahn electrobalance.

Amino acid composition. About 2.5 mg of PhLb*a* or PhLb*b* was hydrolyzed in 2.0 ml of 6 N HCl containing 10 µl of 0.1 M phenol for 20 or 72 h in evacuated, sealed tubes at 110 °C. The amino acid composition was determined on a Beckman Spinco Model 120 B amino acid analyzer according to Spackman *et al.*[7] Values for serine, threonine, tyrosine, and phenylalanine were extrapolated to zero hydrolysis time, and for valine, isoleucine, and leucine the 72 h values were taken to be the final ones. The spectrophotometric method of Spies and Chambers [8] was used for the estimation of tryptophan from unhydrolyzed samples.

Apoleghemoglobin. ApoPhLb*a* and apoPhLb*b* were prepared as described previously.[9] Centrifugation of the apoprotein from acid acetone was performed at −13 °C.

N-*Terminal sequence determination.* The *N*-terminal amino acid sequence of apoPhLb*a* was determined by the dansyl-Edman procedure.[10] The dansylated amino acids were identified by thin layer chromatography on 3 × 3 cm polyamide sheets (BDH).[11]

Hydrazinolysis. Hydrazinolysis was performed according to Braun and Schroeder.[12] Hydrazine

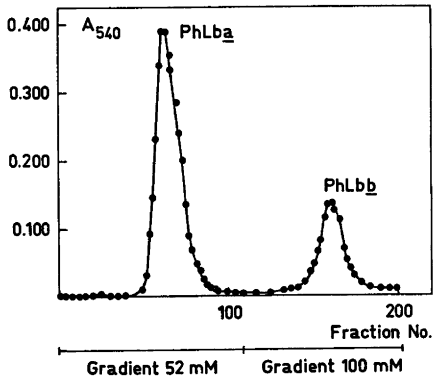

Fig. 1. Fractionation of *Phaseolus vulgaris* leghemoglobin on a DEAE-Sephadex A-25 column (2.5 × 36 cm) equilibrated with 15 mM acetate buffer pH 5.6. Gradient elution was performed with 52 mM and 100 mM acetate buffer pH 5.6. The sample has been purified from 200 g of wet nodules after 1 − 1.5 year's storage at −16 °C.

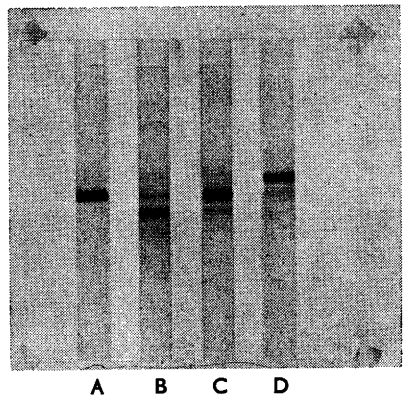

Fig. 2. Polyacrylamide disc electrophoresis (pH 7.5, 7.5 % polyacrylamide) of purified PhLb*a* (A), PhLb*b* (B), purified PhLb*a* after prolonged storage at −16 °C with occasional melting and refreezing (C) and apoPhLb*a* (D). Migration is from top to bottom.

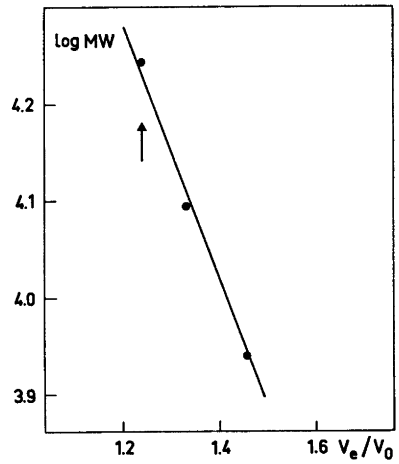

Fig. 3. Estimation of the molecular weight of PhLb*a* by gel chromatography on Sephadex G-50 superfine. The logarithms of the molecular weights are plotted against V_e/V_0. The reference proteins of known molecular weight are: (1) *Pseudomonas aeruginosa* cytochrome *c*-551, 8700;[13] (2) horse heart cytochrome *c*, 12 400;[25] (3) horse myoglobin, 17 600.[26] The position of PhLb*a* is marked by an arrow.

