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Evaluation of sodium dodecyl sulfate non-acrylamide, polymer gel-filled capillary electrophoresis for molecular size separation of recombinant bovine somatotropin

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ABSTRACT

Sodium dodecyl sulfate (SDS) non-acrylamide, polymer gel-filled capillary electrophoresis was examined as an alternative to the high-performance size-exclusion chromatographic (HPSEC) method for the analysis of recombinant bovine somatotropin (bovine growth hormone, rbSt). A calibration curve for the molecular mass of protein standards (M_r 14 000 to 97 000) was linear ($r^2 = 0.998$) when the molecular mass of the proteins and their peak migration time were plotted on a logarithmic (log–log) scale. Relative standard deviation (R.S.D.) of the molecular mass determination was approximately 2–3%. A pre-production as well as a production lot of the SDS gel-filled capillary columns were examined. Performance of both of these columns were equivalent. Peak migration time remained relatively constant beyond 140 sample injections. Gradual loss of theoretical plates was noted over the course of the assay; however, the peak resolution remained adequate for the analysis of rbSt.

Peaks corresponding to monomer, dimer, trimer, and tetramer of rbSt were base-line resolved by the SDS gel-filled capillary electrophoresis. Theoretical plate of the monomer peak was approximately 28 000–30 000 per 40 cm column. Both the monomer and the dimer recovery studies indicated that the calibration curves are linear ($r^2 > 0.993$) and the slopes are no different from one. Amounts of the components in rbSt determined by the SDS gel-filled capillary electrophoresis compared well with those of the HPSEC method. The results of this study indicated that the SDS non-acrylamide gel-filled capillary electrophoresis may be a viable alternative to the HPSEC method for the molecular size separation and analysis of recombinant proteins.

INTRODUCTION

Recombinant proteins are posing a considerable challenge to analytical chemistry. High-performance size-exclusion chromatography (HPSEC) is routinely employed to determine the composition of recombinant proteins. However, peak resolution capability of the HPSEC method is not always ideal and difficulty has been experienced in obtaining reliable/usable columns for the assay of recombinant bovine somatotropin (rbSt). The difficulty was traced to changes made in the column manufacturing processes.

Sodium dodecyl sulfate–polyacrylamide slab gel electrophoresis (SDS-PAGE) is an indispensable technique for the separation of proteins based on their apparent molecular mass. In spite of impressive advances made in recent years [1], electrophoresis still represents a collection of labor-intensive and time consuming techniques and quantification by means of an optical scanning apparatus is often sub-optimum.

Recent developments in the commercialization of high-performance capillary electrophoresis (HPCE) instrument [2–5] have made it possible to exploit potential of SDS gel-filled capillary column for separation of proteins with promises of rapid and automated analysis with improved reproducibility and quantification. Hjertén [6] was the first to demonstrate utility of a polyacrylamide gel filled column for separation of a membrane protein. He utilized an uncoated, home made, ordinary glass capillary column filled with polyacrylamide gel. Cohen and Karger [7] also applied SDS polyacrylamide gel-filled

capillaries for electrophoresis of peptides and proteins, but no quantitative data was presented. Tsuji [8] prepared an SDS polyacrylamide gel-filled capillary column filled with cross-linked acrylamide gel and provided quantitative data for molecular mass separation and analysis of SDS–protein complexes.

Zhu *et al.* [9] expanded molecular sieving action of linear polymers, *e.g.* dextran, methylcellulose, and polyethylene glycol, and used them as additives in the HPCE buffer to facilitate separation of DNA and proteins. Ganzler *et al.* [10] substituted polyacrylamide with a dextran polymer and/or a linear polyethylene glycol polymer network and successfully achieved molecular mass separation of proteins. Just recently, the SDS non-acrylamide, polymer gel-filled capillary column became commercially available [11]. Such columns were examined as an alternative to the HPSEC method for the analysis of rbSt.

EXPERIMENTAL

Instrumentation

A Beckman P/ACE system 2100 HPCE instrument (Beckman Instruments, Fullerton, CA, USA) was used. Each analytical run consists of rinsing a coated capillary column (parts No. 241521, Beckman; 100 μm I.D. × 375 μm O.D.; effective length, 40 cm) with 1.0 *M* HCl for 2 min and the column was filled with an SDS non-acrylamide, polymer gel solution (parts No. 241522, Beckman) for 4 min. Use of a coated capillary column is required to eliminate electroendosmosis.

