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Capillary gel electrophoresis: separation of major erythrocyte membrane proteins

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Abstract

A new separation method of human erythrocyte membrane proteins by sodium dodecyl sulfate capillary gel electrophoresis (SDS–CGE) is described. In this method, a replaceable gel matrix was used. Seven major erythrocyte membrane proteins, α-and β-spectrin, ankyrin 2.1, band 3 (anion-exchanger), 4.1a and b, and 4.2 (pallidin), were separated and identified by SDS–CGE method. High reproducible migration times of these proteins (inter-assay coefficients of variation less than 2%), as well as quantification (inter-assay coefficients of variation less than 11%) were obtained. This new SDS–CGE method may provide important diagnostic evidence for hereditary spherocytosis. It can be a powerful diagnostic tool in place of SDS polyacrylamide gel electrophoresis for erythrocyte membrane protein analysis. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Erythrocyte; Membrane proteins

1. Introduction

Erythrocyte membrane structure is a bilayer of phospholipids intercalated with molecules of unesterified cholesterol and glycolipids. The membrane also contains proteins which are asymmetrically organized. These integral membrane proteins, i.e. band 3 and glycophorins, penetrate or span the lipid bilayer, and interact with the membrane skeleton. The erythrocyte membrane skeleton proteins are composed of spectrin, ankyrin, protein 4.1, protein 4.2 and actin. Many hemolytic disorders result from primary abnormalities of the erythrocyte membrane proteins, but the most commonly encountered are the hereditary spherocytosis (HS) and hereditary elliptocytosis (HE) syndromes [1].

Protein composition of the erythrocyte membrane as well as the characterization of the specific membrane lesion are possible by electrophoretic analysis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on slab gel is one of the most effective methods in protein separation. This powerful analytical tool is used for molecular mass determination. Two main SDS–PAGE systems are used to evaluate membrane protein composition: the discontinuous system described by Laemmli [2] which allows a good separation of proteins 4.1a and b, but not of ankyrin 2.1 from spectrin [3], and the continuous one described by Fairbanks [4] which allows the

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separation of ankyrin 2.1 from spectrin; but protein 4.1 presents, however, as an unique band.

In the last decade, capillary gel electrophoresis (CGE) methods have been developed for protein analysis. In the late 1980s, Cohen and co-workers applied CGE in separation of SDS–protein complexes [5] and several methods were developed using SDS–CGE with different types of gels. The gel matrix used in SDS–CGE can be a chemical gel or a physical gel. The chemical gel, like cross-linked polyacrylamide [6], provides well defined pore size but suffers from short life time of the gel-filled capillary and limitation of applied voltage. In order to avoid gel shrinkage, bubble formation and matrix collapse, physical gels such as non-cross-linked polyacrylamide [7,8] have been applied. Besides, a number of hydrophilic polymer solutions, called entangled polymer solutions [9–11], can also be used as the sieving media if the concentrations are higher than the overlap thresholds. Their low viscosity allows to push them into the capillary but also to replace them. Another advantage is their high UV transmittance. In addition, Guttman et al. [12] investigated the separation mechanism of SDS-protein complex in SDS–CGE with a replaceable gel matrix.

In this article, we report a new CGE method used to separate and quantify major erythrocyte membrane proteins.

2. Experimental

2.1. Materials

SDS run buffer, molecular mass standard proteins, Coomassie Brilliant Blue R250 and LPA coated capillary (I.D.=50 μm) were purchased from BioRad Laboratory (Richmond, CA, USA). Orange G, β-mercaptoethanol and phenylmethylsulfatefluoride (PMSF) were from Sigma (St. Louis, MO, USA). Vivaspin concentrators were obtained from Vivascience (Binbrook Lincoln, UK). Other chemicals, unless specified, were obtained from Merck (Darmstadt, Germany).

2.2. Samples

Samples were blood samples (collected with heparin as anticoagulant) sent to the laboratory for routine analysis. Samples with normal red cell indices or samples from healthy blood donors were regarded as controls.

2.3. Determination of membrane proteins by SDS–PAGE

Red cell membrane proteins were prepared and purified according to Dodge et al. [13] with minimal modifications. PMSF was dissolved in dimethylsulfoxide (DMSO) and was used as protease inhibitor in all steps.

