

The Primary Structure of Soybean Leghemoglobin

I. The Separation of the Tryptic Peptides of the Apoprotein from the Slow Component and Their Amino Acid Compositions

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Tryptic hydrolysis of the heat denatured apoprotein of the slow component of soybean leghemoglobin gives rise to free lysine and 18 peptides, one of which was found to be ditryptic. They were separated on a preparative scale on Dowex 1 × 2, Dowex 50 × 2, and Sephadex G-50, and by high voltage paper electrophoresis and paper chromatography. All the isolated peptides were sufficiently pure for quantitative amino acid determinations.

Leghemoglobin is unique in being the only hemoglobin-like hemoprotein found in the plant kingdom. It yields two main components on electrophoresis at the pH values studied. Both of these components have been separated and crystallized.¹ Each consists of a single peptide chain with a single hemin group.^{2,3} The molecular weights of these two main components were found to be around 17 000.² They differ, however, in their *N*-terminal amino acids, which is glycine in the faster component and valine in the slower one.³ In contrast, as shown by their peptide maps, most of the tryptic peptides of these two components are nearly identical.⁴

In order to elucidate the structure of leghemoglobin the tryptic peptides of the slow component of soybean leghemoglobin have been studied. The peptides were fractionated and purified by ion-exchange chromatography, gel filtration, high voltage paper electrophoresis and paper chromatography and the purified peptides were subjected to quantitative amino acid analysis on the automatic amino acid analyzer (Beckman/Spinco 120 B) of Spackman, Stein and Moore.⁵

EXPERIMENTAL

Leghemoglobin. The cultivation of the soybean plants and the preparation of the two main components of leghemoglobin as well as the apoprotein of the slow component were performed as described previously.^{1,4}

Trypsin used for the digestion was a crystalline chymotrypsin-free preparation obtained from Serva AG (Heidelberg, Germany).

Tryptic digestion. The apoprotein (usually 250 mg) was denatured by incubation at pH 9.5 and 95°C for 4 min. 0.5 % trypsin was added initially and after 45 min of digestion. Hydrolysis was performed at 37°C by continuous titration to pH 8.0 with 1 N NaOH in a Radiometer pH-stat. After hydrolysis the pH of the clear solution was reduced to pH 5.8, the precipitate that then formed was removed by centrifugation and the supernatant was lyophilized. The digestion of the ditryptic peptide α T8, α T16 was performed with 0.2 μ mol of peptide in 0.2 ml of 0.1 M sodium bicarbonate solution of pH 8.5 and with the addition of 10 μ l trypsin solution (1 mg/ml). The mixture was incubated at 37°C for 18 h and then evaporated to dryness *in vacuo*.

*Peptide separation on Dowex 1 \times 2.*⁸ The digest was dissolved in a small amount of water and the solution adjusted to pH 9.0 before adding to a column of Dowex 1 \times 2 (200–400 mesh) (1.5 \times 80 cm) equilibrated to pH 8.4 at 35°C in a buffer composed of 1 % 2,4,6-collidine and 1 % pyridine (pH adjusted to pH 8.4 with glacial acetic acid). Elution was begun with the pH 8.4 buffer at a flow rate of 25 ml/h and 2.5 ml fractions were collected. After fraction No. 30, a gradient was set up with 300 ml buffer of pH 8.4 in a closed mixing chamber and a 1 % 2,4,6-collidine-, 1 % pyridine acetate buffer, pH 6.5. The elution was continued with 0.5 M acetic acid at fraction No. 170, with 1 M acetic acid at fraction No. 225, and finally with glacial acetic acid at fraction No. 252. After hydrolysis with alkali, a 0.1 ml aliquot from each tube was analyzed by the ninhydrin method as previously described.⁸ The contents of the tubes representing each peak were pooled, evaporated to dryness *in vacuo*, and dissolved in 2 ml of deionized water with occasional addition of acetic acid, collidine or pyridine in order to bring the peptides into solution. A 10 μ l aliquot of each fraction was examined by paper electrophoresis at pH 6.5 and by paper chromatography with the butanol-acetic acid-water system. On the basis of this examination, the course of further purification of the peptides was decided.

Further purification of peptides on Dowex 1 \times 2. Some of the fractions obtained in the resolution of the tryptic digest on the Dowex 1 \times 2 column were further purified on a Dowex 1 \times 2 column (0.9 \times 55 cm) at 35°C. The buffers used consisted of collidine-pyridine-acetic acid mixtures, the exact compositions of which and elution details are given in the legends of the figures.