About 1 mg protein per ml solution was injected for 60 s under nitrogen pressure (total injection volume, *ca.* 60 nl) onto the SDS gel-filled capillary column and the peaks migrating in the capillary were monitored on-column by UV at 214 nm. The column temperature was maintained at 20°C by a circulating coolant to minimize band diffusion for effective size separation. An electrophoretic run was conducted at −14.1 kV (−300 V/cm, 24 μA) using the SDS gel solution as reservoirs at both anode and cathode terminals. The area under the peak was integrated by means of an in-house GC/LC program residing on a VAX mainframe computer and with an electronic integrator (Model 3392A, Hewlett-Packard, Palo Alto, CA, USA).

A series II 1090 Liquid Chromatograph with a low-volume autosampler/injector (Hewlett-Packard) was used for the HPSEC assay. Peaks eluting from the Zorbax GF-250 column (25 cm × 9.4 mm I.D.; MAC-MOD Analytical, Chadds Ford, PA, USA) were monitored at 280 nm.

Reagents

The molecular mass protein standard solution containing hen egg white lysozyme (molecular mass: 14 400), soybean trypsin inhibitor (21 500), bovine carbonic anhydrase (31 000), hen egg white ovalbumin (45 000), bovine serum albumin (BSA, 66 200), rabbit muscle phosphorylase *b* (97 400) was obtained from Bio-Rad Labs. (No. 161-0304, SDS-PAGE Low Range Molecular Weight Standard, Richmond, CA, USA).

The sample buffer solution, containing 1% SDS (Sigma, St. Louis, MO, USA) in 0.12 *M* Tris–HCl, pH 6.6 (part No. 241525, Beckman), was used to dilute the protein sample. The 2-mercaptoethanol (Sigma) was used to reduce the protein sample.

For the HPSEC assay, a thoroughly de-gassed mobile phase composed of 150 m*M* NaCl (Mallinckrodt, Paris, KY, USA), 25 m*M* NaH_2PO_4 (J.T. Baker, Phillipsburg, NJ, USA) at pH 8.0 and 0.1% SDS was pumped at a flow-rate of approximately 1.0 ml/min.

Preparation of protein samples

Molecular mass protein standard. A 5-μl volume of the molecular mass protein standard solution was diluted in 40 μl of the sample buffer solution. After through mixing, 2 μl of 2-mercaptoethanol was added and the mixture was heated at 80°C for 5 min.

rbSt. Samples of rbSt used in this study were manufactured by The Upjohn Company (Kalamazoo, MI, USA). Approximately 1 mg of rbSt samples was dissolved in 100 μl of a weak ammonium solution [10 μl of NH_4OH (Mallinckrodt) per 10 ml of water (Burdick & Jackson, Muskegon, MI, USA)] and diluted in 900 μl of the sample buffer solution just prior to the

analysis. An aliquot of 20 μl of the dimer-enriched sample (*ca.* 4.5 mg protein per ml) was dissolved in 10 μl of the weak ammonium solution. After through mixing, the solution was diluted in 70 μl of the sample buffer just prior to the analysis.

To reduce and denature the protein, 2 μl of 2-mercaptoethanol was pipetted into a solution with *ca.* 1 mg protein concentration per ml. After through mixing, the sample was heated at 80°C for 5 min.

For a standard addition/recovery study, several different quantities of the dimer-enriched sample was pipetted into the monomer-enriched sample to prepare seven solutions containing the dimer ranging from 5 to 30%.

For the HPSEC analysis, approximately 10 mg of rbSt per ml of the weak ammonium hydroxide solution containing 0.1% SDS was prepared. About 100 μl of the sample solution was injected onto the size-exclusion column.

RESULTS AND DISCUSSION

SDS gel-filled capillary electrophoresis

The SDS non-acrylamide gel-filled capillary electrophoresis separates SDS–protein complexes based on their apparent molecular mass [9–11] similar in the principal as that of the conventional SDS-PAGE technology [12]. A typical HPCE electropherogram indicating separation of the molecular mass protein standards is shown in Fig. 1-I. A linear relationship ($r^2 = 0.998$) existed when the molecular mass of protein stan-