(95+ %, Eastman) was distilled and stored at 4 °C. Thoroughly dialyzed apoPhLb*a* or apoPhLb*b* (0.25 µmol) was lyophilized and dried in an Abderhalden dryer at 64 °C for 24 h, with 50 mg of Amberlite CG-50 resin in the hydrogen form added to the sample. After 72 h hydrazinolysis at 80 °C the liberated amino

acids were separated from hydrazides on a 1 x 25 cm Whatman cellulose phosphate P-70 column equilibrated with 0.4 M pyridine-formate buffer pH 3.2. Small aliquots (about 1/100) of the effluent fractions were analyzed by high voltage paper electrophoresis at pH 1.9.[13] The paper was stained with ninhydrin-cadmium acetate.[14] The fractions containing the neutral or basic amino acids were pooled, lyophilized and analyzed in the amino acid analyzer.

Pyridine hemochrome was determined according to Paul *et al.*[15]

Spectra. Absorption spectra were recorded with a Cary 15 spectrophotometer.

Abbreviations. EDTA = ethylenediaminetetraacetic acid, dansyl = 1-dimethylaminonaphthalene-5-sulfonyl.

RESULTS AND DISCUSSION

Purification. Fig. 1 represents the elution diagram of the PhLb components *a* and *b* from the anion exchanger. The yield of PhLb*a* is about 0.6 mg from 1 g of wet nodules. The amount of PhLb*b* varied from 5 % to 30 % of the amount of PhLb*a*, the smallest value for PhLb*b* being obtained from the brightest red nodules.

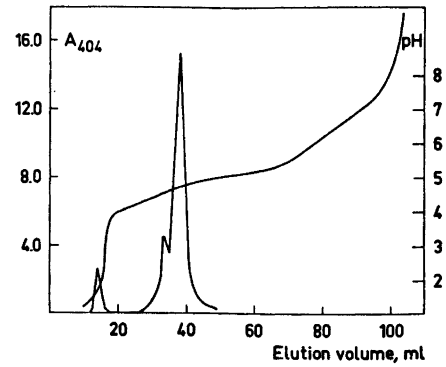

Fig. 4. Isoelectric focusing of the PhLb components in a sucrose density gradient. The experimental conditions are described in Methods section.

Criteria for homogeneity. In the isoelectric focusing column very faint red bands with somewhat higher pI than PhLb*a* can be seen

Table 1. The amino acid compositions of PhLb*a* and PhLb*b*.

Amino acid residue	PhLb*a* Hydrolysis time 20 h	70 h	Extrapolated or average value	Nearest integer	PhLb*b* Hydrolysis time 20 h	70 h	Extrapolated or average value	Nearest integer
Lys	11.98	11.47	11.73	12	11.76	11.98	11.87	12
His	1.82	1.85	1.84	2	1.95	1.84	1.90	2
Arg	1.86	1.60	1.73	2	1.94	2.06	2.00	2
Asp	14.37	14.32	14.35	14	14.67	14.43	14.55	15
Thr	6.68	6.00	6.95	7	6.85	6.14	7.12	7
Ser	9.82	7.95	10.60	11	9.80	8.09	10.60	11
Glu	15.02	15.10	15.06	15	15.10	15.12	15.11	15
Pro	4.24	4.37	4.31	4	4.28	4.39	4.34	4
Gly	7.96	7.71	7.84	8	8.22	7.97	8.10	8
Ala	25.03	24.80	24.92	25	24.62	24.69	24.66	25
Cys	0	0	0	0	0	0	0	0
Val	9.04	10.45	10.45	11	8.73	10.51	10.51	11
Met	0	0	0	0	0	0	0	0
Ile	3.70	3.70	3.70	4	3.81	3.93	3.93	4
Leu	17.02	17.44	17.44	17	16.62	17.07	17.07	17
Tyr	3.41	2.83	3.72	4	3.22	2.93	3.50	4
Phe	6.86	6.64	6.98	7	6.55	6.34	6.70	7
Trp	2.18 [a]		2.18	2	2.21 [a]		2.21	2
Total number of residues				145				
Recovery				97.2 %				93.2 %

[a] Determined spectrophotometrically (see Methods).

close above the major bands. This microheterogeneity possibly represents minor genetic variants of leghemoglobin sequence in a large population. Point mutations have been detected in the soybean leghemoglobin sequence.[16] The purified PhLb*a* migrated as a single band in acrylamide gel electrophoresis (Fig. 2), although in many extensively dialyzed PhLb*a* samples some apoprotein was also detected.

