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PURIFICATION OF SPECTRIN AND ITS SUBUNITS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Technological and methodological advances in the techniques of structural and biological studies of proteins have reduced the required amount of sample. In conjunction with these advances, high-performance liquid chromatography (HPLC) has emerged as a technique of high utility for the purification of complex molecules. Using a combination of size-exclusion and reversed-phase HPLC and ionic buffers containing sodium dodecyl sulfate, the red cell membrane-associated high-molecular-weight polypeptide spectrin and its subunits have been purified. The system described in this paper is fast, reproducible and quantitative.

INTRODUCTION

High-performance liquid chromatography (HPLC) has been extensively employed for the separation of polypeptides and their fragments. However, not many reports have been published for the purification of high-molecular-weight proteins, particularly those which are associated with the plasma membranes. The red cell has attracted much interest as a model of biological membranes. The major proteins of these membranes have been well characterized[1]. On the cytoplasmic surface of red cell membranes is a network of structural proteins, known as the cytoskeleton, of which the major component is spectrin[2,3]. Human erythrocyte spectrin is composed of two high-molecular-weight polypeptide chains: α (240 000 daltons) and β (220 000 daltons). Conventional procedures for the purification of these subunits include preparation of ghosts, low salt extraction and open-column gravity-feed, size-exclusion and ion-exchange chromatography[4,5]. The procedures give good yields but are time-consuming. We have developed an HPLC system for the rapid isolation of pure spectrin and its α-subunit. The procedural details and results are presented in this communication.

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MATERIALS AND METHODS

Chemicals

HPLC chemicals were purchased from EM Science (Cherry Hill, NJ, U.S.A.). Ultrapure Tris[tris(hydroxymethyl)aminomethane; Cat. No. 604205] with no protease activity was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN, U.S.A.). All other chemicals were of highest purity grade obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.), Pierce (Rockford, IL, U.S.A.) and Fischer Scientific (Fairlawn, NJ, U.S.A.). All solvents for HPLC were constituted in water purified through a Milli-Q system and filtered through a 0.2-μm filter before use.

Preparation of red cell ghosts

Normal human red cells were washed three times with physiological saline and lysed in 30–40 times their volume of lysis buffer (10 m*M* sodium phosphate pH 7.0). The membranes were pelleted by centrifugation at 30 000 *g* for 30 min at 4–5°C and repeatedly washed with cold lysis buffer, until white or light pink.

Extraction of spectrin

Spectrin along with some low-molecular-weight proteins was extracted from ghost membranes prepared from freshly drawn normal human blood by osmotic lysis in dilute phosphate buffer[6]. Ghost membranes were suspended in ten volumes of 0.1 m*M* EDTA pH 8.0 containing 0.4 m*M* phenylmethyl sulfonyl fluoride (PMSF) and incubated at 37°C for 30 min as described by Marchesi[6]. Spectrin which was released in the medium was separated from membrane fragments by centrifugation at approximately 30 000 *g* for 45 min at 4°C, and lyophilized.

Purification of spectrin by size-exclusion HPLC

Spectrin was purified free of small-molecular-weight proteins which co-extract with spectrin by gel-permeation HPLC.

The size-exclusion TSK-4000-SW column (60 × 0.76 cm I.D.) was purchased from Sci-Con (Winter Park, FL, U.S.A.) while the TSK-4000-PW column (60 × 0.76 cm I.D.) was a gift from Dr. Yoshio Kato (Toyo Soda, Japan).

The columns were developed at a flow-rate of 0.2 or 0.4 ml/min with 10 m*M* Tris pH 6.5 for SW and pH 8.5 for PW columns containing per litre 168 mg EDTA, 0.2 g sodium azide, 87 mg PMSF, 1.0 g sodium dodecyl sulfate (SDS) and 50 μl mercaptoethanol. The effluent was monitored at 280 nm in an LKB 2138 Uvicord S detector. A Waters Model 6000 A pump was employed as a solvent delivery system and a Model 7125 Rheodyne injector was used for sample loading.

Lyophilized extract was dissolved in HPLC eluting buffer (2 mg/ml) and up to 1 mg fractionated on a TSK-4000 column as described above. Peaks were collected manually, lyophilized and analyzed for purity on SDS-polyacrylamide (gradient 5–20%) slab gel electrophoresis (SDS-PAGE).