The erythrocyte membrane proteins were analyzed by a continuous buffer system of Fairbanks with an acrylamide linear concentration gradient gel of 4 to 12% [14]. Proteins were stained with Coomassie Brilliant Blue R250 and gels were scanned using a laser densitometer.

2.4. Purification of membrane protein fractions by ultracentrifugation

The purification method described by Tyler et al. [15] was applied with minor modifications. To deplete spectrin, the crude extracts of erythrocyte membrane, namely membrane ghosts, from 25 ml of washed red cells were suspended in 40 volumes of a phosphate buffer (0.3 m*M*, pH 7.6) containing 0.2 m*M* EDTA and 0.3 m*M* PMSF (PMSF was added just before usage), and centrifuged at 12 500 rpm (DuPont SM24 rotor) at 4°C for 30 min. The pellet was suspended in 30 volumes of the same buffer (pre-warmed to 37°C). The pH was adjusted to 7.6. The membrane ghosts were incubated at 37°C for 30 min. After the incubation, the suspension was centrifuged at 12 500 rpm for 30 min. Most of the spectrin was left in the supernatant which was discarded. The precipitant was then washed once in ten volumes of the same buffer.

In order to determine the end of the band 3 tail, band 3 was eliminated in the following steps. The spectrin-depleted ghosts were re-suspended in 15 ml of phosphate buffer (5 m*M*, pH 8.0) containing 1 m*M* EDTA. To this suspension, 15 μl of aprotinin (10 000 UIK/ml), 50 μl of PMSF (300 m*M* in DMSO), 30 μl of leupeptine (1 g/l), 30 μl of pepstatin (1 g/l) and 1.1182 g of dry KCl (1 *M*).

The pH was adjusted to 7.6 with 1 *M* borate buffer (pH 8.5) and incubated at 37°C for 30 min. The solution was then centrifuged at 12 500 rpm for 60 min. All of band 3 was thus precipitated while part of spectrin, ankyrin 2.1, 4.1a and b as well as 4.2 were left in the supernatant. The supernatant was collected and dialyzed overnight at 4°C against 2 l of a phosphate buffer (5 m*M*, pH 7.6) containing 1 m*M* EDTA, 20 m*M* KCl and 0.5 m*M* β-mercaptoethanol. The dialyzed supernatant was concentrated using a Vivaspin concentrator (10 kD cut-off).

2.5. Purification of ankyrin 2.1 and spectrin by SDS–PAGE

SDS–PAGE was performed with a continuous buffer system (Fairbanks system) in a 4% to 8% acrylamide linear concentration gradient gel. The gel fractions containing ankyrin 2.1 and spectrins, as indicated by the standard protein positions, were cut, broken and suspended in ten volumes of a 100 m*M* Tris–acetate buffer with 0.1% SDS (pH 8.0) and incubated at 37°C overnight. The suspension was then centrifuged at 3000 rpm to precipitate the gel. The supernatant was concentrated using a Vivaspin concentrator (10 kD cut-off).

2.6. SDS–CGE instrumentation

The analyses were carried out with a Beckman P/ACE 5500 capillary electrophoresis system equipped with a diode array detector (Beckman, Fullerton, CA, USA). Peaks detected at 220 nm were integrated with the P/ACE station software. The LPA-coated capillary, 50 μm I.D.×27 cm (effective length=20 cm), was enclosed in a cartridge format and maintained at 20°C by liquid cooling.

2.7. SDS–CGE procedure

The membrane proteins or molecular mass standard protein mixture were dissolved in a sample buffer containing 60 m*M* Tris–HCl (pH 7.0), 2% SDS, 5% β-mercaptoethanol and 0.05% Orange-G. In each analysis, the capillary was first rinsed with 0.1 *M* HCl for 5 min followed by a 7-min rinse with running buffer (138 kPa). The sample was electrokinetically injected into the capillary at 8 kV for 10 s (180 s for proteins purified by SDS–PAGE). The separation was carried out at 13.5 kV (500 V/cm) for 17 min. The typical current level was 16.0 μA.

