Original

Automatic Protein Separation by Microchip Electrophoresis Using Quartz Chip

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Abstract

MCE using a quartz microchip and an LIF detector was applied to the analysis of proteins. In this study, the experimental conditions were optimized to achieve faster separation and higher resolution of SDS−denatured proteins chemically labeled with fluorescent dye. Proteins (MW 5.7−116. 0 kDa) were completely separated (Rs=1.1−4.4) within 160 s under the optimized conditions using 4.4% linear polyacrylamide at 100 V/cm and the plate number was 0.1−1.4×10^6 plates/m.

Increasing the separation voltage decreased analysis time (70−90 s at 200−240 V/cm) but decreased the resolution relative to the optimal conditions. The detection limit achieved (0.13 ng/μL) is almost 150 times lower than that of the conventional MCE system.

Keywords: Protein / Microchip Electrophoresis / Quartz microchip / Linear polyacrylamide / Sequential analysis

Abbreviations: **BR**, buffer reservoir; **BW**, buffer waste; **HTS**, high−throughput screening; **LIF**, laser−induced fluorescence; **LPA**, linear polyacrylamide; **MCE**, microchip electrophoresis; **MW**, molecular weight; **PMMA**, polymethyl methacrylate; **Rs**, resolution; **SDS−PAGE**, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; **SR**, sample reservoir; **SW**, sample waste

1. Introduction

Electrophoretic separation techniques using microchips have been dramatically improved and several commercial MCE systems have been developed. For example, the Agilent Technologies 2100 Bioanalyzer system is commercially available with a protein separation chip and reagent kit that enables rapid and highly efficient protein separation based on their molecular size [1]. Separation of protein mixtures in ten samples can be achieved in approximately 30 minutes using the disposable 10−sample separation glass microchip. For more numerous samples or automatic separations, however, the chip must be replaced for each set of 10 samples. In contrast, Shimadzu Corporation has developed the MCE−2010 system based on a quartz microchip, which is capable of sequential and automatic analyses [2]. Once the samples are set and programmed, 96 sequential samples can be processed without further handling. Two types of detection methods (UV and LIF) are available in models of MCE 2010 and MCE 2010 LIF, respectively. DNA analysis can be performed using either UV or LIF [3], amino sugar analysis can use LIF [4], and analysis of monosaccharide derivatives can be performed using UV detection [5]. Since the detectability of proteins in UV detection is 10−100 times lower than that in LIF detection, on−line sample preconcentration on single channel [6] or cross channel [7] techniques were developed and applied to higher sensitive detection of low concentration protein mixtures that are not detectable with UV methods. In this report, we describe the optimization of size−based protein separation on

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quartz chip using the MCE−2010 system equipped with LIF detection.

2. Experimental

2.1 Materials and Chemicals

Bovine insulin (5.7 kDa), ribonuclease (13.68 kDa), lysozyme (14.4 kDa), trypsin inhibitor (21.5 kDa), trypsin (23.0 kDa), bovine serum albumin (66.5 kDa), phosphorylase B (97.0 kDa), and β−galactosidase (116.0 kDa) (Bio−Rad Laboratories, Richmond, CA, and Sigma Chemical, St. Louis, MO) were used as standard proteins for separation on the basis of molecular size. Aspartic acid, SDS, and Tris−HCl buffer solution were purchased from Sigma Chemical Co. (St. Louis, MO). Alexa Fluor 488 carboxylic acid succinimidyl ester was acquired from Molecular Probes (Eugene, OR). Acrylamide, ammonium persulfate, and (N,N,N',N')−tetramethylethylenediamine (TEMED) were purchased from Bio−Rad (Hercules, CA, USA). 2−Mercaptoethanol was purchased from Kanto Chemical Co. Inc. (Tokyo, Japan).

2.2 MCE instrumentation

MCE−2010 LIF systems and quartz microchips were obtained from Shimadzu Corporation (Kyoto, Japan). Figure 1 shows a schematic diagram of the MCE−2010 LIF systems. The quartz microchips used had dimensions of 35 mm × 12.5 mm × 1.25 mm, with cross−channels 110 μm in width and 50 μm in depth. The channel length between the sample reservoir (SR) and the sample waste (SW) was 15 mm, while the length between the buffer reservoir (BR) and the buffer waste (BW) was 40.5 mm. MCE−2010 LIF control software (Shimadzu Corporation, Kyoto, Japan) was used. The mobility of the proteins was measured at 21 mm from the center of the intersection. LIF detection was performed at 530 nm.