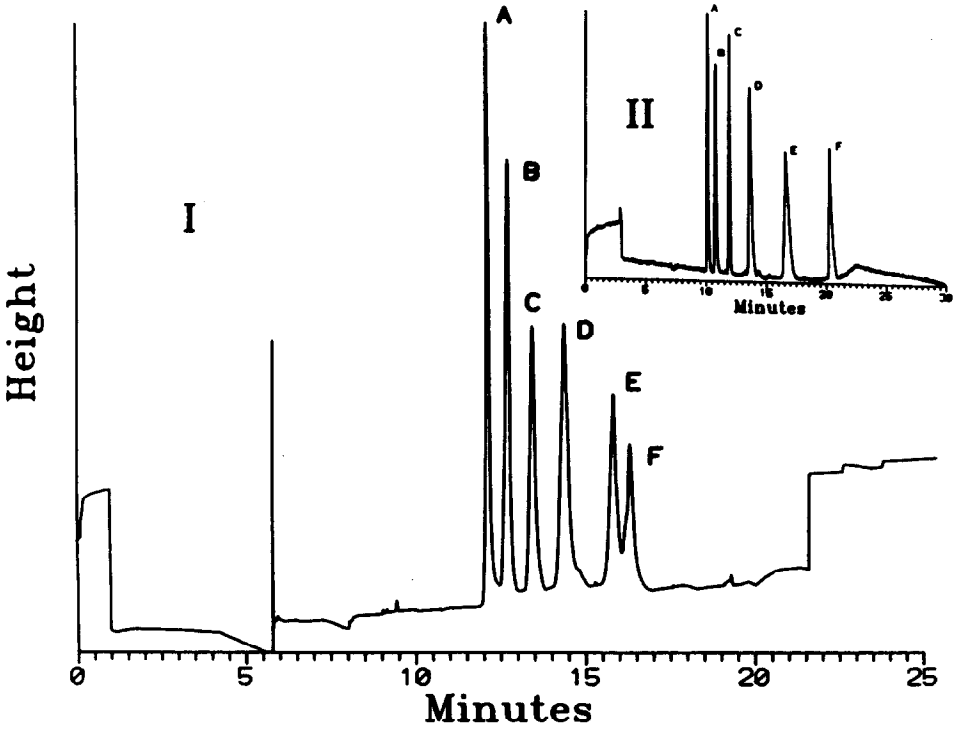

Fig. 1. SDS gel-filled capillary electrophoretic separation of the molecular mass protein reference standards as monitored at 214 nm. Peak migration order, A = lysozyme (M_r 14 400); B = trypsin inhibitor (21 500); C = carbonic anhydrase (31 000); D = ovalbumin (45 000); E = BSA (66 400); and F = phosphorylase *b* (97 400). (I) SDS non-acrylamide, polymer gel-filled capillary column [8,9]. Conditions, −300 V/cm (24 μA); column temperature: 20°C; effective peak migration distance: 40 cm; coated capillary: 100 μm I.D.; running buffer: SDS non-acrylamide, polymer gel solution. (II) SDS polyacrylamide gel-filled capillary column [7]. Conditions, −83 V/cm (12 μA); column temperature: 25°C; peak migration distance: 7 cm; coated capillary filled with 5% T [T = (5 g acrylamide + 0.13 g N,N′-methylenebisacrylamide)/100 ml solution] polyacrylamide gel: 75 μm I.D.; running buffer: 375 m*M* Tris, pH 8.8, 0.1% SDS, 2.5 *M* ethylene glycol.

dards, ranging from approximately 14 000 to 97 000, was plotted against their electrophoretic mobilities or peak migration time on a logarithm (log–log) scale. Unlike the excellent peak resolution efficiency of the SDS polyacrylamide gel-filled capillary column (Fig. 1-II) [8], complete separation of BSA (M_r 66 200) from phosphorylase *b* (M_r 97 400) was not attained nor minor components in carbonic anhydrase and ovalbumin resolved by this commercially available SDS non-polyacrylamide gel-filled capillary column (Fig. 1-I).

Beckman uses a proprietary, non-polyacrylamide, hydrophilic, low-viscosity, linear polymer as the gel matrices to obtain size separations [11]. Use of a low-viscosity linear polymer as a sieving matrices has an advantage over a cross-linked polymer in that the low-viscosity polymer can be removed and the column rinsed and re-filled with the fresh polymer for each sample analysis. Thus, the problem of column instability due to accumulation of slow eluting impurities experienced by Tsuji [8] on a cross-linked polyacrylamide gel-filled capillary column has been effectively eliminated.