2.8. Reproducibility tests

Six normal samples were selected to test the reproducibility of migration times and ratios of 4.1a/4.1b, α-spectrin/β-spectrin, (α+β spectrin)/band 3, ankyrin 2.1/band 3, 4.2/band 3 and (ankyrin+α-spectrin)/band 3. The samples were analyzed over five days, once a day and each analysis performed in duplicate. The mean values were used to calculate the inter-assay coefficients of variation.

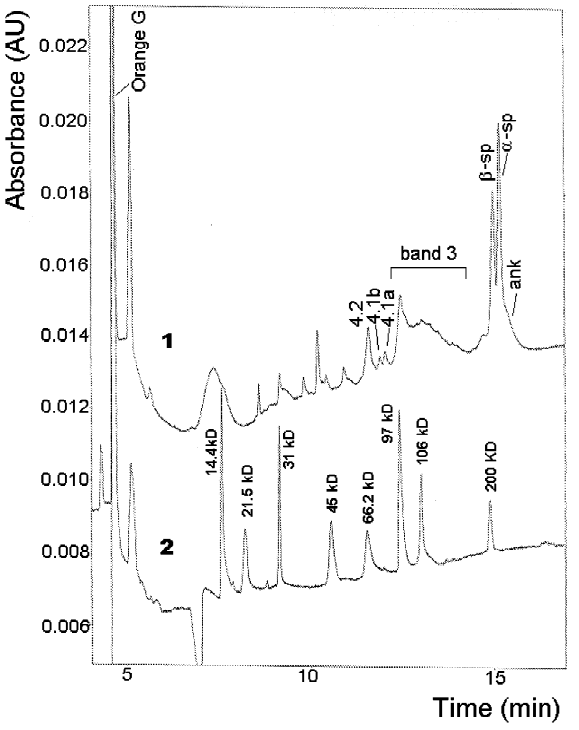

Fig. 1. Separation of erythrocyte membrane proteins and molecular mass protein standards by SDS–CGE. Orange G was used as an internal standard for migration time. Elution profile (1) erythrocyte membrane proteins: protein 4.2 (4.2), protein 4.1b (4.1b), protein 4.1a (4.1a), band 3 (band 3), β-spectrin (β-sp), α-spectrin (α-sp) and ankyrin 2.1 (ank); elution profile (2) molecular mass protein standard: lysozyme (14.4 kD), trypsin inhibitor (21.5 kD), carbonic anhydrase (31 kD), ovalbumin (45 kD), serum albumin (66.2 kD), phosphorylase (97 kD), β-galactosidase (116 kD) and myosin (200 kD). Separation conditions are described in the Experimental section.

3. Results

3.1. Separation of major erythrocyte membrane proteins

Fig. 1 shows the separation of erythrocyte membrane proteins and a group of molecular mass protein standards. Orange G was used as the internal standard for migration time. At least seven membrane proteins can be separated. These are protein 4.2, protein 4.1b and 4.1a, band 3, β- and α-spectrins, and ankyrin 2.1. By SDS–CGE method, band 3 was the biggest peak, with a sharp front and a long tail, ranging from 86 to 183 kD. The two major peaks after band 3 were β- and α-spectrins. By this method, protein 4.1 was separated into two peaks, 4.1b and 4.1a. Protein 4.2 migrated faster than protein 4.1b while ankyrin 2.1 was partially separated from α-spectrin.

A linear relationship between the logarithm of molecular mass and migration time of the standard proteins was obtained (R^2=0.99). This linear relationship was used to calculate the apparent molecular masses of the erythrocyte membrane proteins (Table 1).

