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Electrophoretic analysis of di- and oligosaccharides derived from glycosaminoglycans on microchip format

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Abstract

Microchip electrophoresis is a powerful tool for fast analysis of nucleic acids and has expanded its applicability to the analysis of various biological materials including proteins and carbohydrates. Glycosaminoglycans have intrinsic negative charges, and are good targets for electrophoretic analysis. In the present paper, we developed a method to analyze oligosaccharides and unsaturated disaccharides derived from some glycosaminoglycans after digestion with specific enzymes followed by derivatization with 2-aminoacrydone (AMAC) by reductive amination. The method described here allowed rapid analysis of oligosaccharides derived from glycosaminoglycans within 150 s with high sensitivity. We show an application of the present technique to the glycosaminoglycan analysis in cultured HeLa cells.

Keywords: Microchip electrophoresis; Capillary electrophoresis; Glycosaminoglycan; Oligosaccharide

1. Introduction

Capillary electrophoresis on microchip format is a new technology that promises fast and sensitive analysis in genome areas [1]. It has a potential to simultaneously analyze hundreds of samples in a matter of minutes or less using multi channels. Electrophoresis on microchip is now applied to the routine analysis of nucleic acids, proteins. Some instruments for microchip electrophoresis are commercially available.

Microchip electrophoresis has been applied to the analysis of some proteins labeled with fluorescein [2], fluorescein isothiocyanate-labeled amino acid enantiomers [3], carbohydrates [4,5] and DNA fragments [6]. Separation performance of microchip electrophoresis is basically similar to that of capillary electrophoresis, but microchip electrophoresis can be performed on a time scale of seconds.

A variety of modern chromatographic techniques such as high-performance liquid chromatography, polyacrylamide gel electrophoresis and capillary electrophoresis have been reported for the analysis of carbohydrates [7]. Capillary electrophoresis has revealed the ability in separation of carbohydrates, although most carbohydrates must be converted to ion forms prior to the analysis. Furthermore, carbohydrates should be labeled with chromophores or fluorophores for sensitive detection [8].

Glycosaminoglycans such as heparin, chondroitin/dermatan sulfate, or keratan sulfate are the constituents of proteoglycans. Hyaluronic acid (hyaluronan) is an important constituent of tissue matrices. Chondroitin sulfate (ChS) and dermatan sulfate (DS), usually categorized as galactosaminoglycans composed of repeating disaccharide units of an uronic acid and an aminosugar are highly charged polymers due to the presence of sulfate groups [9]. Sulfation of hydroxyl and amino groups plays a key role in determination of physiological characteristics of proteoglycans. Altered sulfation patterns are often encountered in pathological state [10]. Hyaluronic acid is a macromolecular anionic polysaccharide, and widely used for medical use. Oligosaccharides derived from hyaluronic acid show some interesting biological activities such as angiogenesis and inhibition of cell apoptosis [11,12].

In the analysis of glycosaminoglycans in biological materials, glycosaminoglycans are collected by a combination of precipitation methods using quaternary ammonium salts such as cetylpyridinium chloride and ethanol [13], and exam-

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ined mainly by two methods. The one is an electrophoretic method using cellulose acetate membrane, and can examine glycosaminoglycans as native state after staining with specific reagents. The other uses specific enzymes such as lyases and hydrolases. Identification and quantitation of the oligosaccharides thus obtained are performed using gel electrophoresis [14], high-performance liquid chromatography [15,16] or capillary electrophoresis [9,17].

In the present report, we propose a rapid method to analyze di- and oligosaccharides derived from glycosaminoglycans using microchip electrophoresis after derivatization with 2-aminoacrydone (AMAC).

2. Materials and methods

2.1. Chemicals

Hyaluronic acid (*Streptococcus zooepidemicus*, HA) was obtained from Nakalai Tesque (Nakagyo-ku, Kyoto, Jpana). Hyaluronidase (sheep testis) was obtained from Roche Diagnostics (Minato-ku, Tokyo, Japan). AMAC was obtained from molecular probes (Eugen, OR). Chondroitin 4- and 6-sulfate and chondroitinase ABC and standard samples of unsaturated disaccharides were from Seikagaku Kogyo (Nihonbashi, Chuo-ku, Tokyo, Japan). Pronase was obtained from Calbiochem (San Diego, CA, USA). Polyethyleneglycol (MW 70,000) was obtained from Wako Pure Chemicals (Dosho-machi, Osaka, Japan). A chemically-modified capillary (DB-1) was purchased from J&W Scientific (Folsom, CA, USA). Other reagents and solvents were of the highest grade commercially available.

2.2. Preparation of oligosaccharides derived from hyaluronic acid (HA) and chondroitin sulfate (ChS)

Oligosaccharides derived from HA (HA oligomers) were prepared by digestion of HA with hyaluronidase. Briefly, HA (*S. zooepidemics*, 100 mg) was dissolved in 150 mM citrate buffer (pH 5.3, 10 ml). Hyaluronidase (sheep testis, 10 mg) was added to the solution, and kept at 37 °C for 24 h. After keeping the mixture in a boiling water bath for 10 min, the mixture was centrifuged and the supernatant was collected. The supernatant (ca. 10 ml) was mixed with 95% ethanol (30 ml) containing 1.3% potassium acetate and 0.27 mM EDTA and kept on an ice bath for 2 h. The precipitate was collected, dried under reduced pressure and used as the mixture of HA oligomers. The mixture of oligosaccharides contained from tetrasaccharide to polymers containing ca. 50 monosaccharides. Oligomers having a defined size were purified by HPLC on an amine-bonded silica column (YMC-PAC PA5) using NaH_2PO_4 as eluent according to the method reported previously [17].