Precision of the protein peak migration time was determined by repeatedly injecting a rbSt solution at approximately 1 mg protein per ml. Relative standard deviation R.S.D. for the monomer peak migration time is approximately 0.3% (Table I).

TABLE I

PRECISION OF THE SDS GEL-FILLED CAPILLARY ELECTROPHORESIS FOR THE DETERMINATION OF MOLECULAR MASS OF MONOMER IN AN rbSt SAMPLE

Non-reduced rbSt sample was used.

Sample injected	Monomer	
	Peak migration (min)	M_r
1	12.88	21 870
2	12.82	21 300
3	12.82	21 300
4	12.79	21 000
5	12.77	20 800
6	12.75	20 700
7	12.77	20 800
8	12.76	20 700
Average	12.80	21 070
R.S.D. (%)	0.3	1.9

Analysis of rbSt

Baseline resolution of peaks corresponding to the monomer, dimer, trimer, and tetramer of rbSt is typified by the SDS gel-filled capillary electrophoresis of a dimer-enriched sample (Fig. 2). When sensitivity of the detector near the minor, monomer peak region of a typical electropherogram of a rbSt sample (Fig. 3-I) was increased, the sample was shown to contain fragment, dimer, and trimer (Fig. 3-II). The dimer peak was further resolved into four peaks; the molecular masses of which differ by approximately 1000. Approximately M_r 1000 was determined to be the limit of the peak resolution capability of the SDS gel-filled capillary electrophoresis (Fig. 3-II) [8].

Performance of the SDS gel-filled capillary column was examined by use of a pre-production and a production lots of the capillary columns. As shown in Table II, performance of these two

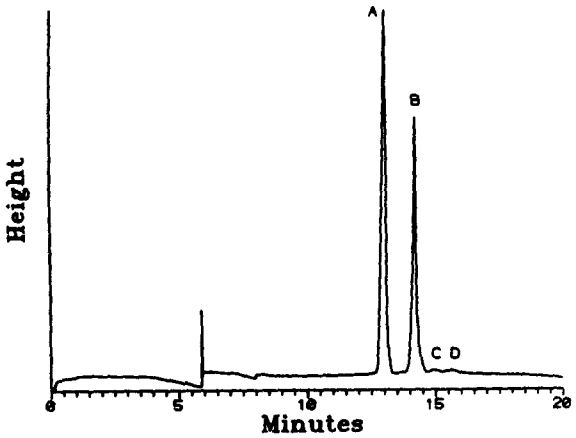

Fig. 2. SDS gel-filled capillary electropherogram of a dimer-enriched rbSt sample indicating a base-line resolution of monomer, dimer, trimer, and tetramer peaks. Conditions, −300 V/cm (24 μA); detector: 214 nm; column temperature: 20°C; migration distance: 40 cm; coated capillary: 100 μm I.D.; running buffer: SDS non-acrylamide, polymer gel solution. Peaks: A = monomer; B = dimer; C = trimer; D = tetramer.

