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High-performance capillary electrophoresis of proteins using sodium dodecyl sulfate–poly(ethylene oxide)

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Abstract

High-performance capillary electrophoresis using the replaceable poly(ethylene oxide) (PEO) polymer network in the presence of sodium dodecyl sulfate (SDS) is shown to be a viable alternative to SDS–polyacrylamide gel electrophoresis. The effects of the PEO molecular mass and polymer concentration on the separation of proteins were studied. The 100 000 PEO polymer provided good resolution of the protein standards and was selected for detailed studies. The preparation of this gel at various concentrations is relatively simple and makes it feasible for Ferguson analysis. Various recombinant proteins were analyzed using a universal calibration curve generated by the Ferguson analysis. The accuracy of the estimated molecular mass were very much protein dependent, in general it was about 10% off to their sequence based molecular mass.

1. Introduction

Capillary electrophoresis of proteins and peptides has become a successful and well documented analytical method [1]. The traditional method of choice for purity assessment of protein preparations is sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). It is also often used for molecular mass estimation of proteins. The popular slab SDS-PAGE is time consuming and requires visualization of the separated protein bands by staining/destaining procedures. The capillary version of SDS-PAGE has been described and is currently undergoing development [2–4]. Presently, it is believed that 25 to 100 μm I.D. capillaries, filled with replaceable viscous polymer solutions offers more potential than crosslinked gels [5,6]. The capillaries are coated or uncoated depending on the nature of the sieving polymer network. In capillary electrophoresis, on-line detection is possible when the separation buffer, coating material or separation polymer network are transparent at the detection wavelength. It appears that replaceable high-molecular-mass dextrans [7], poly(ethylene oxides) [8], linear polyacrylamides [9], and other polymers are appropriate for detergent mediated protein separations [10].

The replaceable polymer network method also allows for easy preparation of separating polymers at a variety of concentrations, thus specific optimization can be tailored for various protein separations. Additionally, capillary electrophoresis offers the possibility of quantitative analysis [11].

Our study focused on the utilization of linear poly(ethylene oxide) (PEO) polymer solutions to evaluate the molecular mass of various recombinant proteins [12]. The separation buffers were UV transparent, thus allowing direct protein

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detection at the low UV (214 nm) region. The most practical advantage of the PEO based solutions is that they do not require coated capillaries. The objective was to optimize protein separations and to study the effects polymer molecular mass and concentration in a defined separation buffer had on the separations. The systematic study was performed with commercially available standard proteins. The feasibility of the method is illustrated with the analysis of recombinant proteins, glycosylated and non-glycosylated, molecular mass ranging from 13 000 to 30 000 and covering a wide range of p*I* values (*ca.* 4.5–10.5).

2. Materials and methods

2.1. Instrumentation

A Beckman P/ACE System 2000 (Beckman Instruments, Fullerton, CA) automated capillary electrophoresis instrument, with System Gold instrument control and data evaluation software was used for analysis. The detection wavelength was 214 nm. The temperature of the analysis was set at 20°C unless it is stated otherwise.

2.2. Materials

The electrophoresis calibration kit for low-molecular-mass proteins was purchased from Pharmacia (Piscataway, NJ, USA) and contained the following proteins: phosphorylase *b* (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 100) and α-lactalbumin (14 400). Crosslinked hemoglobin was from Sigma (St. Louis, MO, USA). The 10 000 polyethylene glycol (PEG), 100 000 and 1000 000 PEO polymers were obtained from Sigma and Aldrich (Milwaukee, WI, USA), respectively. Sodium dodecyl sulfate (SDS) stock solution was from Amres (Solon, OH, USA). Recombinant brain derived neurotrophic factor (BDNF), granulocyte stimulating factor (GCSF), erythropoetin (EPO) produced in *E. coli* or Chinese hamster ovary, platelet derived growth factor (PDGF-BB), consensus interferon (CON-IFN) are from Amgen.

2.3. Preparation of PEO or PEG polymer stock solutions

The PEO polymer stock solutions were prepared by dissolving the appropriate amount of polymer in 0.1% ethylene glycol containing distilled water. The aqueous polymer solutions were filtered through a 5.0-μm Acrodisc filter. The polymer content of the filtered solutions were determined by gravimetry.