Peptide separation on Dowex 50 \times 2. The procedure described by Schroeder *et al.*⁷ was employed. A Dowex 50 \times 2 (200–400 mesh) column, 0.9 \times 55 cm, was equilibrated with 0.2 M pyridine acetate buffer of pH 3.1 at 35°C. A gradient in respect to both pyridine concentration and pH was applied with 2.0 M pyridine acetate buffer of pH 5.0. The closed mixing chamber contained 500 ml of the pH 3.1 buffer. The runs were evaluated in a similar way as that described for Dowex 1.

Gel filtration of peptides on Sephadex G-50. Sephadex G-50, fine grade (Pharmacia, Uppsala, Sweden) was suspended in 30 % acetic acid and the suspension was allowed to settle to remove the very fine particles. A column (2.5 \times 97 cm) was poured in portions and washed with 30 % acetic acid over night. The sample was applied in 3 ml of 50 % acetic acid and eluted at room temperature with 30 % acetic acid at a flow rate of 24 ml/h. 2 ml fractions were collected. A 0.1 ml sample of every second fraction was analyzed by the ninhydrin method after hydrolysis with alkali⁸ and the appropriate fractions were pooled. After evaporation of the acetic acid the fractions were analyzed in the same way as those obtained by chromatography on Dowex 1.

Paper chromatography. The solvent system used in the chromatography of the peptides was butanol–acetic acid–water (4:1:5 by volume) and the paper Schleicher & Schüll No. 2043b. After a descending run of about 20 h the dried papers were treated either with a ninhydrin solution containing 0.1 % ninhydrin, 5 % collidine and 95 % butanol or with a ninhydrin-cadmium reagent prepared by adding 12 ml of a 5.5 % solution of cadmium chloride in 33 % acetic acid into 100 ml of a 1 % solution of ninhydrin in acetone. The color was developed by heating the chromatogram at 80°–85°C for 30 min. In some cases, the Cl₂-starch-KI reaction of Rydon and Smith⁸ was used. In addition, the Ehrlich reaction, the Pauli reaction, the nitrosonaphthol reaction and the Sakaguchi reaction were employed to detect peptides containing tryptophan, histidine, tyrosine, and arginine, respectively.⁹ The chromatographic mobilities of the peptides were determined relative to leucine, and are denoted by R_{Leu} .

Preparative descending paper chromatography was performed on Schleicher & Schüll

No. 2043b or on Whatman 3 MM paper using the butanol – acetic acid – water system. The sample was applied onto the paper as a band on the starting line. After development of the chromatogram, 0.2 – 0.3 cm wide guide strips were cut on both sides of the sample area. The guide strips were treated with the ninhydrin reagent and strips corresponding to the ninhydrin positive bands on the guide strips were cut out from the chromatogram and eluted with deionized water.

Paper electrophoresis. High voltage paper electrophoresis was carried out by the method of Ryle *et al.*¹⁰ in a modification of the apparatus of Michl.¹¹ The buffers used were: pH 6.5 (pyridine – acetic acid – water, 100:3:900, by volume); pH 3.5 (pyridine – acetic acid – water, 1:10:190, by volume); pH 1.9 (acetic acid – formic acid – water, 4:1:45, by volume). Electrophoresis was generally performed using a potential gradient of 60 V/cm at pH 6.5 for 50 min, at pH 3.5 for 60 min, and at pH 1.9 for 40 min. Toluene was used as coolant with the pH 6.5 buffer, and light petroleum (“Varsol”, Esso) with the other buffers. The dried electropherograms were usually treated with the ninhydrin – cadmium reagent or with the Cl_2 -starch-KI reagent of Rydon and Smith. The specific reagents used to detect the various amino acids were identical with those used in the paper chromatography. The electrophoretic mobilities were determined relative to lysine or aspartic acid and are denoted by E_{Lys} or E_{Asp} . Caffeine was used to measure the electroendosmotic mobility. Preparative high voltage paper electrophoresis was performed on Whatman 3 MM paper. The peptides from these electropherograms were isolated in the same way as that used in the preparative paper chromatography as described above.

Amino acid composition of peptides. Each peptide was hydrolyzed in 2 ml of 6 N HCl in evacuated sealed tubes for 18 hours and the amino acid composition was determined with an amino acid analyzer (Beckman/Spinco 120 B) as described by Spackman *et al.*⁵ The values of serine and threonine were not corrected for decomposition during hydrolysis.