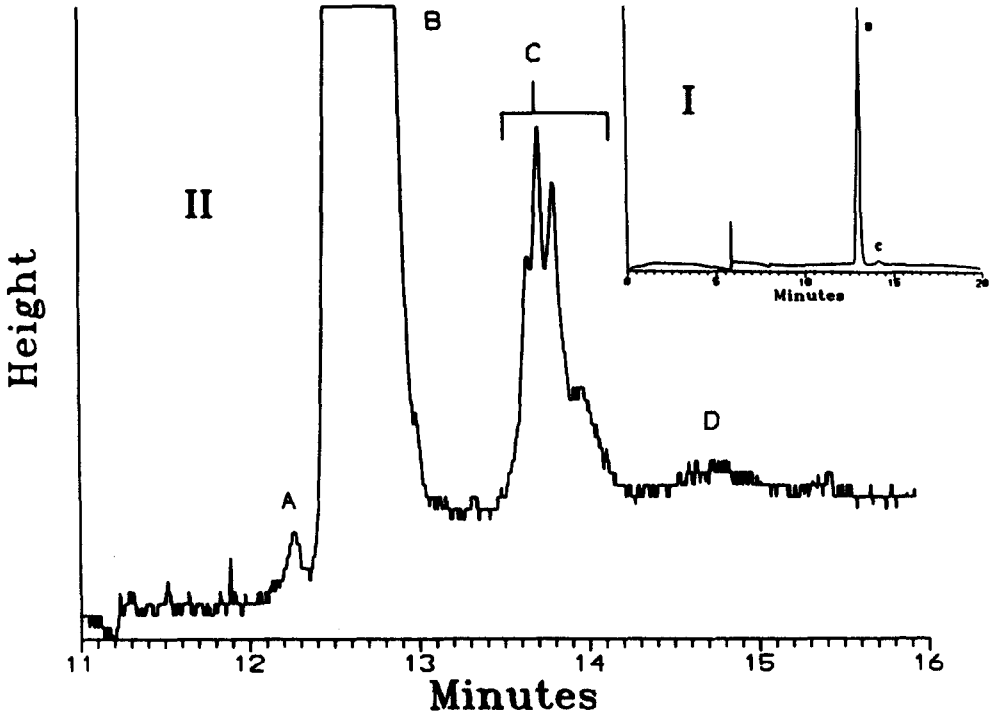

Fig. 3. (I) A typical SDS gel-filled capillary electropherogram of an rbSt sample as monitored at 214 nm. Peak identification, B = monomer; C = dimer. (II) Expanded electropherogram of the rbSt sample indicating resolution of the dimer peak into 4 sub-peaks differing by $M_r \approx 1000$. Peaks: A = fragment; B = monomer; C = dimer; D = trimer. Conditions, −300 V/cm (24 μA); column temperature: 20°C; peak migration distance: 40 cm; coated capillary: 100 μm I.D.; running buffer: SDS non-acrylamide, polymer gel solution.

columns were equivalent as indicated by similarities of the theoretical plate number (28 000 to 30 000 per 40 cm), the R.S.D. of the assay (R.S.D.: 14 to 19%), peak capacity factor (data not shown), and resolution ($R_s > 1$) between the monomer and the dimer peaks.

Molecular mass determination. Using a dimer-enriched sample against the molecular mass protein reference standard, the molecular mass of the components in rbSt (Fig. 3-II) were calculated. The molecular mass of the fragment, monomer, dimer, trimer, and oligomer were calculated to be approximately 15 000–16 000, 20 000–21 000, 46 000–47 000 and >62 000, respectively, with R.S.D.s of 2 to 3% for the monomer and the dimer determination. The observed masses determined by the HPSEC for the monomer, dimer and oligomer were 19 200, 43 500 and >68 000, and 20 400, 41 800 and >126 000 by the conventional SDS-PAGE technique, respectively. The theoretical, average mass of the rbSt is 21 812. The protein reference standard solution used to construct the calibration curve for the determination of the molecular mass was reduced and denatured but not the rbSt samples (refer to the following paragraph for the reason of not reducing the rbSt sample). Discrepancy of the observed molecular mass of the monomer from its theoretical value may be due to the non-reduced state of the rbSt molecule. Unless the protein is completely reduced to disrupt its secondary structure, the protein cannot unfold to full length and tend to run faster than expected resulting in lower observed molecular mass in an SDS-gel system [1]. For a precise measurement of the molecular mass of the protein, a separate technique, such as a capillary electrophoresis interfaced with an electrospray mass spectrometer [13], must be utilized.

When reduced and denatured, the majority of

TABLE II

THEORETICAL PLATE NUMBER OF THE SDS GEL-FILLED CAPILLARY ELECTROPHORESIS FOR THE DETERMINATION OF rbSt

Theoretical plate (N) of the monomer peak was calculated and expressed as N per the effective column length of 40 cm. About 60 nl of a rbSt sample solution at 1 mg protein per ml was injected onto the SDS gel-filled capillary column, 47 cm × 100 μm I.D. Migrating peaks were monitored at 214 nm; column temperature, 20°C; −300 V/cm.

Sample injection	Theoretical plates per 40 cm column	
	Pre-production column	Production column
1	23 100	29 700
2	22 900	37 100
3	22 900	26 600
4	25 000	33 000
5	31 100	26 500
6	34 900	33 000
7	31 100	24 000
8	34 800	29 500
Average	28 200	29 900
R.S.D. (%)	18	14

TABLE III

PRECISION OF THE SDS GEL-FILLED CAPILLARY ELECTROPHORESIS FOR THE DETERMINATION OF MONOMER AND DIMER CONTENTS IN AN rbSt SAMPLE

About 60 nl of a rbSt sample at 1 mg protein per ml was repeatedly injected onto the SDS gel-filled capillary column, 100 μm I.D. with an effective length of 40 cm. Migrating proteins were monitored at 214 nm; column temperature, 20°C; −300 V/cm.