Table 1
Apparent molecular masses of the major erythrocyte membrane proteins

Protein	Molecular mass (kD) SDS–CGE	Molecular mass (kD) SDS–PAGE [14]
α-Spectrin	237	240
β-Spectrin	223	220
Ankyrin 2.1	248	214
Band 3 protein	90	90
Protein 4.1a	78	
		80
Protein 4.1b	75	
Protein 4.2	67	72

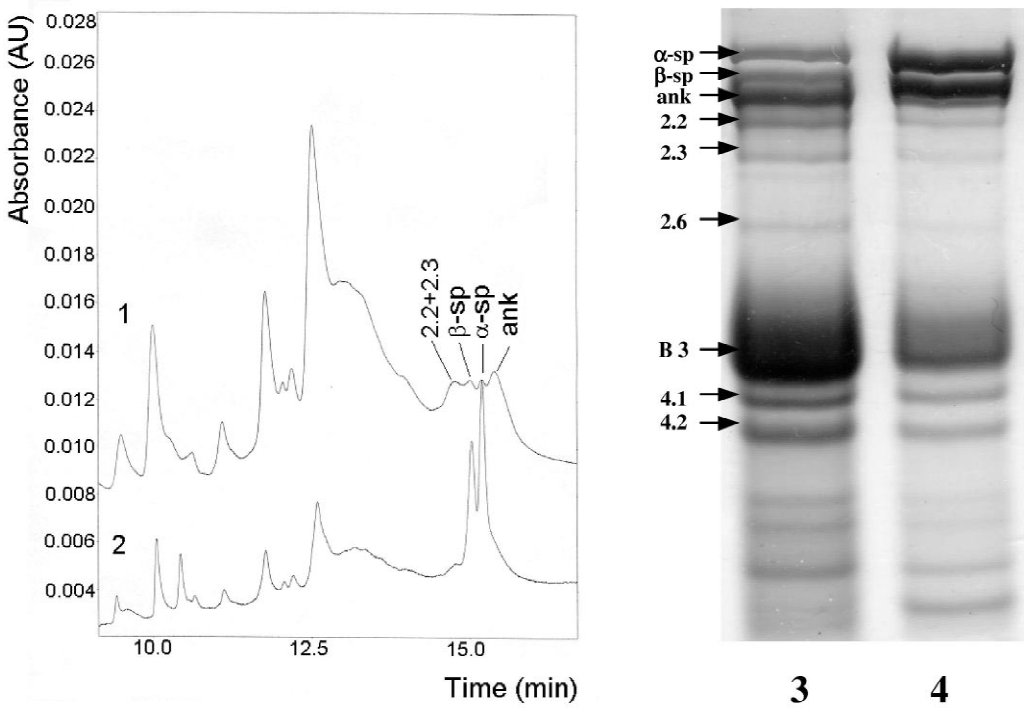

Fig. 2. Separation of spectrin-depleted ghosts and total membrane ghosts by SDS–CGE and SDS–PAGE. Elution profile 1 and lane 3: spectrin-depleted ghosts, elution profile 2 and lane 4: total membrane ghosts. Possible positions of β-spectrin (β-sp), α-spectrin (α-sp) and ankyrin 2.1 (ank) as well as ankyrin isoforms (2.2, 2.3) in SDS–CGE are indicated in this figure. Separation conditions are described in the Experimental section.

3.2. Identification of erythrocyte membrane protein fractions separated by SDS–CGE

Depletion of over 90% of spectrins was obtained by the low-ionic strength extraction method. Comparison of the separation of these spectrins depleted ghosts fractions with the total ghosts fractions either by SDS–CGE or by SDS–PAGE are shown in Fig. 2. By SDS–CGE, the minor peaks left before and after α- and β-spectrins might be ankyrin 2.1 and its isoforms (elution profile 1), band 2.2 and 2.3 which have been separated by SDS–PAGE (lane 3).

In order to confirm the position of ankyrin 2.1 as well as those of α- and β-spectrins, these proteins were extracted after their separation by SDS–PAGE. As demonstrated in Fig. 3, various mixtures of ankyrin 2.1, α-spectrin, and β-spectrin were analyzed. On lane 4, depletion in α-spectrin is accompanied with the disappearance of the middle peak in SDS–CGE (elution profile 1). Depletion in ankyrin 2.1, as demonstrated in lane 6, conducts to the disappearance of the last peak in SDS–CGE (elution profile 3).

After depletion in band 3 and partially in protein 4.2, as seen in elution profile 2 and lane 4 from Fig. 4, peak position of band 3 can be clearly defined in SDS–CGE. It is situated between peak of protein 4.1a and ankyrin 2.1 isoforms, and peak position of protein 4.2 can be clearly demonstrated before protein 4.1b.