2.4. Buffers

The separation buffers, which contained 100 m*M* Tris–2-(N-cyclohexylamino)ethanesulfonic acid (CHES) (pH 8.5), 0.1% SDS and PEO polymer at various concentrations were prepared by the combination of the 1.0 *M* Tris–CHES buffer (pH 8.5), 20% SDS and polymer stock solutions. All stock solutions were stored at 4°C when not in use.

2.5. Samples

The final sample buffer contained 0.06 *M* Tris–HCl, pH 6.6 with 5% 2-mercaptoethanol and 1% SDS. The samples were boiled for 5 min, then cooled on ice for 3 min, followed by centrifugation.

2.6. Capillaries

The separations were performed in 100 μm I.D. and 375 μm O.D. fused-silica capillaries from Polymicro Technologies (Phoenix, AZ, USA). Most of the work was done with capillaries of 20 cm effective and 27 cm total length. The capillaries were first washed with 1 *M* NaOH, HPLC-grade water, 1 *M* HCl and then conditioned with the separation buffer. Between runs the capillary was washed with 1 *M* HCl and water to remove surface adhered material.

2.7. Electrophoresis

For the evaluation of the separation characteristics of the PEO polymers the Pharmacia low-molecular-mass electrophoresis calibration kit proteins were used. The background buffer concentration, pH, as well as the SDS concentration were kept constant in all experiments, as described in the buffer and sample section above, no attempt was made to optimize the separations. The electrophoresis was performed in uncoated capillaries at 300 V/cm field strength. The samples were introduced by pressure injection and the duration of injection varied between 2 and 20 s according to the viscosity of the separation polymer network.

2.8. Calculations

The relative migration time (RMT) is calculated by dividing the migration time of the protein by the migration time of Orange G. The standard curve for molecular mass estimation is constructed by plotting the logarithm of the molecular mass as a function of 1/RMT. Linear regression provides the slope and intercept of the standard curve used for molecular mass estimation of unknown proteins. The Ferguson graphs are constructed by plotting the logarithm of 1/RMT of the individual proteins in different polymer solutions as a function of the polymer concentration. Linear regression provides the slope, which is the negative value of the retardation coefficient (K_r). The universal calibration curve is than drawn by plotting the logarithm of molecular mass as a function of the square root of K_r. Linear regression provides the slope and intercept for calculations used for the protein molecular mass estimation.

3. Results

3.1. Effect of polymer molecular mass on the separation of SDS–protein complexes

Solutions of high-molecular-mass PEO as a sieving network for capillary SDS electrophoresis has been used before in coated capillaries, but our preliminary experiments showed that they can be used in uncoated capillaries as well. Separation in uncoated capillaries is reproducible and the R.S.D. of migration times were less than 2% up to 15 to 20 consecutive runs. Separation in coated capillaries does not change the performance of the polymer network but increases the lifetime of the column. Consequently, for the following study PEO and PEG polymers were selected, which are commercially available in a wide range of molecular masses, have good UV characteristics and are soluble in the relevant buffers. The general formula for PEO and PEG is $-(O-CH_2-CH_2)_n-$. The difference is in the end groups and the length of the polymer, the shorter being PEG. For sake of simplicity, we choose to use the name PEO for both polymers.

The wide range of the available PEO polymers can be divided into three subgroups according to the polymer–protein molecular mass ratio using a median protein molecular mass (44 750). We selected one polymer from each group. In the first group the ratio was below one, for our 10 000 PEG it was 0.22. The ratio in the second group was 2.23 for the 100 000 PEO and in the third group the molecular mass ratio was 22.35 using the 1000 000 PEO.

Fig. 1A displays the electropherogram of standard proteins using a 3% (w/v) solution of the 10 000 PEG for their separation. The protein standards are poorly separated; both the resolution and efficiency are low. The two sharp peaks eluting prior to the proteins correspond to Orange G (larger) and its degradation product (smaller).

The second PEO (100 000) exhibited relatively good separation at the 1% concentration. The peaks are acceptable and distinguishable though the resolution is not satisfactory as shown in Fig. 1B.

The electropherogram using the 1000 000 PEO at 1% concentration is shown in Fig. 1C. All components of the standard mixture are baseline separated.