Sample injected	Monomer		Dimer	
	Area	%	Area	%
1	3 276 000	96.1	134 100	3.9
2	3 375 000	96.3	129 900	3.7
3	3 315 000	96.4	123 700	3.6
4	3 286 000	96.0	135 800	4.0
5	3 197 000	96.2	125 100	3.8
6	3 202 000	96.4	119 900	3.6
7	3 237 000	96.3	124 900	3.7
Average	3 270 000	96.3	127 600	3.7
R.S.D. (%)	1.9	0.2	4.6	4.1

the dimer in the dimer enriched sample reverted to monomer indicating that the dimer is formed by an intra-molecular disulfide bonding during the protein re-folding processes. However, approximately 15–16% dimer remained after the treatment suggesting an existence of a dimer species with another bonding mechanism [14].

Peak area measurement. Precision for the determination of rbSt was examined by repeated injection, eight times, of a rbSt sample solution at the protein concentration of approximately 1 mg/ml. The R.S.D.s for measurement of the area under the monomer and the dimer peaks were approximately 2 and 5%, respectively (Table III). Since the sample was injected under a nitrogen pressure into the column filled with a highly viscous polymer solution, variability in quantitative injection may be an inherent nature of the SDS gel-filled capillary electrophoretic assay operation. Variability in the sample injection did not affect precision for the determination of components when expressed in the relative percentage, however.

When a sample with the protein concentration of over 2 mg/ml was analyzed, the gel-filled capillary column severely over-loaded resulting in a skewed peak shape with a significant loss of theoretical plate, 6300 from 29 000 per 40 cm. Theoretical plate number of the monomer peak nearly doubled, from 29 000 to *ca.* 60 000 per 40 cm, when a sample with the protein concentration of less than 0.5 mg/ml was analyzed. However, variability (R.S.D.) of the dimer peak area increased significantly.

Relative response factor. The calibration curve for the monomer peak area is linear ($r^2 = 0.998$) with the linear equation of $y = 6.26x + 1.01$. The calibration curve for the dimer was also linear ($r^2 = 0.999$) with the linear equation of $y = 7.07x - 1.03$. From the ratio of the slopes of the monomer and the dimer curves the relative response factor for the dimer peak was determined to be 1.1. The rbSt sample used contained too small quantities of fragment, trimer, and

tetramer to effectively obtain their relative response factor.

In order to calculate the relative response factors for the dimer, trimer, and tetramer, the dimer-enriched sample solution, ranging from 2 to 0.14 mg protein per ml was injected onto the column. From the regression analysis the relative response factors for the dimer, trimer, and tetramer were calculated as 1.0, 1.0, and 1.2, respectively. Differences in the relative response factor for the dimer between the two studies, 1.1 and 1.0, are within the assay error.

Sensitivity of the SDS gel-filled capillary electrophoresis for detection of the monomer and the dimer is approximately 5 fmol, respectively.

Recovery study. Various amounts of the dimer-enriched sample were added to the monomer-enriched sample creating the solution with the dimer contents ranging from 5 to 30%. These solutions were analyzed by the SDS gel-filled capillary electrophoresis. Calibration curves for recovery of the two components from the preparation were linear ($r^2 = 0.993$ and $r^2 = 0.994$) with the linear regression equations of $y = 1.01x + 0.334$ and $y = 0.998x + 0.209$ for the monomer and the dimer of rbSt, respectively. The slopes of these regression equations were not significantly different from one and the y-intercepts were not significantly different from zero.

Assay precision. Precision of the SDS gel-filled capillary electrophoresis for the determination of isoforms and fragments was examined using both a pre-production and a production lots of the coated capillary column and the non-acrylamide, polymer gel solution. Composition of monomer, dimer, trimer, and fragment contents in a rbSt sample determined by use of the production lot of the coated capillary column were 96, 4, <1, and <1%. R.S.D.s or the determination of monomer and the dimer were 0.1–0.2 and 4%, respectively (Table III). Performance of the pre-production lot of a coated capillary column was similar (data not shown).

SDS gel-filled capillary electrophoresis vs. HPSEC. Table IV presents data obtained by use of the SDS gel-filled capillary electrophoresis and the HPSEC methods for the analysis of rbSt. Percentage of isoforms and fragments in rbSt samples examined by the SDS gel-filled capillary electrophoresis correlated well with those of the HPSEC method. The values determined by the HPSEC were well within the inter-day variability of the SDS gel-filled capillary electrophoresis method.