Fig. 5 shows the comparison between a sample containing high amount of young red cells (elution profile 1) and a normal sample (elution profile 2) analyzed by SDS–CGE. In the first sample, the

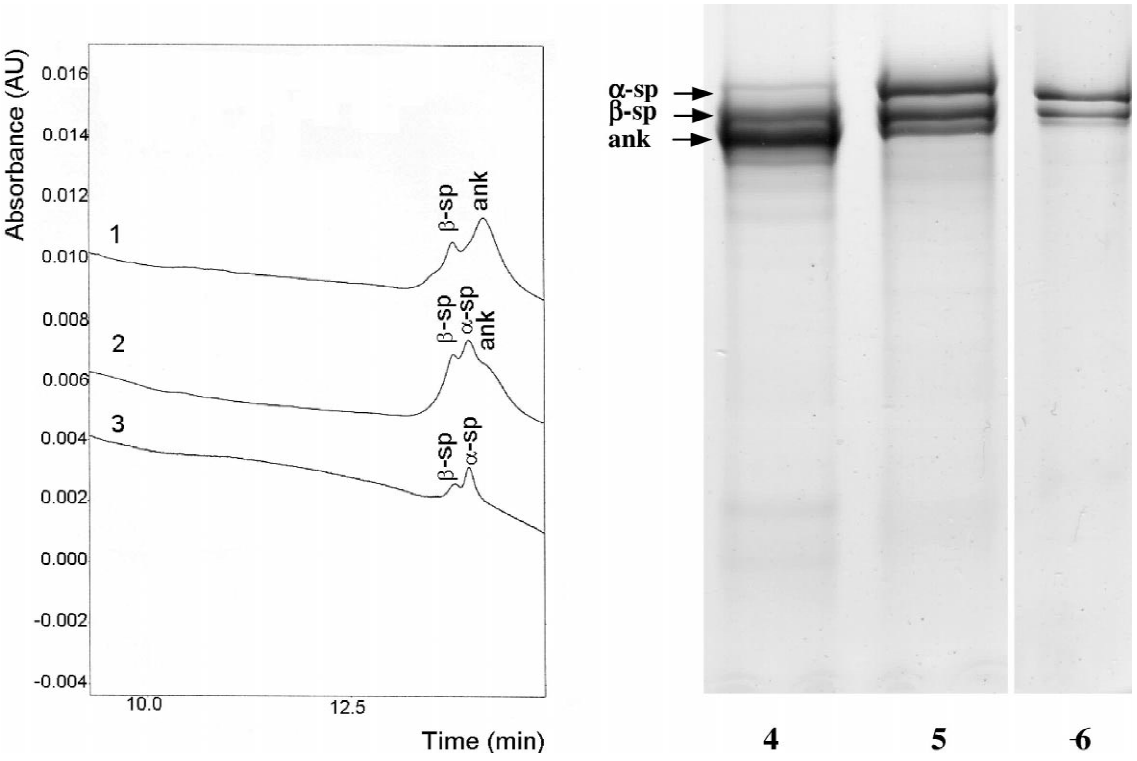

Fig. 3. Separation of mixtures of ankyrin 2.1 and spectrins purified by SDS–PAGE. Elution profile 1 and lane 4: mixture of ankyrin 2.1 and β-spectrin; elution profile 2 and lane 5: mixture of ankyrin 2.1 with α- and β-spectrin; elution profile 3 and lane 6: mixture of α- and β-spectrin. Peak positions of β-spectrin (β-sp), α-spectrin (α-sp) and ankyrin 2.1 (ank) are indicated in this figure. Separation conditions are described in the Experimental section.