Purification of subunits by reversed-phase HPLC

Pure spectrin obtained as above was desalted on a PD-10 column (Pharmacia, Uppsala, Sweden) in 0.1% SDS containing 0.4 m*M* PMSF, and lyophilized. Its α- and β-subunits were separated on a reversed-phase PLRP-S 300 Å, 8-μm column

(150 × 4.6 mm I.D.) manufactured by Polymer Labs. (Shropshire, U.K.) and marketed by Polymer Labs. (Amherst, MA, U.S.A.). Buffer A was 0.1% SDS in 0.5% trifluoroacetic acid (TFA) and buffer B was acetonitrile containing 0.5% TFA.

Separation of subunits was performed on a Waters HPLC system consisting of two 6000A solvent delivery pumps, a U6K Universal septumless injector, Model 440 dual-channel absorbance detector and Model 720 system controller. The column was developed for 5 min at initial condition (100% A) followed by a 5-min linear gradient to 50% A + 50% B, then to final condition of 32% A + 68% B in 35 min and held at final condition for 5 min at a flow-rate of 1.0 ml/min. The effluent was monitored at 280 nm and recorded on a Fisher Recordall Series 5000 recorder using a chart speed of 1.0 cm/min. The peaks as they eluted were collected manually. A 10–15 μl volume of 10 m*M* Tris buffer used for gel-filtration HPLC was added to each fraction, lyophilized and analyzed on SDS-PAGE.

Gel electrophoresis

The separated products were analyzed on a slab of polyacrylamide gel (gradient 5–20%), 0.7 mm thick, as described by Laemmli[7]. Polypeptides were located by staining with Coomassie blue.

RESULTS AND DISCUSSION

The most pragmatic approach for biochemical characterization of a protein molecule capitalizes on the efficiency of techniques available to obtain the protein molecule in (i) high yields, (ii) a highly purified form and (iii) a short period of time.

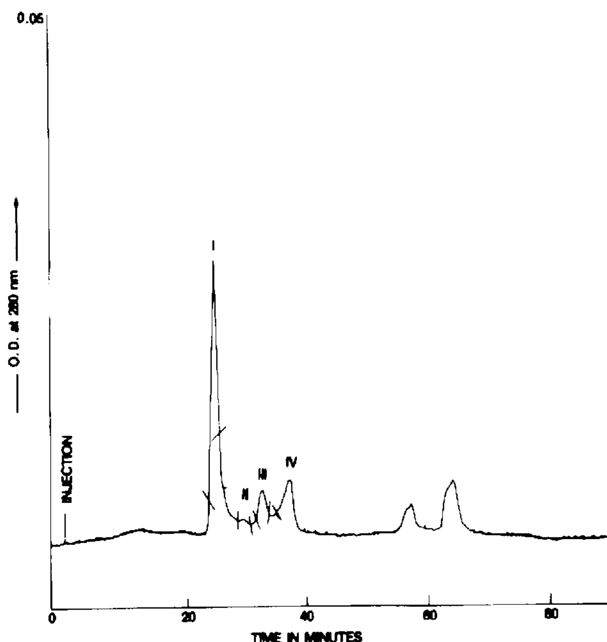

Fig. 1. Elution profile of low salt extract when injected on a TSK-4000-PW column (60 × 0.76 cm I.D.)

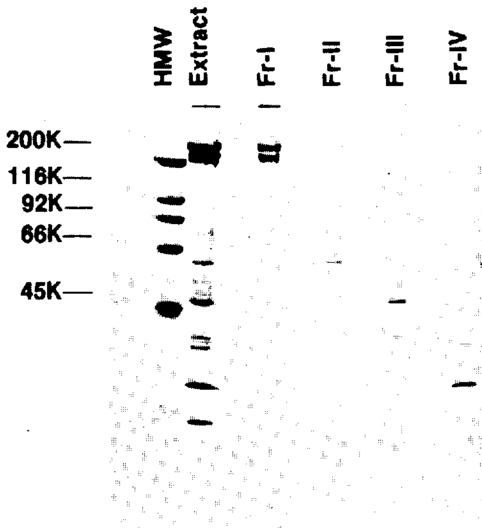

Fig. 2. SDS-PAGE analysis of the four fractions collected from a TSK-4000-PW column. HMW = high-molecular-weight protein standard; Fr = fraction.

Previously, available techniques for the isolation of spectrin and its subunits were time-consuming and required large amounts of starting material.

Fig. 1 shows the protein elution profile of human red cell ghost extract obtained on a TSK-4000-PW column. Each fraction was analyzed on SDS-PAGE and the results are presented in Fig. 2. As expected on the basis of molecular weight, peak I [Fraction I (Fr-I)] contains pure spectrin, while peaks II, III and IV contain low-molecular-weight proteins (Fig. 2). Up to 1 mg of total protein was injected without any loss in resolution. The time between injections was about 60 min. It is interesting to note that the spectrin peak elutes within 25–30 min, which is much faster than classical chromatography.