An improvement of the separation can be seen between the 100 000 and 1000 000 polymer network. The separation window, defined as the

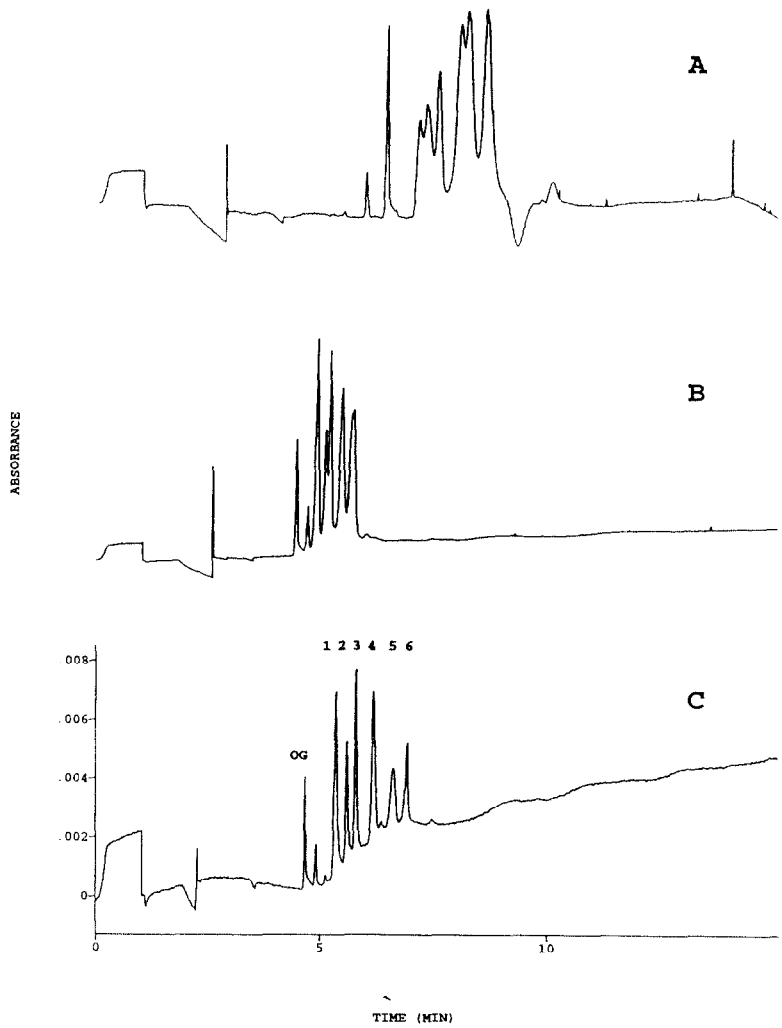

Fig. 1. SDS–PEO capillary polymer network electrophoresis of standard proteins using different molecular mass PEOs. (A) 10 000, (B) 100 000 and (C) 1000 000. The polymer concentration for A was 3% and 1% for B and C. The electrophoresis was performed in a 100-μm I.D. capillary. The effective and total length of the capillary was 20 and 27 cm, respectively. The proteins were separated at reversed polarity, at 300 V/cm field strength. The migration was followed at 214 nm. The sample was pressure injected. OG = Orange G; 1 = α-lactalbumin; 2 = soybean trypsin inhibitor; 3 = carbonic anhydrase; 4 = ovalbumin; 5 = bovine serum albumin; 6 = phosphorylase *b*.

difference between the migration time of the last and first protein peak, approximately doubles. Despite the good separation the increase in viscosity presents a practical filtration problem over 100 000 during the preparation of separation polymer networks. The 100 000 PEO polymer seems to be a reasonable compromise between performance and ease of preparation, thus we focused our further studies on its use for studying other parameters which might effect the separation.

