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Note

Separation of peptides by cellulose-phosphate chromatography for identification of a hemoglobin variant

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This paper describes an automatic procedure of cellulose-phosphate chromatography for the separation of peptides, with an example of the application to the analysis of a human hemoglobin variant.

MATERIALS AND METHODS

Venous blood was obtained, with ethylenediaminetetraacetate (EDTA) as anticoagulant, from individuals either normal or heterozygous for a hemoglobin variant [1]. Isolation of hemoglobin components by DEAE-cellulose chromatography [2], separation and aminoethylation of α- and β-chains [3], and tryptic and chymotryptic digestion of the globins [3] were carried out using standard methods. Cellulose phosphate, 100 g, was suspended in 1500 ml of 0.5 *N* potassium hydroxide, mixed for 30 min, and then filtered through a Büchner funnel [4]. The settled cellulose phosphate was thoroughly washed with deionized water until the filtrate was near neutral; it was then resuspended in 1500 ml of 0.5 *N* hydrochloric acid, mixed for 30 min, filtered, and washed with deionized water as before. The precycling procedures with potassium hydroxide, water, hydrochloric acid and water were repeated once. The well-

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washed cellulose agent was resuspended in 2 l of 0.01 *M* ammonium acetate solution (pH 7.3), and, after standing for 30 min, the unsettled particles were decanted. The wash and decantation procedure was repeated five times. A thin slurry of cellulose phosphate was poured into a glass column, 60 cm × 0.9 cm I.D. Packing was continued under a nitrogen pressure of 0.5 kg/cm^2 until the cellulose-phosphate bed was 55 cm in height. Both equilibration in 0.01 *M* ammonium acetate (pH 7.3) and subsequent chromatography were performed at a constant flow-rate of 30 ml/h, using a liquid chromatographic pump with column pressure varying from 2 to 4 kg/cm^2. Soluble peptides, 4—15 mg, were dissolved in 0.5 ml of 0.01 *M* ammonium acetate solution and were loaded onto the column by nitrogen pressure. The chromatography was run using a linear gradient device, containing 250 ml of 0.01 *M* ammonium acetate (pH 7.3) in the mixing cylinder and 250 ml of 0.4 *M* ammonium bicarbonate (pH 9.0) in the reservoir cylinder. The column effluent was monitored continuously at 215 nm by passage through a 2-mm flow-cell, and collected in a fraction collector. Correspondence between the tubes in the fraction collector and record of the ultraviolet absorption in the effluent monitor was facilitated by the incorporation of an event marker on the side of the recorder which was activated by changes of the collector [5]. A back-pressure of up to 1 kg/cm^2 was applied to eliminate bubbles in the flow-cell. Pooled fractions were lyophilized or evaporated to dryness. Rechromatography was performed on a 50 cm × 1.0 cm I.D. column of Bio-Gel P2. The linear flow-rate of 0.01 *M* ammonium acetate (pH 7.3) was maintained at 10 ml per h per cm^2 of cross-sectional area using a peristaltic pump. The effluent was monitored as described. An aliquot of peptides purified in this manner was analysed for amino acid composition on a Model 835 analyser (Hitachi, Tokyo, Japan) equipped for single-column analysis. High-performance liquid chromatography was carried out as described previously [6].

Trypsin (Lot No. T-TCA 591) and α-chymotrypsin (3× crystallized, Lot CD1-6100-1) were obtained from Worthington Biochemical Co., Freehold, NJ, U.S.A. The cellulose-phosphate agents were from Whatman, Maidstone, Great Britain (Grade P11: Lot Nos. 611514 and 611599, with 25-μm mean fiber length and capacity of 7.4 mequiv/g), or from Serva, Heidelberg, G.F.R. (P-Cellulose: Lot No. 26076, with a capacity of 5.03 mequiv./g). Porous polyacylamidegel beads were supplied by Bio-Rad Labs., Richmond, CA, U.S.A. (Bio-Gel P-2: minus 400 mesh). A Model 034 Liquid Chromatograph Pump and UV—VIS Effluent Monitor were obtained from Hitachi. A Model P3 pump and a glass column were purchased from Phamacia, Uppsala, Sweden.