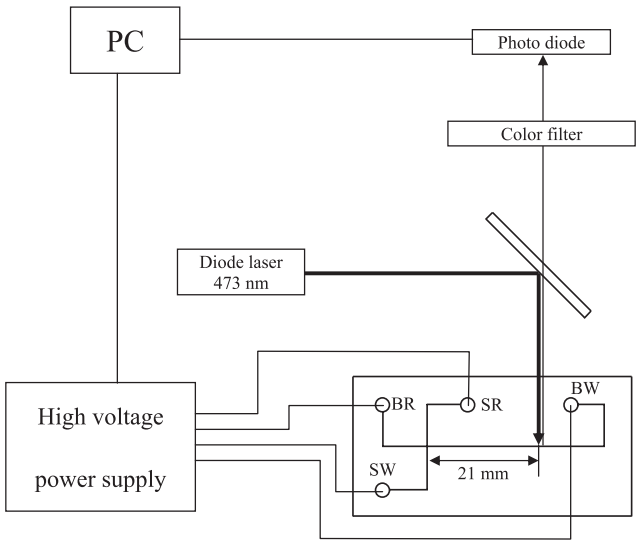

Figure 1. Schematic diagram of MCE−2010 LIF.

2.3 Preparation of the separation buffer

The solution of linear polyacrylamide (LPA) used to fill the quartz microchannel was prepared as follows. 0.4−3.2 g of acrylamide (1.0−8.0% w/v final concentration) and 0.038 g of ammonium persulfate were dissolved in 10 mL of water; this solution was mixed with 20 mL of the SDS buffer and the volume was adjusted to 40 mL (the final concentration of the SDS buffer was 0.05 M Tris, 0.035 M aspartic acid, 0.1% w/v SDS, pH 8.0) ; immediately 6−60 μL of TEMED were added to start polymerization. The solution was degassed by an ultrasonic bath and left overnight at 30 ℃to accomplish polymerization.

2.4 Preparation of the protein sample solution

The protein solution containing the eight molecular weight standards was prepared as follows. The proteins were labeled with a fluorescence dye, Alexa Fluor 488 (the wavelengths of absorption and fluorescence maxima are 494 nm and 519 nm, respectively), for measurement using MCE−2010 LIF. Free dye in the solutions of labeled proteins was removed using gel filtration. Eight protein solutions, which total volume was 16 μL, were mixed together with 16 μL of sample buffer composed of 100 mM Tris−HCl buffer (pH 8) and 2% w/v SDS. 1 μL of 2−mercaptoethanol was added to the solution, which was then denatured at 99℃ for five minutes. The sample solution was diluted to one−tenth with buffer solution (50 mM Tris−HCl, 35 mM aspartic acid, pH 8) after denaturation. The final concentrations of the eight proteins in the sample solution were 8.8−5.4 ng/μL. Protein separation was performed in a separation buffer solution (1.0−8.0% LPA, 0.1% w/v SDS, 50 mM Tris−HCl, 35 mM aspartic acid, pH 8) on the quartz microchip.

2.5 Separation of proteins

Loading of the protein solution into the microchannel was performed using a simple pinched injection method as shown in Figure 2A. Figure 2A(b) and 2A(c) show the pinched sample loading step. A 350 V (340 V/cm) potential was applied to SW for sample loading while SR, BR and BW were grounded (conditions before 0 s in Fig. 2B). Figure 2A(d) shows the sample injection and separation step. The voltage program for protein separation was 220 V (70 V/cm), 220 V (70 V/cm), and 500 V (100 V/cm) for SR, SW, and BW, respectively, while BR was grounded (Fig. 2B). Optimized voltage programs were summarized in Table 1.

2.6 Safety Considerations

2−mercaptoethanol and monomer acrylamide are toxic, therefore gloves should be worn when the solution is handled.