3.2. Effect of polymer concentration on the separation

Fig. 2 displays the electropherograms of the protein standards using 2, 3, 4 and 5% solutions of 100 000 PEO. The most dramatic effect is the

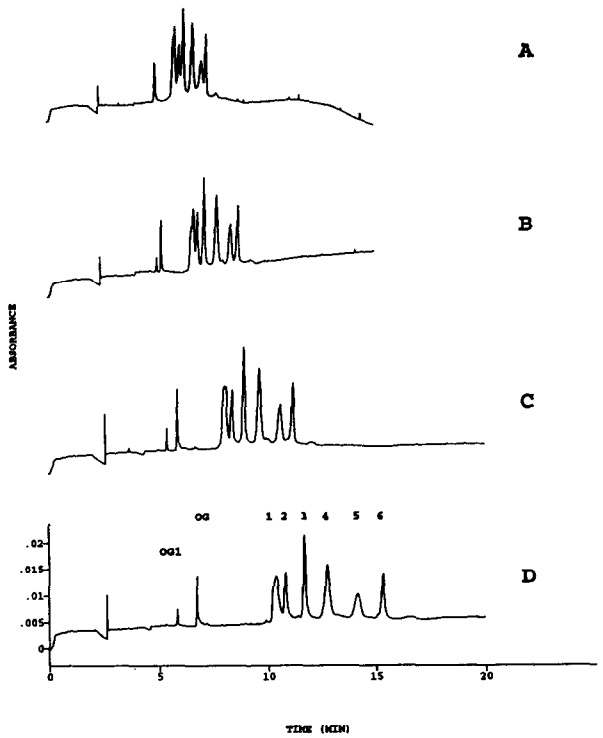

Fig. 2. The effect of polymer concentration on the SDS–PEO electrophoresis of proteins. The concentration of the 100 000 PEO polymer was (A) 2%, (B) 3%, (C) 4% and (D) 5%. For other conditions see Fig. 1.

apparent increase of the separation window and consequently the resolution of the samples. Below 1% the separation window was around 1.5 min (data not shown), which then increased to about 3 min at 3%. The separation window increases to about 6 min at 5% PEO concentration. It seems that the separation window increases exponentially with the concentration of the polymer network.

It should be mentioned that the peak efficiency for the protein peaks does not seems significantly change over the studied PEO concentration range. The efficiency is around 50 000 plates per meter, which is about one order of magnitude lower than expected, based on free solution data. In free zone electrophoresis the inclusion of polymers in the running buffer eliminates some of the diffusion related peak broadening. The lower efficiency is due to interactions between the PEO network [13,14], SDS [15,16] and the proteins. The SDS–protein–PEO system is complex with multiple equilibrium, the identification of all the factors involved in the peak broadening should be further investigated.

3.3. Protein molecular mass determinations

Theoretically, the free solution mobility of all SDS–protein complexes should be the same. Consequently, the migration of proteins in a sieving matrix should depend only on their hydrodynamic radius. The traditional method for molecular mass estimation is to use a standard curve constructed by plotting the migration times of standard proteins against their known molecular mass. Consequently, most errors in the molecular mass determination using the single standard curve method originate from discrepancies of the free solution mobility [17]. The usual source of error is the anomalous binding of SDS to the protein.

One way to avoid errors related to the single standard curve method is to measure the migration times at different polymer network concentrations, construct Ferguson plots and a universal calibration curve [18]. The use of relative migration times further improves the run-to-run reproducibility of the analysis. The Ferguson plot is the logarithm of the relative migrations plotted as a function of polymer concentration. The slope of the curve represents the retardation coefficient (K_r) while the intercept at zero polymer concentration corresponds to the free solution mobility of a protein. The logarithm of the molecular mass *vs.* the square root of the retardation coefficient of proteins provide a universal standard curve which then can be used for the estimation of the molecular mass of unknown proteins. The Ferguson analysis for traditional SDS-PAGE is extremely time consuming, especially because the analysis is recommended to be performed at at least six different gel concentrations [19]. Consequently this method of analysis is practically abandoned. The method, however, is in revival because the use of replaceable polymer networks makes the analysis more feasible again [20].

We prepared separation polymer networks

containing 2, 3, 4 and 5% PEO and analyzed the same protein mixture under otherwise identical conditions. Fig. 3A and B show the Ferguson plots for six proteins and the constructed universal standard curve. All regressions show acceptable linearity, the r^2 values are larger than 0.98. The intercepts are different and the intersection points located in the negative PEO concentration range. These anomalies could be the consequence of differences in free solution mobilities of the SDS–protein complexes (for example glycoproteins), sample preparation or any combination of the above. In an attempt to find the reason(s) behind this anomalous behavior, the following experiment was designed.

