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Purification by hydrophobic chromatography of soluble cytochrome b_5 of human erythrocytes

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Soluble cytochrome b_5 of human erythrocytes was purified very effectively by hydrophobic chromatography using a butyl-Toyopearl 650 column. Cytochrome b_5 was adsorbed tightly on the column in the presence of 60% saturated ammonium sulfate, and was eluted at 40% saturation of ammonium sulfate in the elution buffer. The chromatography gave a good yield of cytochrome b_5 of the highest purity so far reported as estimated from the 414 nm to 280 nm absorbance ratio of the oxidized form of the cytochrome b_5. The value obtained with the cytochrome b_5 purified in this study was 6.57, and is higher than the previously reported highest value of 6.4 (Hultquist, D.E., Dean, R.T. and Douglas, R.H. (1974) Biochem. Biophys. Res. Commun. 60, 28–34). Spectral properties including molecular absorption coefficients were determined using the cytochrome b_5 purified by this method.

Introduction

Butyl-Toyopearl 650 was recently introduced by Shin et al. [1] as a new hydrophobic adsorbent for the purification of water-soluble proteins. Since the mechanism of chromatography of the hydrophobic adsorbent is completely different from those of ionic exchange or gel permeation, the combination of these chromatographies is expected to yield further purified proteins. Cytochrome b_5 in human erythrocytes was shown by Hultquist to be soluble and plays a role as an electron carrier to reduce methemoglobin by NADH–cytochrome-b_5 reductase, formerly called methemoglobin reductase [2]. Soluble cytochrome b_5 in human erythrocytes was partially purified first by Passon et al. [3], and then purified further by Hultquist et al. [4] using DEAE-Sephadex. A minimal molecular weight of the cytochrome was reported to be 11 113 based on the amino acid sequence [5], but the estimation of the molecular weight by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate gave an extremely high value, 15 300 [4]. The introduction of hydrophobic column chromatography on butyl-Toyopearl to the combination of ionic exchange chromatography on DEAE-cellulose and gel filtration on Sephadex G-75 gave a good yield of highly purified cytochrome b_5. Spectral properties of the highly purified cytochrome b_5 of human erythrocytes were determined.

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Materials and Methods

DEAE-cellulose and Sephadex G-75 were purchased from Brown and from Pharmacia Fine Chemicals, respectively. Butyl-Toyopearl 650 M, a butyl derivative of Toyopearl HW-65, was kindly provided by Toyo Soda Mfg. Co., Tokyo. Other chemicals were obtained commercially. Out-dated blood samples obtained from local hospitals were used as soon as possible. Hemolysate was prepared as described previously [6] after washing blood twice with saline to remove serum and buffy coats.

Electrophoresis of proteins on polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS-PAGE) was carried out by the method of Laemmli [7]. Spectrophotometric determinations were performed with a Union Spectrophotometer model SM-401 (Union Giken, Osaka, Japan).

Amino acid composition of the purified cytochrome b_5 was analyzed by the method of Yokote et al. [8]: about 1 nmol of the purified cytochrome b_5 was hydrolyzed at 166 °C for 25, 50 and 75 min with a trifluoroacetic acid/HCl mixture (1 : 2, v/v) containing 5% thioglycolic acid. The hydrolysis within the short period of time gave a good yield of tryptophan, methionine and other unstable amino acids.

Molecular absorption coefficients of the purified cytochrome b_5 were determined by the pyridine hemochromogen method (20% pyridine and 0.2 N NaOH) based on the molecular absorption coefficient of 34.4 $mM^{-1} \cdot cm^{-1}$ at 557 nm of protoheme IX [9] as described in the text. The concentration of cytochrome b_5 during purification and the rate of reduction of cytochrome b_5 with NADH and NADH–cytochrome-b_5 reductase of human erythrocytes [10] were determined using the value 124 $mM^{-1} \cdot cm^{-1}$ at 424 nm, this being the difference in molecular absorption coefficient between the reduced and oxidized form.