Determination of tryptophan. Tryptophan was determined on samples of unhydrolyzed peptides by the method of Spies and Chambers.¹²

Nomenclature of leghemoglobin components. For brevity, leghemoglobin is abbreviated Lb and the different components are designated *a*, *b*, *c*, and *d* in the order of their elution from the DEAE-column as described previously.¹

Nomenclature of peptides. The peptides isolated from the different fractions were numbered with Arabic numerals in the order of elution from the Dowex 1 column. If a fraction eluted from Dowex 1 was found to contain several peptides, depending on the purification procedure, they were numbered in the order of elution from Dowex 50, in order of increasing R_{Feu} value when paper chromatography was used, or in their order of location from the anode when separation was performed electrophoretically. “T” placed before the peptide number indicates a tryptic peptide and *a* before “T” indicates Lba, the main slow component.

RESULTS

The tryptic peptides of Lba were first fractionated by column chromatography on Dowex 1 × 2 as illustrated in Fig. 1. Thirteen ninhydrin-positive fractions, numbered I – XIII, were thus separated and pooled as shown in the figure. Each pooled fraction was evaporated to dryness *in vacuo* and dissolved in 2 ml of deionized water. By paper chromatography and paper electrophoresis, it was found that fractions I and X were artifacts. Fraction I presumably consisted of undigested apoprotein, which emerged from the column in the buffer front. Fraction X emerged after applying the 1 N acetic acid gradient, when a significant decrease in pH occurred as shown by the elution diagram in Fig. 1. The paper chromatogram of this fraction showed several different faint spots, which all seemed to be due to traces of peptides already eluted from the column. It was found that only fractions V and XII were homogeneous. All the remaining 9 fractions, II – IV, VI – IX, XI and XIII, required further fractionation.

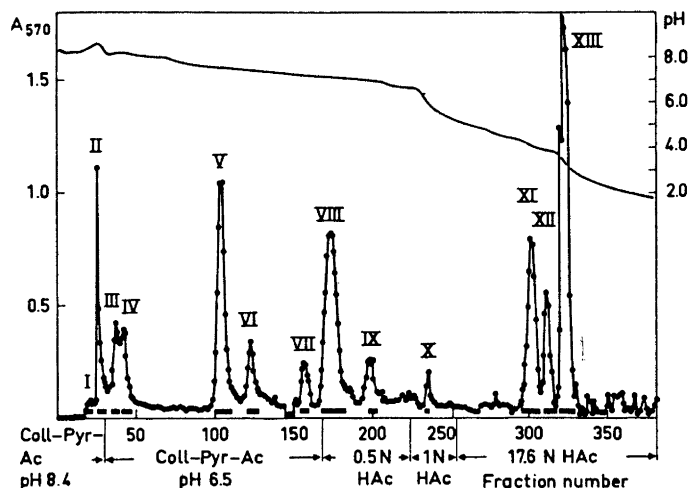


Fig. 1. Fractionation of peptides from a tryptic digest of $16.6 \mu\text{mol}$ of the slow component (Lba) of soybean leghemoglobin on a Dowex 1×2 column ($1.5 \times 80 \text{ cm}$) at 35°C . The flow rate was 25 ml/h and 2.5 ml fractions were collected. Experimental details are given in the text. The pooled fractions are indicated by black bars.

Peptides of fraction II. The fraction II eluted from the Dowex 1×2 column at a pH of about 8.2 gave two spots on paper chromatography. Rechromatography of this fraction on Dowex 50×2 is shown in Fig. 2. The two components thus separated were found to be homogeneous on paper chromatography and paper electrophoresis and were designated *aT1* and *aT2* as indicated in the figure.

Peptides of fraction III. The fraction III eluted from the Dowex 1 column at pH 8.2 gave only one peptide on paper chromatography, but was contaminated by peptides from fraction IV. No purification could be achieved on Dowex 50×2 and therefore the peptide was purified by preparative chromatography. The purified peptide was found to be electrophoretically pure and was designated *aT3*.

Peptides of fraction IV. These peptides were eluted from Dowex 1×2 in the pH range of 8.2–8.1. It was found that they were slightly contaminated by peptide *aT3*. Rechromatography on Dowex 50×2 did not separate them, but separation was effected by preparative paper chromatography. The electrophoretically homogeneous peptides were designated as *aT4* and *aT5* according to their increasing $R_{\text{f,eu}}$ values.