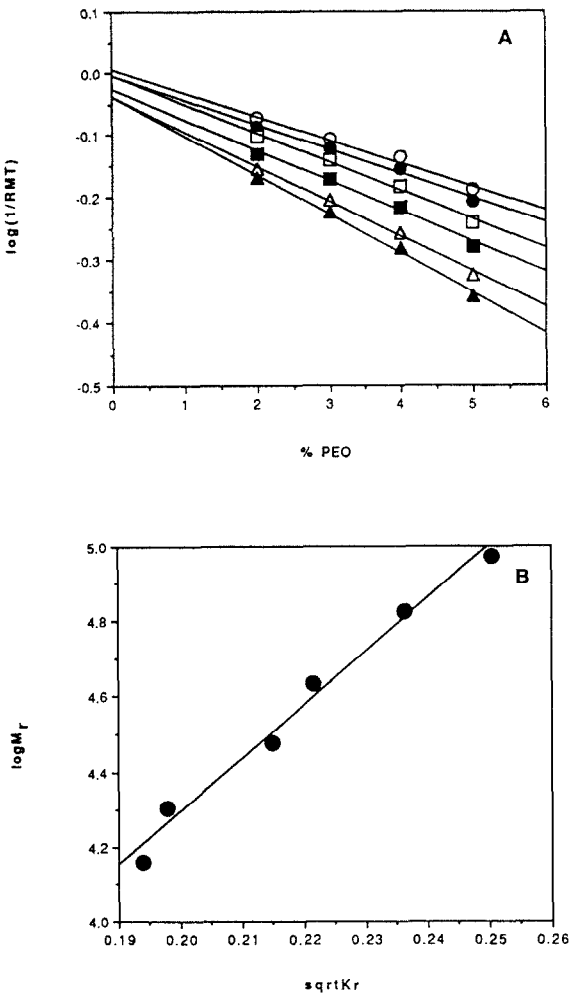

Fig. 3. (A) Ferguson plots of the standard proteins. ○ = α-lactalbumin; ● = soybean tripsin inhibitor; □ = carbonic anhydrase; ■ = ovalbumin; △ = bovine serum albumin; ▲ = phosphorylase *b*. (B) Universal calibration curve constructed based on the Ferguson plot.

It is assumed that polymeric protein samples containing the same subunits will behave similarly during sample preparation. The free solution mobility of these polymeric proteins should be the same or very similar. In order to check the performance of the SDS–PEO system, eliminating some of the sample related variances, cross-linked bovine hemoglobin's were selected. Such polymeric proteins are commercially available and contain monomers, dimers, trimers and tetramers. Fig. 4 displays their electropherograms at different PEO concentrations. The resolution again improves with increasing polymer network concentration. It is apparent that penta, hexa and heptamers are also present in the sample. The Ferguson plots of the hemoglobin "polymers" are displayed in Fig. 5. The linearity for each compound is excellent. The difference in the slopes proves that the separation is based on size differences between the molecular species. The intercepts at zero polymer network concentration are different, but the intersection points of the lines are in good agreement. Since we assumed that the model compounds have identical free solution mobility, the lines should intercept in close proximity to each other. Apparently SDS–PEO does not behave ideally, and the samples appear to interact with the PEO network. The electric field known to orient the molecules, can be a potential source for unusual migration behavior. Anomalous protein migration can be the source of major errors in the estimation of the molecular mass and the use of a universal calibration curve should eliminate some of these errors.

3.4. Molecular mass determination of recombinant proteins

The applicability of the SDS–PEO polymer network electrophoresis was further tested by estimating the molecular mass of recombinant proteins (BDNF, PDGF, CON-IFN, GCSF, EPO). Fig. 6 is the Ferguson plot constructed for