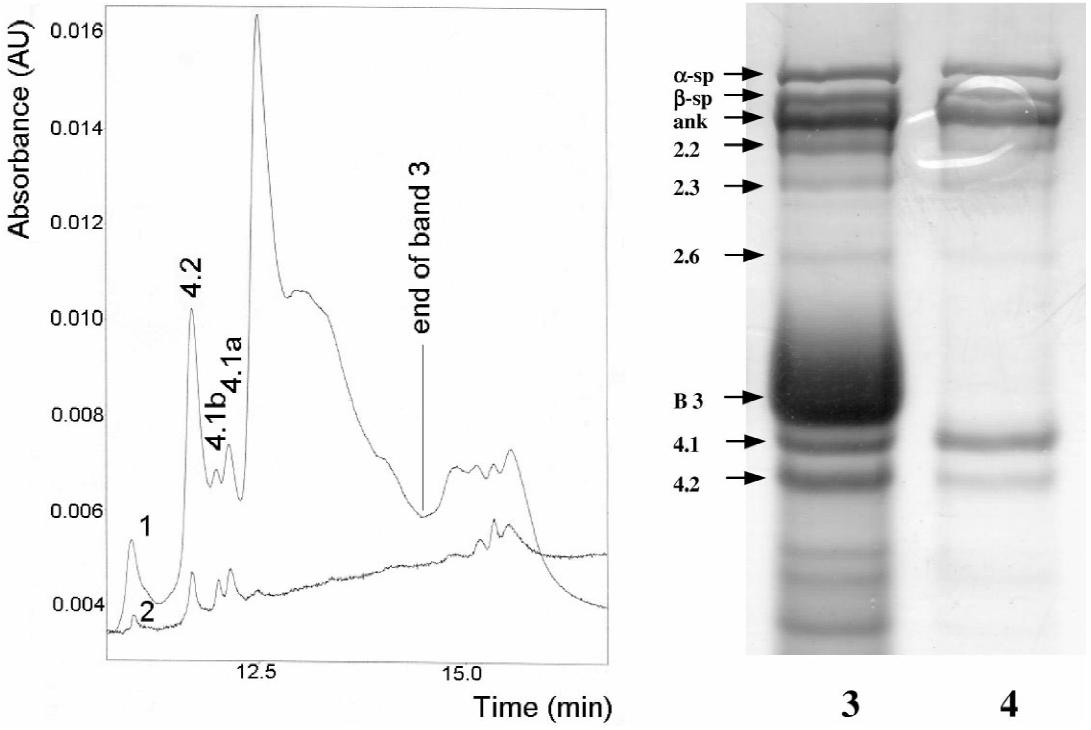

Fig. 4. Separation of spectrin-depleted ghosts before and after extraction of band 3 by SDS–CGE and SDS–PAGE. Elution profile 1 and lane 3: spectrin-depleted ghosts (before extraction of band 3); elution profile 2 and lane 4: spectrin-depleted ghosts (after extraction of band 3). Positions of protein 4.2 (4.2), protein 4.1b (4.1b), protein 4.1a (4.1a) and the end of band 3 are indicated in this figure. Separation conditions are described in the Experimental section.

amount of the second peak after band 3 is higher than the first one while this profile is reversed in the second sample.

These different techniques of purification and extraction of erythrocyte membrane proteins allow the definition of the separation sequence of these proteins by SDS–CGE as: protein 4.2, proteins 4.1b and a, band 3, β-spectrin, α-spectrin, and ankyrin 2.1.

3.3. Reproducibility tests

The inter-assay coefficient of variability of the migration times of the various peaks and ratios between the different proteins are summarized in Tables 2 and 3. The C.V.s for migration times and ratios were always less than 2% and 11%, respectively.

4. Discussion

In this article, we presented a new SDS–CGE method for separation of major erythrocyte membrane proteins. This method allows resolution similar to that obtained with conventional SDS–PAGE but faster and with higher reproducibility.

Our approach demonstrates the possibility of separating and quantifying erythrocyte membrane proteins in one step by SDS–CGE. The gel matrix is replaceable and can provide a sieving effect by the physical interactions among polymer molecules. Unlike in chemical gels, i.e. cross-linked polyacrylamide, the pores size is dynamic depending on the polymer concentration and temperature [11]. In our method, the linear relationship between the protein mobility and logarithm of the molecular weight proved that a molecular sieving effect exists. In the presence of high SDS concentrations, protein