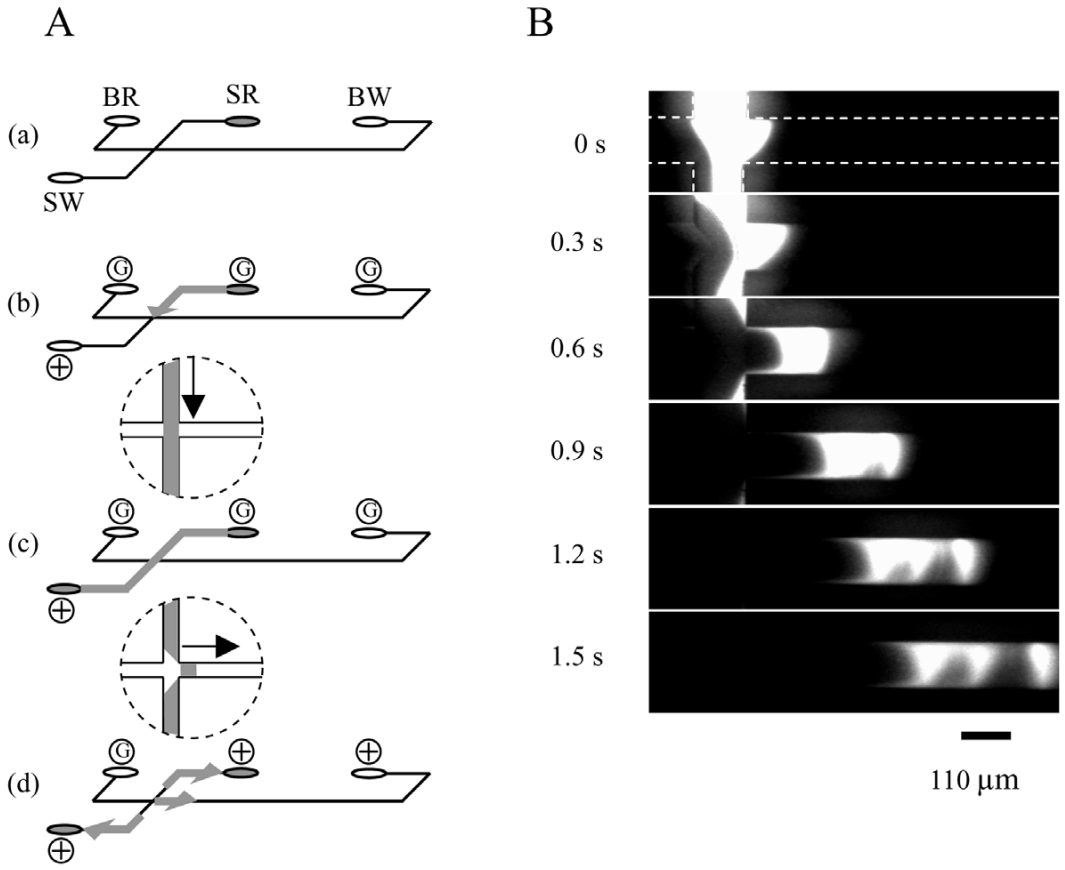

Figure 2. Images of the channel of quartz microchip used in the protein separation experiment.
(A) Schematic diagrams of sample injection and separation procedures. (B) Fluorescence images of sample loading in the channel. Channels are 110 μm wide and 50 μm deep. Injection channel length is 15 mm, and separation channel length is 40.5 mm. The proteins were labeled with Alexa Fluor 488. Conditions in the separation buffer (5.0% LPA, 0.1% w/v SDS, 50 mM Tris–HCl, 35 mM aspartic acid, pH 8.0). In sample loading, a 350 V (340 V/cm) potential was applied to SW for sample loading while SR, BR and BW were grounded. In sample separation, the voltage program was 220 V (70 V/cm), 220 V (70 V/cm), and 500 V (100 V/cm) for SR, SW, and BW, respectively, while BR was grounded.

Table 1. Sample loading and separating voltage application and electric field strengths

		Voltages (V) (Electric field strengths (V/cm))				
		Reservoirs				Time (s)
	Voltage application name[a)]	SR	SW	BR	BW	
Sample loading phase	350–1200 V	0 (160)	350 (340)	0 (160)	0 (30)	60
Sample injection and separation phase	350 V	190 (70)	190 (70)	0 (200)	350 (60)	480
	400 V	200 (70)	200 (70)	0 (220)	400 (80)	480
	450 V	210 (70)	210 (70)	0 (230)	450 (90)	480
	500 V	220 (70)	220 (70)	0 (240)	500 (100)	480
	1000 V	440 (140)	440 (140)	0 (490)	1000 (200)	480
	1200 V	540 (180)	540 (180)	0 (590)	1200 (240)	480

a) The symbols 350 V–1200 V have the same meanings as in Figure 4.