Wide-bore (7.6 mm) columns have an advantage that large sample volumes (1

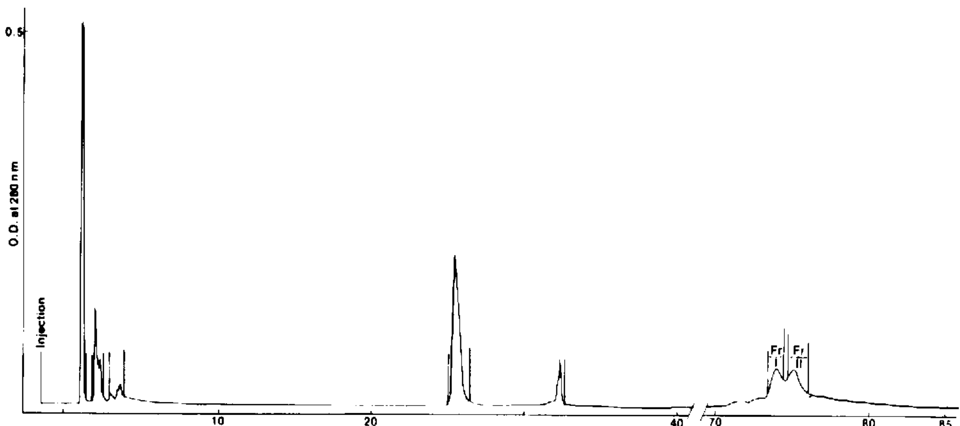

Fig. 3. Elution profile of spectrin (Fr-I; Fig. 1) on a reversed-phase column (15 × 0.46 cm I.D.). For details see text.

TABLE I

PARAMETERS FOR RP-HPLC SEPARATION OF SPECTRIN SUBUNITS

% B		*Time (min)*
From	*To*	
0	0	5
0	50	5
50	68	35
68	68	5
68	0	5

ml) can be injected to make them semi-preparative columns, but a slow flow-rate (< 0.5 ml/min) must be maintained in order to avoid the column compression creating a void at the column inlet which is a major drawback of these types of columns. We have been using these columns for more than a year without any loss in the resolution.

Fig. 3 shows the separation profile when pure spectrin is injected in a reversed-phase column and column developed as shown in Table I. Fr-I was found to be pure α-chain while Fr-II contains β-chain with 10–15% α-chain when analyzed on SDS-PAGE (Fig. 4). Previously the subunits of spectrin had been separated by treatment with high urea followed by classical chromatography on hydroxylapatite[8], DEAE-cellulose[5] or gel filtration[4]. However, use of 4 *M* urea in our hands produced little or no success under the conditions used in this study. In the present report we describe

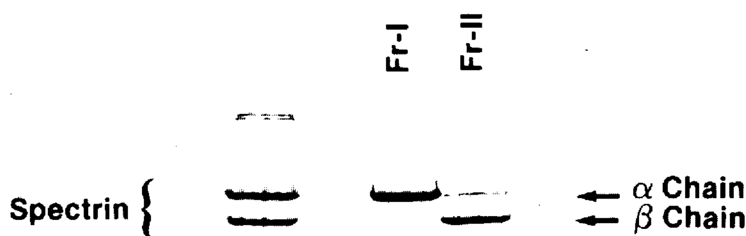

Fig. 4. SDS-PAGE analysis of Fr-I and Fr-II (Fig. 3) obtained on reversed-phase HPLC of spectrin.

for the first time an HPLC procedure for the separation of spectrin and its subunits, which incorporates all the advantages of an HPLC system, such as speed and reproducibility. In addition, both the HPLC systems employed in this study are quantitative. Based on the assay of Lowry *et al.*[9] the yields of pure spectrin and α-subunits have been 80–90%. We have observed that α- and β-subunits, when lyophilized, irreversibly stick to the walls of the test tubes. However, addition of 100–200 μl of 10 m*M* Tris buffer (employed for gel-filtration HPLC) to these fractions before lyophilization helps to avoid this problem.

Due to refinements in the techniques of structural and biological studies of protein molecules, the amount of sample needed is decreasing. In view of this, it is our belief that the HPLC method and buffer system presented in this communication will be a helpful aid for purification and structural and biological studies of this and other high-moecular-weight proteins, particularly in phylogenetic studies of spectrin from related and different species. In addition, we have further established that the use of ionic buffers containing SDS, in conjunction with or without organic modifers, has no detrimental consequences on the column life and instrument performance. The procedure has been successfully employed to obtain these molecules from erythrocyte membranes of other species.

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