Peptide of fraction V. This fraction was eluted from the Dowex 1×2 column in the pH range of 7.6–7.5. It was found to be homogeneous by paper chromatography and paper electrophoresis. This histidine containing peptide was designated *aT6*.

Peptide of fraction VI. The peptide of fraction VI emerged from the Dowex 1×2 column in the pH range of 7.5–7.4. This peptide was slightly con-

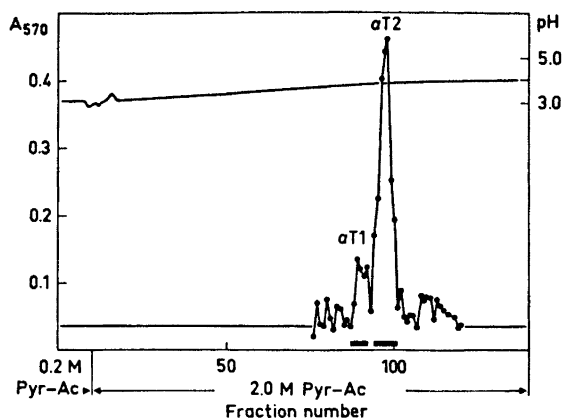


Fig. 2. Chromatography of fraction II obtained from Dowex 1 \times 2 (Fig. 1) on a Dowex 50 \times 2 column (0.9 \times 55 cm) at 35°C. The column was equilibrated in 0.2 M pyridine acetate buffer of pH 3.1. Gradient elution with 2.0 M pyridine acetate buffer of pH 5.0 started at fraction No. 10. The flow rate was 20 ml/h and 2 ml fractions were collected. The pooled fractions are indicated by black bars.

taminated by α T6 and was therefore purified further on Dowex 50 \times 2 under the experimental conditions described in the legend of Fig. 2. Only one peak emerged from the column and eluent fractions 69–79 were collected. The peptide was found to be homogeneous on chromatography and electrophoresis on paper and was designated α T7.

Peptides of fraction VII. The peptide of this fraction emerging in the pH range of 7.3–7.2 from the Dowex 1 \times 2 column was slightly contaminated when tested by paper chromatography and paper electrophoresis and was therefore rechromatographed on Dowex 50 \times 2 under the experimental conditions described in the legend of Fig. 2. Only one peak emerged from the column and the eluent fractions 96–106 were combined. The peptide was found to be chromatographically and electrophoretically homogeneous and was designated α T8.

Peptides of fraction VIII. This fraction was eluted from the Dowex 1 \times 2 column in a pH range of 7.2–7.1. It was found to contain three components, which were separated and purified on Dowex 50 \times 2 as shown in Fig. 3 and then found chromatographically and electrophoretically homogeneous. They were designated α T9, α T10, and α T11, the last of which was found to be a histidine-containing peptide.

Peptide of fraction IX. This peptide was eluted from Dowex 1 \times 2 at pH 7.0–6.9. Because of some contamination the peptide was rechromatographed on Dowex 50 \times 2 under the conditions described above. It emerged in fractions 136–147 as a homogeneous peak and was designated α T12.

Peptides of fraction XI. This fraction, eluted in the pH range 4.4–4.1 from Dowex 1 \times 2, was found to contain three main components. Attempts to separate them by rechromatography on Dowex 1 \times 2 were unsuccessful. They

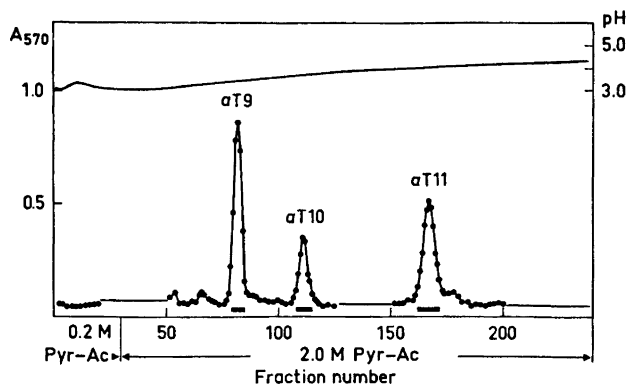


Fig. 3. Chromatography of fraction VIII obtained from Dowex 1×2 (Fig. 1) on a Dowex 50×2 column (0.9×55 cm) at 35°C . The experimental conditions are identical with those given in the legend of Fig. 2, except that the gradient elution was started at fraction No. 30. The pooled fractions are indicated by black bars.

were therefore purified by preparative high voltage paper electrophoresis (3000 V, 60 min) at pH 3.5. Three peptides were obtained which were chromatographically homogeneous and were numbered from the anode to the cathode αT13 , αT14 , and αT15 . The last of the peptides contained tyrosine but was found in only one leghemoglobin preparation.