RESULTS

Electrophoresis on thin-layer starch gel or cellulose-acetate sheet detected a hemoglobin variant in hemolysate from an apparently healthy Japanese man aged 35 years. When the relative mobility was calculated by the method of Schneider and Barwick [7], the mobility of the variant was +3.0. The abnormal component comprised 35% of the total hemoglobin. The resolving power of the cellulose-phosphate chromatography and identification of the peptides are illustrated in Fig. 1. The yields of peptides from the column were essentially

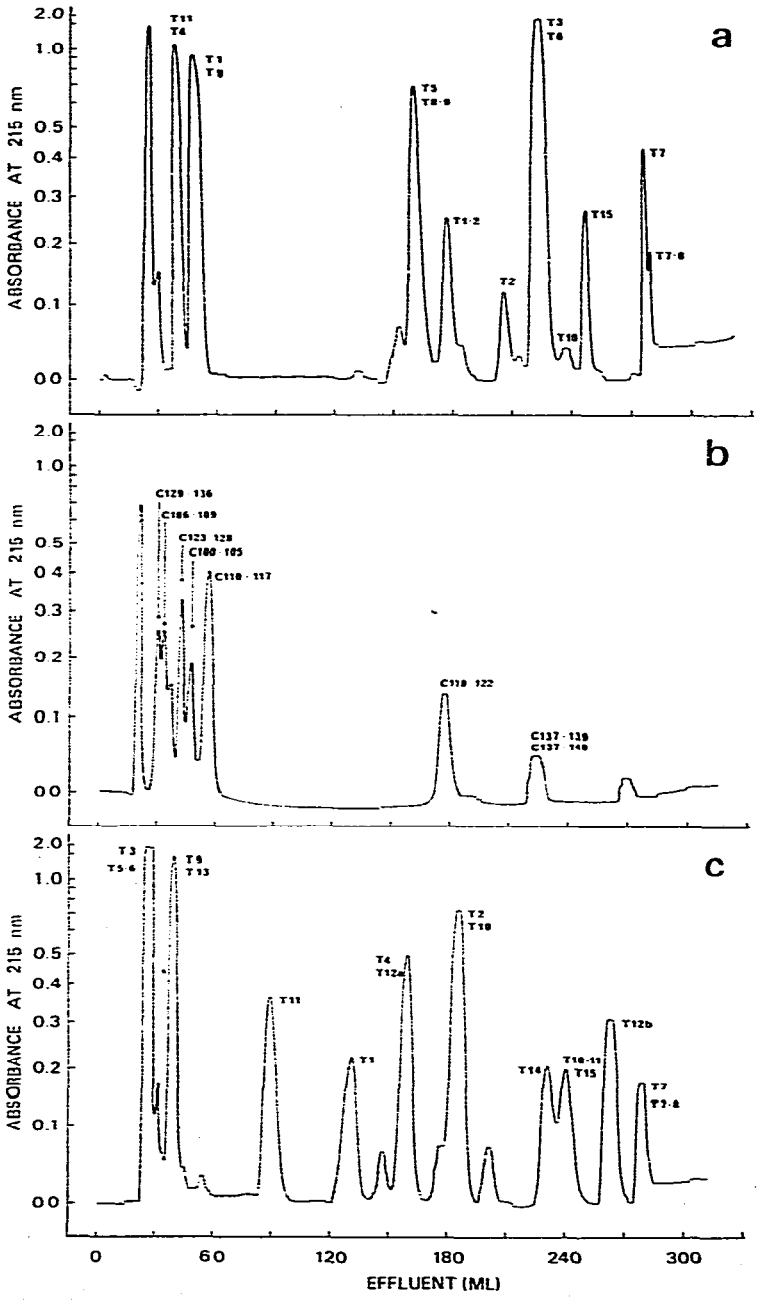

Fig. 1. The chromatographic separation of peptides produced by (a) tryptic digestion of th non-derivatized α-chains (11 mg), (b) chymotryptic digestion of the α-chain core peptid which had undergone performic-acid oxidation (4 mg), and (c) tryptic digestion of th aminoethylated β-chains (11 mg) from a hemoglobin variant. Normal βT5 peptide co-chro matographs with βT3, and the βT6 peptide with βT14. Tryptic peptides (T) are numbered i the sequence in which they occur in the polypeptide chains. Numbers beside the chymo tryptic peptides (C) denote the amino- and carboxyl-terminal residues of the peptide

TABLE I

AMINO ACID COMPOSITION OF THE β-CHAIN TRYPTIC PEPTIDES OF HEMOGLOBIN J LOME

Data are molar ratios of amino acids recovered after hydrolysis in 6 *N* HCl. The fragments are numbered in the sequence in which they occur in the polypeptide chain. Numbers in parentheses are the values expected from the normal β-chain sequence.

Amino acid	T1	T2	T3	T4	T5,6	T7	T8,9
Aspartic acid			1.97 (2)		3.94 (3)		3.20 (3)
Threonine	1.02 (1)	0.96 (1)		1.07 (1)	1.06 (1)		
Serine		0.42 (1)			1.87 (2)		1.07 (1)
Glutamic acid	2.09 (2)		2.10 (2)	1.12 (1)	1.05 (1)		
Proline	0.99 (1)			0.51 (1)	1.99 (2)		
Glycine		1.08 (1)	3.42 (3)		1.71 (2)	1.09 (1)	2.08 (2)
Alanine		2.19 (2)	1.08 (1)		1.08 (1)	0.90 (1)	1.91 (2)