TABLE IV

COMPARATIVE ANALYSIS OF rbSt SAMPLES BY THE SDS GEL-FILLED CAPILLARY ELECTROPHORESIS (HPCE) AND THE HIGH-PERFORMANCE SIZE-EXCLUSION CHROMATOGRAPHY (HPSEC)

rbSt			Composition (%)			
			Fragments	Monomer	Dimer	Trimer
Sample 1	HPCE	day 1	<1	94.6	4.1	1.1
		day 2	<1	95.8	3.7	<1
	HPSEC	day 1	<1	96	4.2	<1
		day 2	<1	95.6	4.0	<1
Sample 2	HPCE		<1	95.9	2.8	1
	HPSEC		<1	98	2.3	<1
Sample 3	HPCE	day 1	<1	96.7	2.1	1.2
		day 2	<1	97.5	2.1	<1
	HPSEC		<1	98	1.7	<1
Sample 4	HPCE		<1	97.0	2.1	<1
	HPSEC		<1	98	1.9	<1

Ruggedness

Ruggedness of the SDS gel-filled capillary electrophoresis system was evaluated during the course of the assay operation. In order to examine reliability of the coated capillary column, both a pre-production and a production lots of the columns were examined. Performance of both of these columns was equivalent in terms of the theoretical plate number and precision of the assay (refer to Table II). As shown in Fig. 4, peak migration time of the molecular mass protein standards remained relatively constant for approximately 140 sample injections. Stability of the peak migration time may be due to the use of new gel matrices for each assay operation. The peak area also remained relatively constant throughout the experiment.

Theoretical plate of the protein standards gradually decreased during the course of the sample analysis (data not shown). Decrease of the theoretical plate number may be caused by the protein slowly coating/accumulating on the surface of the column, even though the column was rinsed with 1 *M* HCl after each assay operation. The peak resolution remained adequate for the analysis of rbSt, however.

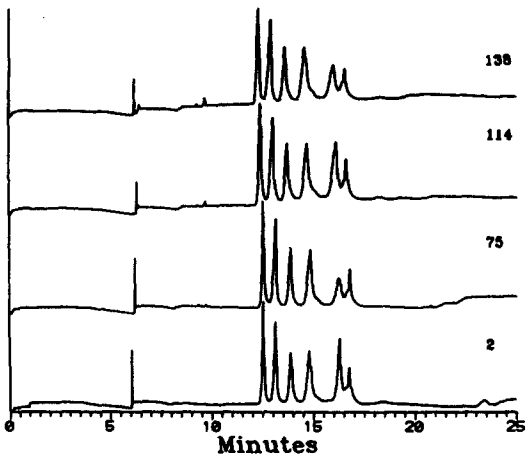

Fig. 4. Composite electropherograms of the molecular mass protein reference standard solution analyzed by the SDS gel-filled capillary electrophoresis indicating ruggedness of the coated capillary column. Number on each electropherogram indicates the number of samples injected on the column. Peak migration order: lysozyme, trypsin inhibitor, carbonic anhydrase, ovalbumin, BSA and phosphorylase *b*.

Because of accumulation of slow eluting compounds, the custom made SDS polyacrylamide gel-filled capillary column of Tsuji [8] requires periodic back-washing. During the column back-washing operation by reversal of the polarity, bubbles frequently formed in the capillary making the column useless. This limits the life of the custom made column life to 20–40 sample injections.

CONCLUSIONS

Peak resolution capability of the commercially available SDS non-polyacrylamide gel-filled capillary column is less than the custom-made SDS polyacrylamide gel-filled capillary column. Conventionally, linear range for the determination of the molecular mass may be extended by modifying the composition and the degree of cross-linking of the polyacrylamide gel matrix [1,8,11]. However, no such provision has yet to be made for the non-polyacrylamide gel-filled capillary column. Nevertheless, the SDS non-polyacrylamide gel-filled capillary column represents significant advantages over the custom-made SDS polyacrylamide gel-filled capillary column for convenience, adequate peak resolution of proteins with M_r values ranging from 10 000 to 50 000, and above all longevity of the column life.

Results obtained in this study indicated that the SDS gel-filled capillary electrophoresis may be a viable alternative to the HPSEC method for the analysis of recombinant proteins.

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