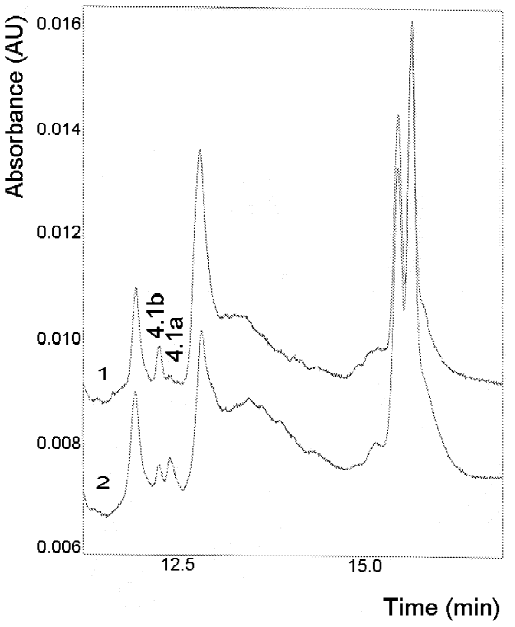

Fig. 5. Separation of erythrocyte membrane proteins from a patient with reticulocytosis (elution profile 1, reticulocyte count= 120/1000 red cells) and a normal sample (elution profile 2). Separation conditions are described in the Experimental section.

mixtures were thus fractionated according to their molecular masses. Similar to SDS–PAGE, this linear relationship can be used to calculate molecular masses of proteins.

It should be stressed that the molecular mass calculated in this way is the apparent molecular mass which is often different from the molecular mass calculated via amino acids sequence. In fact, the apparent molecular mass of the same protein may vary from system to system. In the case of erythrocyte membrane proteins, the behaviors of some proteins, like ankyrin 2.1, are different in different electrophoretic systems. In SDS–PAGE with the discontinuous system described by Laemmli [2], ankyrin 2.1 is not separated from α-spectrin, while with the continuous one described by Fairbanks [4], ankyrin 2.1 is separated from β-spectrin. In SDS–CGE, ankyrin 2.1 migrates slightly after α-spectrin. Obviously its apparent molecular mass is quite different by the above methods but the linear relationships of molecular masses vs. migration times still exist.

Since this is the first SDS–CGE method described for the erythrocyte membrane proteins analysis, identification of each clinically important protein were performed. Several protein fractions were depleted from the original membrane proteins of the erythrocyte ghosts and analyzed by SDS–CGE and SDS–PAGE. Several proteins were also extracted from the SDS–PAGE and analyzed by SDS–CGE. Using these methods, all major erythrocyte protein fractions separated by SDS–CGE were identified and the sequence of their separation profile was established.

The separation order of the erythrocyte membrane proteins obtained in SDS–CGE was very close to the separation profile obtained in the SDS–PAGE method described by Laemmli [2] including the resolution of protein 4.1 in two peaks 4.1 a and b. In contrast to the Laemmli method, a partial separation of α-spectrin and ankyrin 2.1 peaks was achieved in the SDS–CGE method because of a high resolution power combined with a relative low injection amount.

In clinical practice, Laemmli [2] and Fairbanks [4] electrophoretic methods are both used for the diagnosis of HS. The relevance of the Fairbanks method is its capacity to separate ankyrin 2.1 from β-spectrin and the relevance of the Laemmli method is its ability to separate proteins 4.1a and b isoforms, giving an indication of the presence of a high reticulocyte count within the sample tested. Indeed, a high reticulocyte content causes an increase of the isoform 4.1b over 4.1 a [16], but it results also in an apparent increase in the amounts of ankyrin 2.1 and spectrin which could mask the deficiency of these proteins. In such cases, normal relative amounts of ankyrin 2.1 and spectrin must warn of a possible deficiency of these proteins [17].

By the current SDS–CGE method, we obtained good reproducibility of the quantification of all major proteins, including protein 4.2, protein 4.1a and b, band 3, α- and β-spectrin as well as ankyrin 2.1. Meanwhile, the presence of the reticulocytosis can be detected in the same run since 4.1a and b are well separated and can be quantified. This method might thus be utilized to detect protein deficiencies related to HS, or to identify protein 4.1 and spectrin abnormalities in HE, or to identify underglycosylated band 3, or be used for biochemical researches related to membrane proteins.