Cysteine*							
Valine	0.86 (1)	1.23 (1)	3.23 (3)	2.36 (2)	1.61 (2)		0.98 (1)
Methionine					0.59 (1)		
Leucine	1.08 (1)	1.11 (1)	1.11 (1)	2.54 (2)	1.11 (1)		4.17 (4)
Tyrosine				1.04 (1)			
Phenylalanine					3.01 (3)		1.04 (1)
Tryptophan**		(+)		(+)			
Lysine	1.09 (1)	1.02 (1)			1.03 (2)	1.03 (1)	1.60 (2)
Histidine	0.87 (1)					0.98 (1)	0.94 (1)
Arginine			1.06 (1)	0.37 (1)			

*Estimated as aminoethylcysteine.
**Not quantitated.

quantitative. The fractions from the cellulose-phosphate chromatography contained only single or two major tryptic peptides, with variable degrees of contamination by minor peptides produced by incomplete or non-specific cleavage by trypsin. Gel-filtration chromatography on Bio-Gel P-2 was then applied for further purification of these fractions. Separation of two peptides from the β-chain, βT3 and βT5, was insufficient even after the rechromatography. Therefore, either high-performance liquid chromatography or paper chromatography was necessary to purify them.

The peptide composition of the α-chains from the variant was identical to that of the normal α-chains. The β-chains, on the other hand, had a different composition as compared to the normal, in that the βT5 peptide had the amino acid composition of residues 41—61 with substitution of either an aspartyl or an asparaginyl for a lysyl residue at position 59 (Table I). As a result of this substitution, the peptide bond between βT5 and βT6 (valyl-lysine) was not cleaved by trypsin, so that βT6 was absent in the chromatogram. The substitution of an asparaginyl rather than an aspartyl residue for a lysyl residue is in accord with the electrophoretic behaviour of the variant. Thus, we conclude that the variant is identical to Hemoglobin J Lome [8]. Two-dimensional paper chromatography and electrophoresis (fingerprinting) of the tryptic peptides confirmed the identification of the structure.