Results and Discussion

Purification of cytochrome b_5

Hemolysates (8-fold diluted) were applied onto a DEAE-cellulose column equilibrated with 5 mM phosphate buffer (pH 7.0) containing 2 mM 2-mercaptoethanol. The column was extensively washed after passing all the hemolysates through the column with the equilibrating buffer to remove hemoglobin. Tightly adsorbed cytochrome b_5 on the column was eluted with 0.2 M KH_2PO_4 containing 0.2 M KCl, after eluting NADH–cytochrome-b_5 reductase with 0.1 M phosphate buffer (pH 7.0) containing 0.1 mM dithiothreitol. Eluates were collected batchwise every 300 ml, and cytochrome b_5-rich fractions were spectrophotometrically identified by enzymatically reducing cytochrome b_5. Cytochrome b_5-rich fractions were concentrated on an Amicon PM10 membrane and then applied to a Sephadex G-75 column in order to thoroughly remove hemoglobin. The eluates containing cytochrome b_5 from the Sephadex G-75 column were brought to 60% saturation of ammonium sulfate by slowly adding saturated ammonium sulfate solution in 20 mM Tris (pH 7.5). Supernatant of the mixed solution was charged onto a butyl-Toyopearl 650 M column (2.5 × 9 cm) equilibrated with 60% saturated ammonium sulfate in 20 mM Tris (pH 7.5). The column was then extensively washed with the buffered 60% saturated ammonium sulfate to remove impurities before decreasing ammonium sulfate concentration stepwise as shown in Fig. 1. Most of the cytochrome b_5 was eluted at 40% saturation of ammonium sulfate with high yield (more than 50% as seen in Table I) and a minor peak was obtained at 35% saturation (Fig. 1). A summary of the purification is shown in Table I.

Purity of cytochrome b_5

Main fractions (fraction numbers 98–110 in Fig. 1) were pooled and concentrated on an Amicon PM10 membrane. Purity of the cytochrome b_5 was examined by SDS-PAGE, which showed only a single band of the purified cytochrome b_5 as shown in Fig. 2. Molecular weight estimated from Fig. 2 was 15000, a similar value to that (15300) reported [4]. These values are, however, apparently artifactual judging from that calculated from the amino acid sequence (11113) [5], or from that of heme content (10549) described below. The purification procedure by hydrophobic chromatography using the butyl-Toyopearl 650 column is simple and significantly effective compared with the conventional chro-