Table 2
Reproducibility test of migration times of the major erythrocyte membrane proteins by SDS–CGE

Samples		4.2 (min)	4.1b (min)	4.1a (min)	Band 3 (min)	β-Spectrin (min)	α-Spectrin (min)	Ankyrin 2.1 (min)
Sample 1								
(n=5)	Mean	11.8	12.1	12.2	12.6	15.2	15.4	15.5
	C.V.%	2.1	1.9	2.0	1.9	1.9	1.9	1.8
Sample 2								
(n=5)	Mean	11.7	12.0	12.1	12.5	15.1	15.3	15.4
	C.V.%	1.1	1.1	1.1	1.1	1.1	1.1	1.1
Sample 3								
(n=5)	Mean	11.6	11.9	12.1	12.5	15.0	15.2	15.3
	C.V.%	0.6	0.6	0.6	0.6	0.6	0.6	0.6
Sample 4								
(n=5)	Mean	11.6	11.9	12.0	12.5	15.0	15.2	15.3
	C.V.%	0.4	0.4	0.4	0.4	0.5	0.5	0.5
Sample 5								
(n=5)	Mean	11.6	11.9	12.0	12.5	15.0	15.2	15.3
	C.V.%	0.7	0.7	0.5	0.6	0.6	0.7	0.7
Sample 6								
(n=5)	Mean	11.6	11.9	12.1	12.5	15.0	15.2	15.3
	C.V.%	0.6	0.6	0.6	0.6	0.6	0.6	0.6

Table 3
Reproducibility test of ratios among the major erythrocyte membrane proteins by SDS–CGE

Samples		4.1a/4.1b	α-Spectrin/ β-spectrin	(α+β)-Spectrin/ B 3	Ankyrin 2.1/ B 3	4.2/B 3	(Ankyrin 2.1+α-spectrin)/B 3
Sample 1							
(n=5)	Mean	1.20	0.88	1.36	0.19	0.40	0.70
	C.V.%	5.3	2.4	2.2	2.7	4.4	1.4
Sample 2							
(n=5)	Mean	1.02	0.83	1.24	0.20	0.42	0.65
	C.V.%	6.0	3.0	1.1	7.7	3.5	4.2
Sample 3							
(n=5)	Mean	1.48	0.85	1.26	0.18	0.40	0.66
	C.V.%	8.1	3.8	3.8	5.3	3.4	5.0
Sample 4							
(n=5)	Mean	1.48	0.81	1.24	0.17	0.33	0.62
	C.V.%	7.3	3.5	1.4	6.0	8.5	3.9
Sample 5							
(n=5)	Mean	1.66	0.95	1.31	0.20	0.36	0.74
	C.V.%	4.5	6.2	5.8	10.7	4.7	8.3
Sample 6							
(n=5)	Mean	1.29	0.82	1.29	0.18	0.36	0.64
	C.V.%	6.4	3.2	4.7	5.1	2.9	3.4

In our method, quantification of the proteins is essential since the diagnosis depends on the quantitative information. In SDS–PAGE, quantification of protein can be obtained by staining with Coomassie Blue. The Coomassie Blue staining and de-staining procedure are time consuming and might induce errors in quantity [18]. Immunoassay is more accurate than Coomassie blue staining, but it is rather difficult and expensive which limits its application. In SDS–CGE, on-line UV absorbance detection makes the direct quantification by the peak area possible. It reduces the time consumption as well as the risk of error coming from staining. Other interesting features of the present method include automation, less labor consumption, and easiness of operation. All these make the current SDS–CGE method interesting for detection of membrane protein abnormalities.

5. Conclusion

The current SDS–CGE method opens the possibility of analyzing erythrocyte membrane proteins. The separation and quantification of seven major protein factions, protein 4.2, protein 4.1a and b, band 3, α- and β-spectrins and ankyrin 2.1 were achieved by this method. Compared with the conventional SDS–PAGE methods, we obtained similar separation profiles, but SDS–CGE is less time and labor intensive. Highly reproducible migration times and quantification of each protein made it possible to use this SDS–CGE method for detection of abnormalities related to erythrocyte membrane proteins.

Acknowledgements

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