Prosthetic group. The pyridine hemochrome of PhLb*a* has a spectrum typical of protoheme IX compounds, with absorption maxima at 557 and 526 nm. The iron content of PhLb*a* is 0.313 % and that of PhLb*b* 0.312 % as determined by the pyridine hemochrome method. The theoretical value from the total amino acid analysis, assuming one heme group per molecule, is 0.347 %. The heme of PhLb seems to have a slight tendency to detach from the apoprotein, which can also be seen in disc electrophoresis.

Molecular weight and amino acid composition. The molecular weight of PhLb*a* obtained by analytical gel filtration is 16 900 (Fig. 3). Total amino acid analysis (Table 1) gives a molecular weight of 15 500 for the apoprotein and 16 115 for the hemoprotein. The value for the molecular weight of kidney bean leghemoglobin is close to that of soybean Lb*a* and Lb*c* (15 775 [16] and 16 695,[9] respectively). Values of 15 260 [17] for lupin and 16 400–16 700 [18] for snake bean leghemoglobin have been reported. Molecular weights of 17 500–20 600 for lupin and serradella leghemoglobins [19,20] are considerably higher. Variations in leghemoglobin molecular weights over such wide limits (15 300–20 600) would be somewhat unexpected.

No sulfur containing amino acids are present in kidney bean leghemoglobin. To the nearest integer, the amino acid compositions of PhLb*a* and PhLb*b* are identical except for aspartic acid (Table 1). The average number of aspartic acid residues per molecule is 14.35 in PhLb*a* and 14.55 in PhLb*b*. This difference is within experimental error. Accordingly, it may be concluded that PhLb*a* and PhLb*b* most probably have identical amino acid compositions.

Isoelectric point. The isoelectric point of PhLb*a* is 4.70 and that of PhLb*b* 4.55 at a temperature of 2 °C (Fig. 4). Component *b* is most probably a deamidation product of component *a*. The fraction with an isoelectric point of 1.6–2.0 is a free porphyrin. A low isoelectric point has been a general feature of all leghemoglobins so far studied.[18,19,21]

N- *and* C-*Terminal characterization of PhLba.* The dansyl-Edman procedure recommended for peptides was employed for PhLb*a* apoprotein. According to this method the by-products were removed only after the appropriate number of Edman steps had been performed. The unambiguous results obtained show that the method can also be applied to some small proteins. The *N*-terminal sequence of PhLb*a* is *NH_2-Gly-Ala-Phe-Thr-Glx-*. This sequence has a striking similarity with the first five *N*-terminal residues of human Hb*γ*, *NH_2-Gly-His-Phe-Thr-Glu-*.[22]

The *N*-terminal residue is also glycine in both the main leghemoglobin components of lupin [19] and of serradella [19] and in soybean Lb*c*,[23] while in soybean Lb*a* it is valine.[23] Glycine and valine are the typical *N*-terminal residues in animal globins.[24]

At first, hydrazinolysis of PhLb*a* and PhLb*b* hemoproteins met with some difficulties due to the bulk of the free amino acids bound to the protein. It was found necessary to remove the heme group and dialyze the apoprotein extensively against 50 mg/ml sodium bicarbonate solution and distilled water at 4 °C. After this treatment a 44 % recovery of alanine was obtained in the amino acid analysis, as calculated from the weight of the lyophilized sample before more thorough drying in the drying pistol. The yields of all other amino acids were 0–5 %, the most prominent impurities being serine and glycine. The carboxyterminal residue of PhLb*a* was thus concluded to be *alanine*.

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