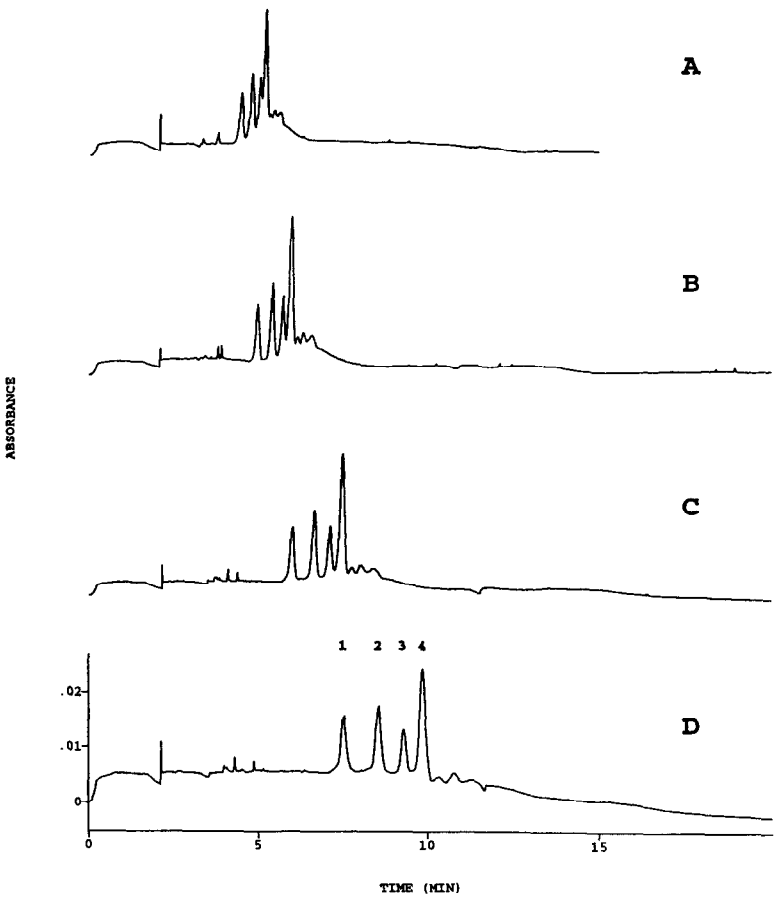

Fig. 4. Electropherograms of hemoglobin polymers using SDS–PEO electrophoresis. The concentration of the 100 000 PEO polymer was (A) 2%, (B) 3%, (C) 4% and (D) 5%. 1 = Monomer; 2 = dimer; 3 = trimer; and 4 = tetramer. All other conditions are as listed in Fig. 1.

some of the selected recombinant proteins. The mobility at zero polymer network concentration varies significantly, and the intersection points are also at different locations. Table 1 compiles the data about the samples with their calculated (literature) and estimated molecular mass using the Ferguson analysis. The single calibration curve method gave estimates with only a few percent difference as shown for the 3% polymer network in Table 1. With the exception of glycosylated EPO, PDGF and BDNF, the error of molecular mass estimation is reasonable.

The molecular mass of *E. coli* derived PDGF-BB is estimated as *ca.* 23 000, which is less than the dimeric molecular mass (26 780) of the native PDGF. PDGF contains two interchain disulfide bridges and it is possible that the general sample treatment used in this work is not appropriate to break the dimeric form of PDGF [21].

The glycosylated and non-glycosylated forms of r-Hu EPO gave two different molecular mass estimates. The *E. coli* derived EPO, which does not contain carbohydrate side moieties migrates as expected. The estimated molecular mass is around 20 000, which is higher than the calculated 18 000 molecular mass [22]. The glycosylated EPO, derived from CHO cells, has a molecular mass of about 30 000 and almost 40% of it is carbohydrate. Using the SDS–PEO

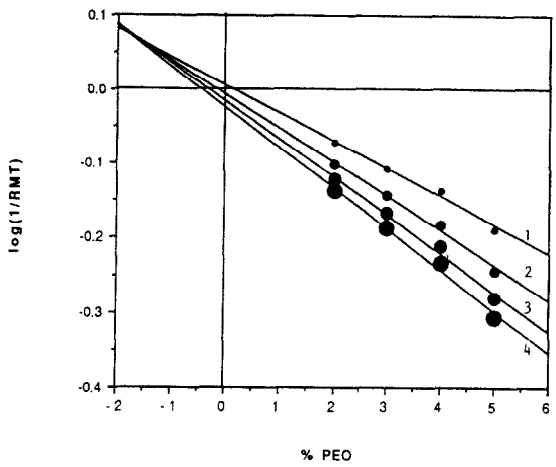

Fig. 5. Ferguson plots of hemoglobin polymers. 1 = Monomer; 2 = dimer; 3 = trimer; and 4 = tetramer.