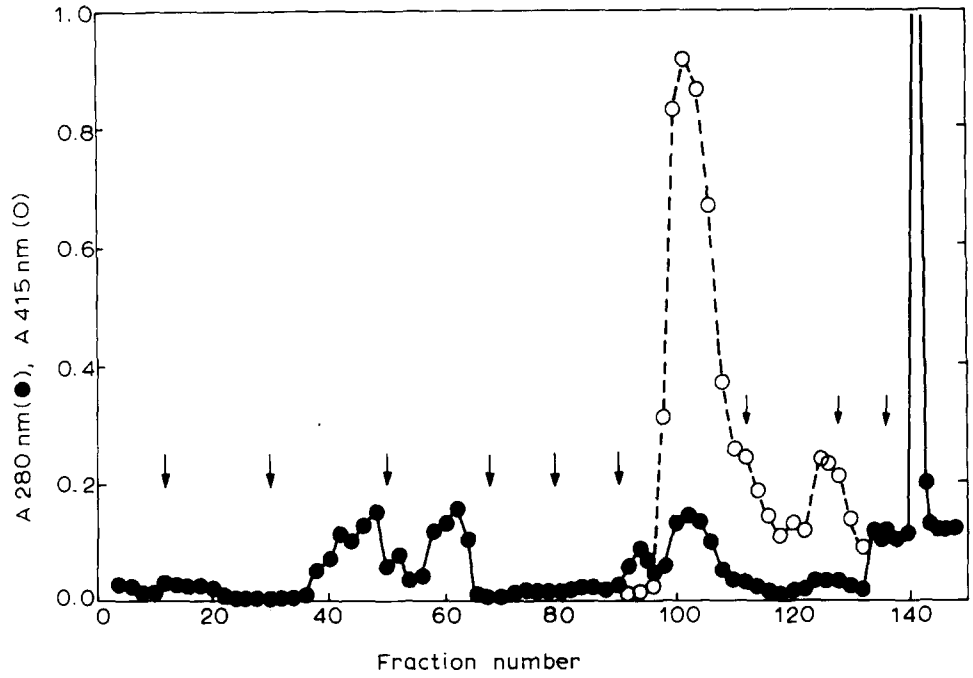

Fig. 1. Elution profile of cytochrome b_5 of human erythrocytes from a butyl-Toyopearl column. Cytochrome b_5 in the supernatant of 60% saturated ammonium sulfate (48 ml) was applied to a butyl-Toyopearl 650 M column (2.5×10 cm), equilibrated with the 60% saturated ammonium sulfate in 20 mM Tris (pH 7.5). Cytochrome b_5 was eluted by stepwise decrease of ammonium sulfate concentration. Changes of the ammonium sulfate concentration are indicated with arrows at 60%, 54%, 50%, 45%, 42%, 40%, 35%, 20%, 0%, from left to right in the figure.

TABLE I

SUMMARY OF PURIFICATION AND SPECTRAL PROPERTIES OF CYTOCHROME b_5 OF HUMAN ERYTHROCYTES

Fractions	Volume (ml)	Protein (mg)	Cytochrome b_5 [a] (nmol)	Yield (%)	414 nm, ox [b] / 280 nm, ox	424 nm, red [b] / 414 nm, ox	424 nm, red [b] / 556 nm, red
DEAE-cellulose eluates (concentrated)	39	975	1585.2	100	– [c]	– [c]	– [c]
Sephadex G-75 eluates	52	219.7	1799.0 [d]	113.5 [d]	1.29	1.44	6.8
Supernatant of 60% saturated ammonium sulfate	38.5	140.2	1133.9	71.5	1.95	1.47	6.85
Butyl-Toyopearl main fractions (concentrated)	4.2	10.0	947.7	59.8	6.57	1.50	6.83

[a] Enzymatically reducible heme of cytochrome b_5 was determined as described in Materials and Methods with NADH and NADH–cytochrome-b_5 reductase.

[b] Absorbance ratios were determined from the absorption spectra of the oxidized and enzymatically reduced form.

[c] Not determined.

[d] As the heme content in the DEAE-cellulose eluates could not be determined accurately, the recovery at the second step became large.

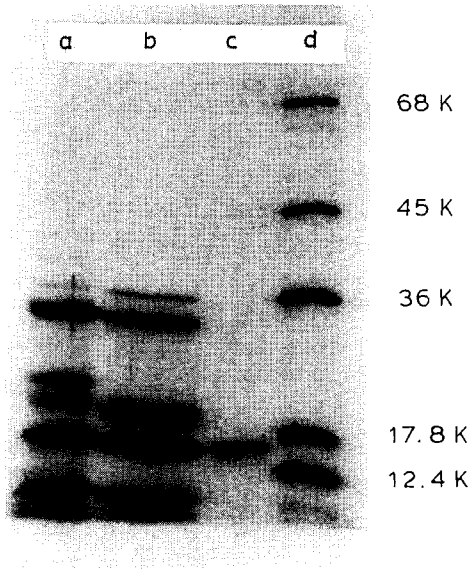

Fig. 2. SDS-PAGE of cytochrome b_5 of human erythrocytes. Purity of cytochrome b_5 was analyzed by SDS-PAGE (15.0% gel). Lanes: a, pooled fractions eluted from Sephadex G-75 column (3.9 μg protein); b, supernatant of 60% saturated ammonium sulfate of (a) (3.4 μg protein); c, purified cytochrome b_5 (pooled eluates from butyl-Toyopearl column (0.6 μg protein); d, marker proteins: bovine serum albumin (68 kDa), ovalbumin (45 kDa), glyceraldehyde phosphate dehydrogenase (36 kDa), sperm whale myoglobin (17.8 kDa), and horse heart cytochrome *c* (12.4 kDa) from top to bottom.

matographic method using DEAE-cellulose or DEAE-Sephadex.