3. Results and Discussion

3.1 Optimization of the concentration of LPA

To perform SDS−protein separations, we first optimize the concentration of LPA. The protein solution, labeled with Alexa Fluor 488 and denatured with SDS as described in Methods, was separated in chips loaded with different LPA concentrations ranging from 1.0%~8.0% as shown in Figure 3. Efficient separation and sensitive detection of proteins were observed by using 4.0−5.0% LPA. Lower LPA concentration (1.0~2.0%) resulted in insufficient resolution and detectability of proteins. Higher LPA concentrations (above 6.0%) decreased the resolution and peak intensity. Generally, higher LPA concentrations can provide higher resolution, but LPA concentrations over 5.0% were difficult to pour into the channel due to high viscosity and were difficult to prepare. Consequently concentrations of 4.0−5.0% LPA appear to be an optimal concentration for the separation buffer.

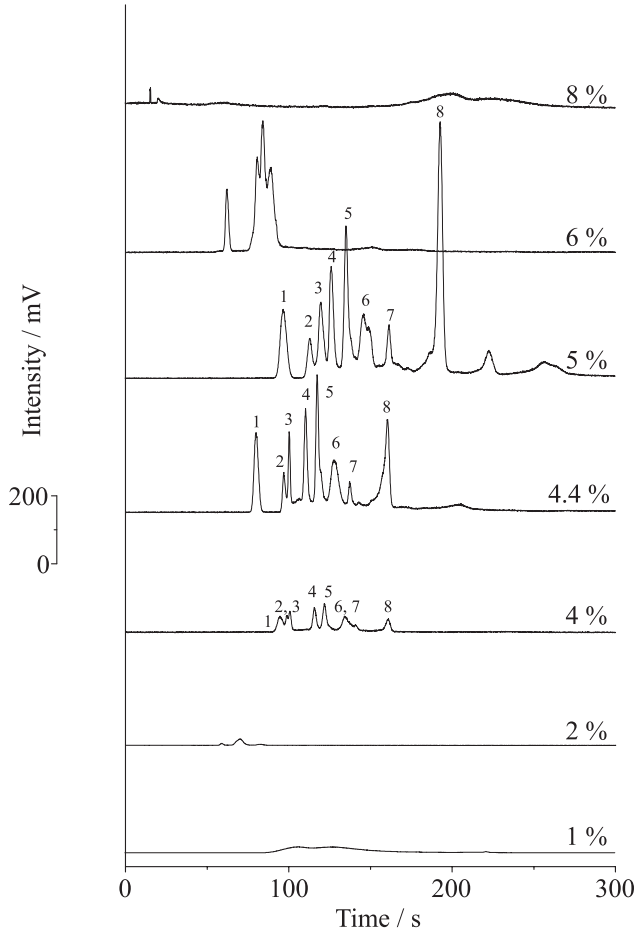

Figure 3. Microchip electropherograms of Alexa Fluor 488 labeled SDS−protein complexes in each concentration LPA (1.0~8.0%) of separation buffer (0.1% w/v SDS, 50 mM Tris−HCl, 35 mM aspartic acid, pH 8.0) on a quartz microchip. The symbols 1, 2, 3, 4, 5, 6, 7, and 8 represent bovine insulin, ribonuclease, lysozyme, trypsin inhibitor, trypsin, bovine serum albumin, phosphorylase B, and β−galactosidase, respectively.

3.2 Optimization of voltage conditions

Generally, higher voltage application can increase the speed of separation, but the resolution will decrease. We examined the different applied voltages for the separation of proteins to realize faster separation as well as higher resolution. Figure 4 shows the electropherograms obtained under various voltage conditions using each LPA (1.0~8.0%) concentrations. As is expected, higher voltage application decreased the migration times. Improvement in separation was not achieved using the concentrations of 1.0, 2.0, 6.0 and 8.0% LPA. Using LPA concentrations of 4.0, 4.4, and 5.0%, higher voltage application decreased the detection intensity and also decreased the resolution because of Joule heat. Efficient separations and higher peak intensity were achieved within 200 s using the LPA concentrations of 4.4 or 5.0% under 500 V (100 V/cm). Peak intensity was lower when the LPA concentrations were high, because the injected sample amount reduced.