Peptide of fraction XII. This peptide was eluted in the pH range 4.1–3.9 from Dowex 1×2 and found to be chromatographically and electrophoretically homogeneous and was therefore not purified further. The peptide was found to be a ditryptic one which contains both arginine and lysine. The peptide was digested with trypsin in sodium bicarbonate solution for 18 h as described in the experimental section. The two peptides formed were isolated by high voltage paper electrophoresis at pH 6.5. The amino acid analyses as well as the chromatographic and electrophoretic properties show that these peptides are identical with αT8 and αT16 . The ditryptic peptide is designated αT8 , αT16 .

Peptides of fraction XIII. The fraction was eluted from Dowex 1×2 in the pH range 3.8–3.0. The fraction was found to be inhomogeneous and contained several peptides. Rechromatography of this material was performed on Dowex 1 (Fig. 4). Four individual chromatographically and electrophoretically homogeneous peptides were isolated. The first one to emerge from the column was an arginine-containing peptide, which also gave a positive test for tryptophan. This peptide was designated αT16 . The second peptide to emerge from the column was also a tryptophan-containing peptide and was denoted αT17 . This was followed by two other peptides of which the first one was found to have the same amino acid composition as αT13 and the second the same composition as αT14 . Therefore it is assumed that they are deamidation products of αT13 and αT14 .

When the amino acid contents of the isolated peptides were compared with the amino acid composition of the intact slow component of Lba¹³ it was

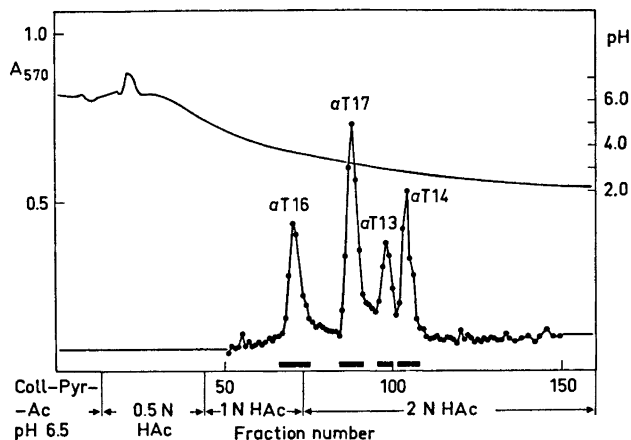


Fig. 4. Chromatography of fraction XIII obtained from Dowex 1×2 (Fig. 1) on a Dowex 1×2 column (0.9×55 cm) at 35°C . The flow rate was 20 ml/h and 2 ml effluent fractions were collected. The first 13 fractions were eluted with a 1% pyridine - 1% 2,4,6-collidine buffer of pH 6.5 adjusted with glacial acetic acid, after which the buffer in the reservoir was replaced by 0.5 N acetic acid. The closed mixing chamber contained 75 ml of the pH 6.5 buffer. At fraction No. 43, the reservoir buffer was replaced by 1 N acetic acid and at fraction No. 73 by 2 N acetic acid. The pooled fractions are indicated by black bars.

evident that all the amino acids in *Lba* were not recovered in the tryptic peptides isolated by the above procedure. Attempts were made to improve the recovery from Dowex 1 column by increasing the ionic strength of the eluting buffer, but without success. We therefore decided to resolve the tryptic digest of *Lba* using Sephadex gel filtration instead of ion exchange chromatography on Dowex 1×2 , which seemed to adsorb some peptides irreversibly.