Amino acid analysis

Amino acid composition of the purified cytochrome b_5 was analyzed and compared with the amino acid sequence data of human erythrocyte cytochrome b_5 reported by Kimura et al. [5] (Table II). The values of the present study are the average of the values obtained from the three kinds of hydrolyses after different time intervals as described in Materials and Methods, and they are in very good agreement with those from the sequence [5]. Molecular weight (11 145) calculated from the amino acid composition is close to that (11 113) determined by Kimura et al. [5].

Spectral properties of the purified cytochrome b_5

Absorption spectra of the purified cytochrome b_5 are closely similar to those reported by Passon et al. [3], and the spectral characteristics are listed in Table I. The 414 nm to 280 nm absorbance ratio of the oxidized form was 6.57 (specific heme content is 94.8 nmol/mg protein, Table I). The ratio is the highest of the reported values, 3.4 [3], 6.4 [4], and 6.34 [11], and a minimal molecular weight calculated (10 549) from the heme content agrees well with that obtained (11 113) from the amino acid sequence [5]. Molecular absorption coefficients for the difference between the reduced and oxidized form determined by the pyridine hemochrome method are 124 $mM^{-1} \cdot cm^{-1}$ at 424 nm and 19.3 $mM^{-1} \cdot cm^{-1}$ at 556 nm (Table III). A difference from the value of steer liver microsomal cytochrome b_5 reported by Strittmatter and Velick [12] is clear at 424 nm, but the values at 424 nm and 556 nm are close to those of hen liver microsomal cytochrome b_5 reported by Ohnishi et al. [13]. It seems now better to use the values obtained in the present study for erythrocyte cytochrome b_5 than to use those of liver microsomal cytochrome b_5 [12,13].

TABLE II

AMINO ACID COMPOSITIONS OF HUMAN ERYTHROCYTE CYTOCHROME b_5

Amino acids	Residues/mol	
	a	b
Asx	10.0	10
Thr	5.4	6
Ser	4.7	5
Glx	15.6	17
Gly	6.5	6
Ala	4.8	5
Val	4.3	4
Met	1.0	1
Ile	3.6	4
Leu	7.5	8
Tyr	2.7	3
Phe	3.1	3
Lys	8.6	8
His	7.0	7
Trp	0.8	1
Arg	3.6	3
Pro	4.8	6
Total residues	97	97
M_r calculated	11145	11113

[a] Values determined in the present study.
[b] Data from Ref. 5.

TABLE III

MOLECULAR ABSORPTION COEFFICIENTS OF CYTOCHROME b_5

Cytochrome b_5 from	Reduced-oxidized		Ref.
	ε_{424} $mM^{-1} \cdot cm^{-1}$ [a]	ε_{556} $mM^{-1} \cdot cm^{-1}$ [a]	
Steer liver microsomes [b]	100	–	10
Hen liver microsomes [c]	121	19	11
Human erythrocytes	124	19.3	this study

[a] ε = molecular absorption coefficient.
[b] Cytochrome b_5 was solubilized by lipase.
[c] Cytochrome b_5 was solubilized by trypsin.

Cytochrome b_5 was purified from a large pool of outdated human red cells stored for a period of 1–2 months in the present study. Thus, the yield of cytochrome b_5 from the DEAE-cellulose column was about 50% of that reported by Passon et al. [3]. Nevertheless, the hydrophobic chromatography gave us a quite high yield of highly purified cytochrome b_5. Function of the highly purified cytochrome b_5 as an electron acceptor from cytochrome b_5 reductase is not damaged by the hydrophobic chromatography. Enzymatically reducible heme in the purified cytochrome b_5 was more than 95% of that reduced by sodium dithionite under aerobic conditions. An advantage of using the hydrophobic column for purification of water-soluble proteins is that all the adsorbed proteins can be removed from the column by washing with distilled water at the last step, and the column is regenerated for use in the next chromatography procedure.

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