3.3 Performance of MCE

We further evaluated the performance of the MCE system, analyzing migration time, peak−width at half−height, plate number, and resolution at 4.0, 4.4, and 5.0% LPA (Table 2). Both of the speed of separation and the resolution at 4.4% LPA were superior to those obtained at the LPA concentration of 4.0 and 5.0%. Thus we concluded that 4.4% LPA and 500 V (100 V/cm) applications are the optimal conditions of MCE−2010 LIF.

In this way, we achieved size−based protein separation up to 116 kDa within 160 s by MCE−2010 LIF using the Shimadzu quartz chip. As for this method, because the sample has been pre−labeled with fluorescent dye, SDS−dilution procedure, which is essential for Agilent 2100 bioanalyer and required to fabricate some complicated microchannel on a chip, is unnecessary. The procedure for separation buffer and sample loading as well as the microchannel design is simpler than the method utilizing the Agilent technologies system as it does not require an SDS−dilution procedure. The estimated detection limit was 0.13 ng/μL and sufficient detection intensity was achieved for 8.8 ng/μL (in comparison, the detection limit of the 2100 Bioanalyzer system is 20 ng/μL), and was achieved without requiring concentration steps such as the electrokinetic supercharging preconcentration method described by Xu *et al.* [6, 7] or special injection methods as described by Tabuchi *et al.* [8]. Therefore, if these methods are applied to the optimized technique described in this report, further sensitivity enhancement could be expected using the MCE−2010 LIF system.