polymer network electrophoresis system the molecular mass of EPO is estimated to be about 70 000. Glycosylated EPO migrates anomalously in size exclusion chromatography, giving an apparent molecular mass of 60 000 [23]. It was established that the anomalous migration is due to an unusually large viscosity radius caused by the glycosylation of EPO. However, in SDS-PAGE under reducing conditions, this anomaly was not observed. The phenomenon suggests that the mechanism of the migration in SDS–PEO polymer network electrophoresis is more similar to size exclusion chromatography than SDS-PAGE.

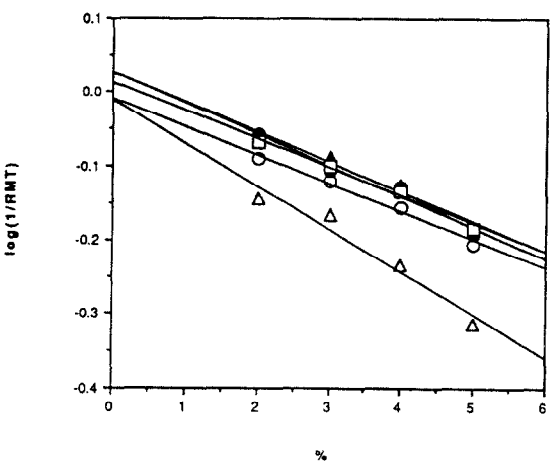

Fig. 6. Ferguson plots of recombinant proteins using SDS–PEO CE. ○ = BDNF; ● = PDGF; △ = EPO; ▲ = CON-IFN; □ = GCSF. The abbreviations can be found in the text. The analysis were performed using standard conditions, see Fig. 1.

4. Conclusions

Poly(ethylene oxides) based replaceable polymer networks are feasible alternatives for the SDS mediated separation of proteins. The separation in general improves with increasing PEO molecular mass and significant resolution improvement was observed with increasing polymer network concentrations. A 3% solution of a 100 000 PEO seems to be a reasonable compromise from a practical point of view. At higher concentrations and molecular mass the filtration of the polymer network stock solution is difficult and time consuming.

Protein molecular mass determination based on a single concentration determination, using log M_r *vs.* 1/relative migration time as a standard curve provides reasonable estimates in general. However the estimated molecular mass can sometimes be off by as much as 20–40%, depending upon the nature of the protein. For a better molecular mass estimation the Ferguson analysis is recommended. Due to the relatively easy preparation of the different concentration PEO solutions, this analysis is more feasible for replaceable polymer networks than for cross linked and immobilized sieving matrices.

Finally, the advantages of the SDS–PEO polymer network electrophoresis can be summarized as follows: uncoated fused-silica capillaries can be used; the polymers are UV transparent; the preparation of polymer stock solutions for SDS–PEO is relatively simple; the unbuffered polymer stock solutions are stable at 4°C for about a month. Separation parameters can be optimized rapidly since the pH, polymer concentration, buffer type, conductivity, viscosity are easy to vary. Optimized separations provide a fast method for molecular mass estimation. At this point the rapid detection of covalent dimers, trimers, etc. appears to be the most promising application

Table 1
Biochemical data and molecular mass estimation of recombinant proteins

	BDNF	PDGF	EPO[a]	EPO	CON-IFN	GCSF
M_r calculated	13 511	13 390	30 400	18 395	19 400	18 791
% Sugar	0	0	40	0	0	0
p*I*	~10.3	~10	4.2–4.6	~8.8	~5.5	~6.1
Native form	D[b]	D[c]	M[d]	M	M	M
M_r by Ferguson	16 978	23 105	75 457	23 934	20 671	17 225
M_r by 3% PEO	19 771	15 496	44 014	19 329	11 201	13 830

[a]Produced in Chinese hamster ovary.
[b]Non disulfide dimer.
[c]Disulfide dimer.
[d]Monomer.

for capillary electrophoresis using the SDS–PEO polymer network.

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