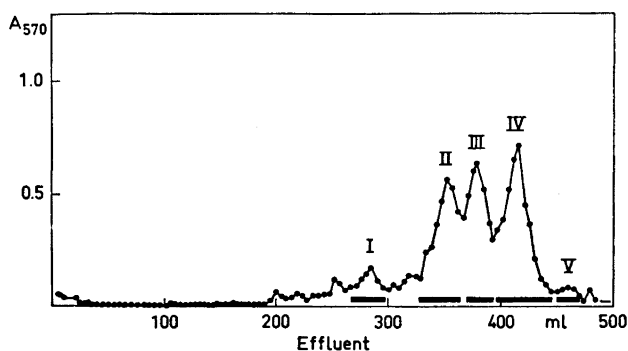


Fig. 5. Gel filtration at room temperature of a tryptic digest of the apoprotein of the slow component (*Lba*) of soybean leghemoglobin on a Sephadex G-50 column (2.5×87 cm) equilibrated with 30% acetic acid. A sample of $6.65 \mu\text{mol}$ of digest in 3 ml of 50% acetic acid was applied. 2 ml fractions are collected at a flow rate of 24 ml/h. The pooled fractions are indicated by black bars.

Table 1. Amino acid compositions of the tryptic peptides of the slow component (Lba) of soybean amino acids during hydrolysis. The yield of peptides was calculated from the micromoles of by elution from

Amino acid	aT1 ^a	aT2	aT3	aT4	aT5	aT6	aT7	aT8	aT9
Aspartic acid						1.02	1.04	1.00	1.02
Threonine					0.99	0.97	1.00		
Serine						1.90			0.95
Glutamic acid						1.05	1.02		1.01
Proline		1.04					1.07		
Glycine						2.03		0.94	1.00
Alanine		2.93	1.06	1.02		5.09	1.00	2.16	1.02
Valine			0.86			2.95	2.74	0.91	
Isoleucine					0.95				
Leucine			2.08			1.00			0.98
Tyrosine									
Phenylalanine			1.08				0.82		
Tryptophan									
Lysine	1.00	1.00		0.98	1.07	1.05	1.00	0.99	1.03
Histidine						0.86			
Arginine			0.92						
No. of residues	1	5	6	2	3	18	10	6	7
Yield (%)	5	19	10	20	12	31	20	16	24
E_{Lys}	1.00	0.55	0.44	0.78	0.58	0.15	neutr.	neutr.	neutr.
E_{Asp}									
$R_{\text{I,eu}}$	0.08	0.12	1.27	0.19	0.39	0.23	0.73	0.09	0.27

^a This peptide was excluded from the total amino acid composition given in the table.

^b 70 h hydrolysis.

Gel filtration of the tryptic digest of Lba on Sephadex G-50. A lyophilized tryptic digest of Lba was dissolved in 50 % acetic acid giving a clear solution indicating complete solubility of all the peptides present in the digest. The peptides were resolved on a Sephadex G-50 column. The elution pattern is shown in Fig. 5. The ninhydrin-positive fractions indicated by the solid bars were pooled. When fraction II was neutralized, a tyrosine-containing peptide precipitated and was designated aT18. Because of its low solubility, the peptide could not be purified by either paper chromatography or paper electrophoresis; in both cases it remained on the starting point. It was therefore purified by repeated (three times) dissolution in 50 % acetic acid, precipitation by neutralization with pyridine and centrifugation of the precipitate formed.

leghemoglobin. The values given in the table are mol ratios and are not corrected for losses of apoprotein digested. α T1– α T17 were isolated by elution from Dowex 1 \times 2 (Fig. 1) and α T18 Sephadex G-50 (Fig. 5).

α T10	α T11	α T12	α T13	α T14	α T15 ^a	α T8, α T16 ^a	α T16	α T17	α T18	Total
			1.09	3.85	1.11	2.13	1.23	1.21	1.13	12
	0.99	1.00		1.03					1.00	7
1.09			2.82	1.10	2.95	1.90	1.83		1.82	11
	1.05	1.09	2.02		2.08	1.17	1.18	2.13	2.05	13
				2.14					1.12	5
	1.02			1.02		0.99				6
1.08	1.04	1.04	2.01	1.19	2.14	2.10		5.05	1.57	26
		1.00	0.94	1.06	1.00	1.08		1.02	1.40(2)	15
								0.81	1.87	4
2.04	0.99		0.97	1.89	0.98	1.05	0.95	1.08	1.23	13
					1.86			0.94	1.63	3
		0.89	1.82	1.73					1.13	8
						1.00	1.00	1.00		2
0.79	0.97	1.03	1.00	1.00	0.88	1.03		0.77	1.05	15
	0.94									2
						0.60	1.00			2
5	7	6	13	16	13	13	7	14	17	142
27	33	22	11	18	12	17	18	23	10	
neutr.	0.33	neutr.							(neutr.)	
			0.24	0.23	0.20	0.20	0.25	0.34		
0.73	0.11	0.37	0.77	0.53	0.43	0.17	0.26	0.53	—	