4. Conclusions

We have optimized a size−based protein separation utilizing

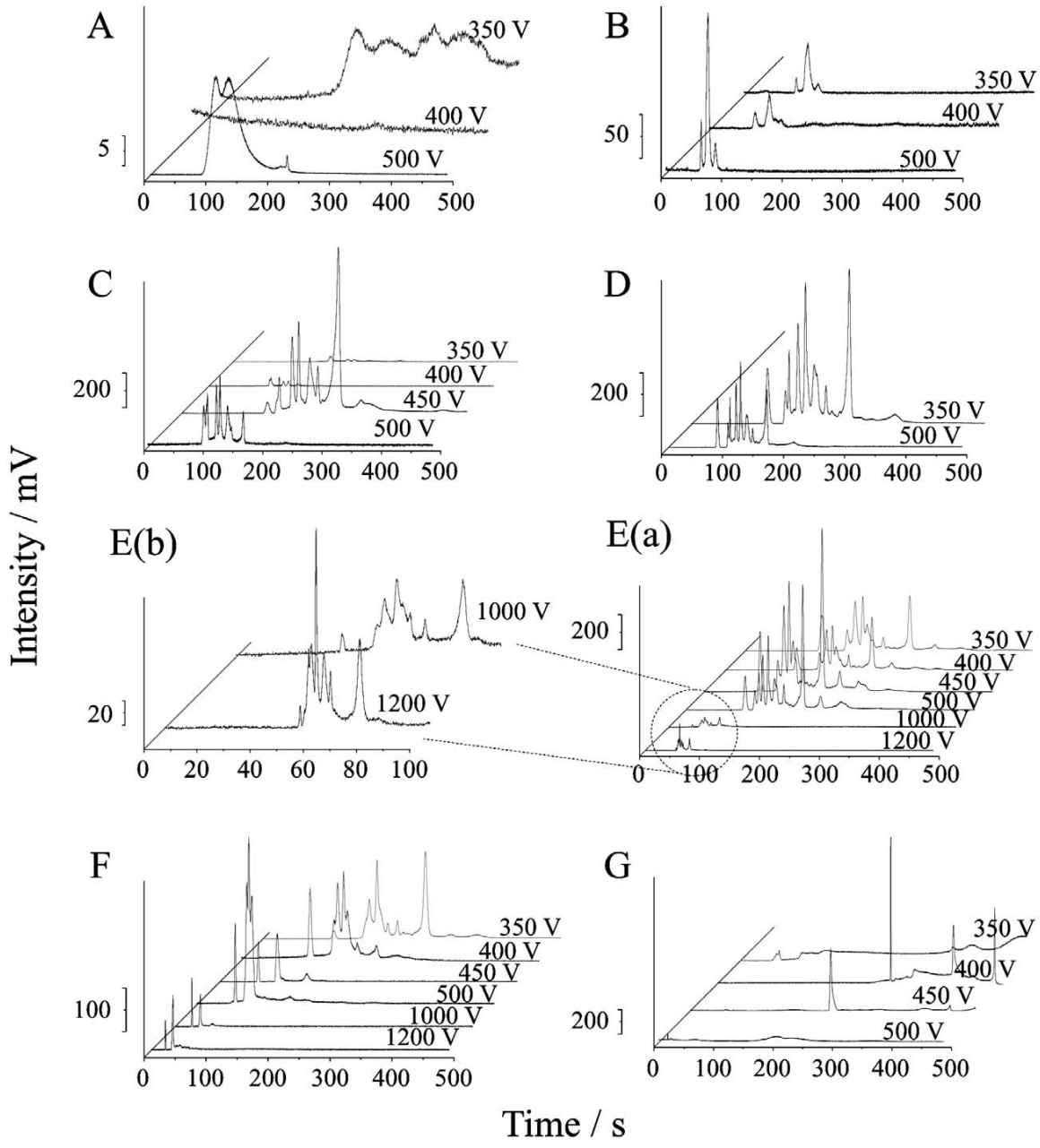

Figure 4. Microchip electropherograms of Alexa Fluor 488 labeled SDS−protein complexes under each voltage application. The voltage conditions are identified on each electropherogram. (A) 1.0%, (B) 2.0%, (C) 4.0%, (D) 4.4%, (E(a), (b)) 5.0%, (F) 6.0%, and (G) 8.0%. 350 V−1200 V were voltage application name in Table 1.

the Shimadzu MCE−2010 LIF equipped with a quartz microchip. Optimal separation and detection were achieved using 4.4% LPA and the applied voltage of 500 V (100 V/cm). SDS−protein complexes in the size range of 5.7 kDa to 116.0 kDa were completely separated within 160 s and detected without SDS dilution procedure on the microchip. The sequential and automatic analysis of up to 96 samples is possible using MCE−2010 LIF. This improvement in separation efficiency, detection limit and sample through−put will be extremely useful for applications where small volumes of dilute samples need to be analyzed. Finally, we achieved sequential and automatic analysis of up to 96 samples using the quartz chip in the MCE−2010 LIF system, simplifying low−volume sample analysis required for applications such as proteome research.

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Table 2. Performance of MCE

polymer concentration (%)	marks in Figure 3[a)]	migration time (s)	peak width at half−height (s)	plate number ($\times 10^5$ plates/m)	resolution
5.0	1	96.9	4.6	1.17	(1/2) 2.29
	2	112.9	3.6	2.59	(2/3) 1.11
	3	119.8	3.7	2.77	(3/4) 1.14
	4	126.2	2.9	5.00	(4/5) 1.89
	5	135.2	2.7	6.61	(5/6) 1.22
	6	146.1	7.8	0.93	(6/7) 1.66
	7	161.6	3.2	6.73	(7/8) 5.49
	8	192.9	3.5	8.01	
4.4	1	79.9	3	1.87	(1/2) 3.84
	2	96.9	2.2	5.12	(2/3) 1.08
	3	100.2	1.4	13.50	(3/4) 3.26
	4	110.2	2.2	6.62	(4/5) 1.96
	5	117.2	2	9.06	(5/6) 1.47
	6	127.3	6.1	1.15	(6/7) 1.34
	7	137.2	2.6	7.35	(7/8) 4.43
	8	160.2	3.5	5.53	
4.0	1	94.3	4.7	1.06	(1/2) 0.79
	2	99	2.3	4.89	(2/3) 0.44
	3	100.6	2	6.67	(3/4) 3.73
	4	115.5	2.7	4.83	(4/5) 1.30
	5	121.6	2.8	4.98	(5/6) 1.47
	6	133.9	7	0.97	(6/7) 0.79
	7	140.8	3.2	5.11	(7/8) 3.37
	8	160.9	3.8	4.73	

a) The symbols 1, 2, 3, 4, 5, 6, 7, and 8 have the same meanings as in Figure 3.

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