T10	T11	T12a	T12b	T13	T14	T15
1.20 (1)	2.06 (2)	0.95 (1)			1.11 (1)	
1.70 (2)				1.02 (1)		
1.06 (1)						
1.22 (1)	1.07 (1)			2.99 (3)		
	0.89 (1)			1.69 (2)		
0.93 (1)		1.03 (1)	1.10 (1)		1.26 (1)	
0.99 (1)		1.14 (1)	1.14 (1)	2.03 (2)	4.44 (4)	
0.96 (1)		0.42 (1)				
	1.06 (1)	2.03 (2)	1.08 (1)	1.14 (1)	2.31 (3)	
2.16 (2)	0.96 (1)	3.00 (3)	0.96 (1)		1.05 (1)	
				0.74 (1)		0.97 (1)
0.79 (1)	1.00 (1)		0.87 (1)	1.13 (1)		
0.96 (1)			0.97 (1)	1.23 (1)	0.98 (1)	
1.03 (1)	0.98 (1)		1.85 (2)		0.85 (1)	1.03 (1)
	0.98 (1)					

DISCUSSION

A combination of ion-exchange chromatography and molecular-sieve chromatography is employed for the separation of peptides from hemoglobin variants. With the use of strong cation-exchange cellulose and a very sensitive non-destructive detection system, substantial efficiency and reproducibility as well as loading capacity are achieved. The reasonable speed, versatility and inexpensive cost of performance make this technique well suited for routine assays of hemoglobin variants. Cellulose-phosphate chromatography, demonstrated to be a versatile technique for the effective separation of peptides [4, 9], has received only limited attention for solving structural variations in abnormal hemoglobins. Thus, the present study was initated as an attempt to evaluate its usefulness, introducing an element of simplicity and sensitivity to the procedure. The applicability of cellulose phosphate as the sole procedure is limited because several peptides elute together. However, molecular-sieve chromatography on microparticulate polyacrylamide gels, coupled with the same detection system, achieves the necessary repurification of peptides very efficiently. The separation of the peptides seems to be excellent sometimes but it is also often inferior to that observed by high-

performance liquid chromatographic methods [10] and by the more laborious ion-exchange resin procedures [5]. The superb resolution of high-performance liquid chromatography must be offset, in part, by the inherent disadvantages including limited loading capacity and variabel recoveries [10]. Multiple runs may be required to obtain an adequate amount of peptide for a satisfactory amino acid analysis and sequence determination, with considerable loss of peptides. On the other hand, standard procedures for peptide chromatography on ion-exchange resin columns must rely primarily on destructive postcolumn detection techniques. The use of ammonium acetate and ammonium bicarbonate in the developers, without pyridine, affords sensitive non-destructive detection at 215 nm. Neither colorimetric determination with ninhydrin reaction, nor repurification of organic chemicals is necessary in this system.

Several points germane to the general use of this technique are worth noting. Since cellulose-phosphate agents are supplied dry by the manufacturer, some ion-exchange sites become inaccessible to large molecules by the formation of new hydrogen bonds within shrunken cellulose (Whatman Bulletin No. 202). These structures may not be fully opened up by immersion of the cellulose in developer. Hence, extreme care in multistep precycling procedures is necessary to attain maximum resolution and reproducible chromatograms. Cellulose-phosphate materials obtained from Whatman appeared to give reproducible results as well as those from Serva (Lot No. 26076). However, a later supply from Serva (Lot No. 14128, with seemingly longer fiber length) was not satisfactory, in spite of the even larger capacity than that of the previous one. New developments in the elution programs, such as the use of more acidic starting buffer [9], may be possible. As shown, the difference in the peptide composition between the normal and the variant globins may be immediately clear by comparing the chromatograms. Hence, this cellulose-phosphate chromatography, designed to analyse materials of the order of 4—15 mg, may be suitable for both preparative and analytical purposes. The high recoveries, and ease of sample collection and preparation are especially suited for handling a quantity of peptides for determination of the amino acid sequence using conventional facilities. Cellulose-phosphate chromatography has been effective in determining the nature of the substitution in several other examples found in this laboratory. In any event, these procedures appear to be powerful techniques for the assay of structural variations in hemoglobins and other proteins.

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