A rechromatography of the peptide on Sephadex G-50 did not improve the purity of the peptide, which still seemed to contain traces of other peptides. In addition to α T18, fraction II was found to contain the peptides α T6, α T14, and α T17, which were isolated from the supernatant after precipitation of α T18 and identified by amino acid analysis, paper chromatography, and paper electrophoresis. Fraction III, when similarly analyzed, contained peptides α T3, α T7, α T13, α T16 and the ditryptic peptide α T8, α T16. Fraction IV consisted of the peptides α T2, α T5, α T8, α T9, α T10, α T11, α T12 and fraction V of α T1 and α T4 only.

Amino acid compositions of the purified peptides. The amino acid compositions of the purified peptides isolated from the tryptic digest of Lba are pre-

sented in Table 1. The yields of the various peptides indicated in the table were calculated from the amino acid analyses of the purified substances and were not corrected for losses during the various separation steps and for samples taken for identification. In the steps involving preparative paper chromatography, these losses were estimated to be high. The table also includes R_{Leu} values for the different peptides in the butanol – acetic acid – water system. The values E_{Lys} and E_{Asp} for the basic and acid peptides, respectively, at pH 6.5 are also given.

The molecular weight of Lba. The total number of amino acid residues contained in peptides $aT1$ – $aT18$ is 142, if $aT15$ and the ditryptic peptide $aT8, aT16$ are excluded. However, free lysine, $aT1$, is assumed to be an artifact which is assumed to be formed either as a result of a slight chymotryptic activity of trypsin or to be tightly bound free lysine contaminating the protein. Thus the peptides $aT2$ – $aT18$ are considered to be primary peptides released in the tryptic digestion of *Lba*. The molecular weight of *Lba* calculated from the amino acid compositions of these peptides was 15 159 for the apoprotein and 15 775 for the intact hemoprotein. The value of 15 775 calculated for the intact *Lba* molecule, deviates only slightly from the value of 15 400 obtained from sedimentation and diffusion measurements.² The theoretical iron content of *Lba* was calculated to be 0.354 % of the total protein.

DISCUSSION

As shown in the chromatogram (Fig. 1), the tryptic peptides of *Lba* were fairly well separated into thirteen fractions. Most of these, however, contained several peptides as shown by the paper chromatograms and electropherograms. For most of the peptides the losses during the purification steps are reasonable, except when paper chromatography was employed. The total loss of peptide $aT18$ on Dowex 1 \times 2 was unexpected. Until recently, it has been generally assumed that Dowex 1 does not adsorb peptides irreversibly¹⁴ which has been observed to occur with Dowex 50. However, recent observations have shown that hydrophobic peptides have a tendency to adsorb irreversibly on Dowex 1.¹⁵

The amount of free lysine recovered from the hydrolysate was only 5.1 % which corresponds to 0.06 mol per mol of apoprotein of *Lba*. Compared with the recovery of other peptides, this indicates that the original peptide chain contains no such sequences as -Lys-Lys- or -Arg-Lys-. It is known that lysine at the *N*-terminal position of a peptide chain is not released by trypsin.¹⁶ If the carboxyl side of arginine in a sequence like -Arg-Lys- or the first lysine in a sequence like -Lys-Lys- is initially split by the enzyme, a peptide is formed with lysine at the *N*-terminal position, which is not enzymatically released. However, no peptide was found in the tryptic digest of *Lba* which contains two lysine residues, one of which should be *N*-terminal. It is therefore assumed that free lysine might be liberated by chymotryptic activity still present in the trypsin preparation or that the apoprotein of *Lba* is contaminated by free lysine. It is known that some proteins adsorb free amino acids tightly. Yeast alcohol dehydrogenase has been found to contain adsorbed free amino acids even after 19 recrystallizations of the preparation.¹⁷ It was shown that glycine

and alanine were easily removed by recrystallization, whereas amino acids like tyrosine, glutamic acid, and lysine were much more tightly bound. Similar observations on adsorbed free amino acids have also been made with horse liver alcohol dehydrogenase.¹⁸ It should be noted that Lba is an acidic protein with an isoelectric point of about 4.6,¹⁹ which makes adsorption of a basic amino acid like lysine still more natural.

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