Oligosaccharides derived from chondroitin sulfates (1 mg) were also obtained after digestion with hyaluronidase (sheep testis, 0.5 mg) in the similar manner as above.

2.3. Fluorescent labeling of oligosaccharides with AMAC

Labeling reaction of oligosaccharides with AMAC was performed using the method reported previously [9]. A mixture of oligosaccharides or the standard sample of an oligosaccharide (100 μg) was dissolved in 100 mM AMAC in a mixture (10 μl) of dimethylsulfoxide-acetic acid (17:3, v/v). Into the solution, was added 1 M sodium cyanoborohydride (10 μl) in the same solvent. After keeping the mixture at 90 °C for 30 min, water (500 μl) and chloroform (500 μl) were to the reaction mixture and mixed vigorously by a vortex mixer. After removing the chloroform layer, the aqueous phase was washed with chloroform (500 μl). After evaporation of the aqueous phase, the residue was dissolved in water (100 μl) and a portion was used for microchip electrophoresis.

2.4. Digestion of AMAC-labeled HA 20 mer (HA_{20})

We examined time course of digestion of HA_{20} with hyaluronidase. Preparation of HA_{20} and fluorescent labeling reaction with AMAC were performed as described above. An aqueous solution (10 μl) of AMAC-labeled HA_{20} (20 μg/ml) was mixed with 200 munits of hyaluronidase (10 μl) in 10 mM acetate buffer (pH 5.3), and kept at 37 °C. At specified intervals, the mixture was analyzed by microchip electrophoresis.

2.5. Digestion of chondroitin sulfates with chondroitinase ABC

Chondroitin 4-sulfate, chondroitin 6-sulfate or the equal amount of the mixture (100 μg each) was dissolved in 20 mM Tris–hydrochloric acid buffer (pH 8.0, 100 μl), and an aqueous solution of chondroitinase ABC (100 munits, 4 μl) was added. After incubating the mixture overnight at 37 °C, the mixture was kept in a boiling water bath for 10 min. After cooling, the mixture was evaporated to dryness by a centrifugal evaporator (SpeedVac, Servant). The residue was dissolved in a mixture (10 μl) of dimethylsulfoxide-acetic acid (17:3, v/v), and labeling reactions with AMAC was performed as described above.

2.6. Microchip electrophoresis (ME) of AMAC-labeled oligosaccharides

ME was performed on a Hitachi Microchip electrophoresis apparatus (Type SV1100) with an LED detector. The excitation wavelength of the LED detector is preset at 470 nm by the manufacturer, and a detection filter for 580 nm or longer wavelengths is installed. The chip made of polymethylmetacrylate (PMMA) has a simple cross channel of 100 μm width and 30 μm depth. The distances from the channel intersection to the sample, sample waste, buffer and buffer waste wells are 5.25, 5.25, 5.75, and 37.5 mm, respectively. The effective length for separation is 30 mm.

Buffer solutions were introduced into the microchannels with a syringe. All reservoirs on the microchips were filled with either running buffer or a sample solution prior to analysis. In the sample loading step, 300 V was applied to the sample well, and separation was performed by applying the potential of 750 V (130 V at the sample introduction side).

2.7. Capillary electrophoresis (CE) of AMAC-labeled oligosaccharides

CE was performed on a Beckman P/ACE MDQ Glycoprotein System fitted with an LIF detector, a laser module (3 mW, air-cooled argon laser with $\lambda_{ex} = 488$ nm). Detection was performed using a 520 nm filter. Separation was carried out at 25 °C using a DB-1 capillary (50 μm i.d., 60 cm length) in 100 mM Tris–acetate buffer (pH 7.5) containing 1% polyethyleneglycol. The sample solution was injected by electrokinetic method (15 kV, 10 s). The applied voltage for separation was 25 kV.

2.8. Glycosaminoglycans from HeLa cells

HeLa cells were cultured in DMEM containing 10% newborn calf sera (NCS) under 5% CO_2 atmosphere at 37 °C. The cells were collected with a cell scraper, and washed with phosphate buffered saline (PBS, 1 ml) several times. Cells (10^7 cells) were suspended in 20 mM phosphate buffer (pH 7.0, 1 ml) and incubated for 20 min at room temperature to remove culture medium. The cells were homogenized in the same buffer (1 ml) with a Teflon-glass homogenizer, and centrifuged at 8000 × *g* for 10 min at 4 °C. The supernatant was collected and diluted with 0.5 M Tris–hydrochloric acid buffer (pH 8.0, 1 ml), and the mixture was digested with pronase (2 mg) at 37 °C overnight. After keeping the mixture in a boiling water bath for 10 min, the mixture was centrifuged at 8000 × *g*, and the supernatant was collected. Ninety-five percent ethanol containing 1.3% potassium acetate and 0.27 mM EDTA (6 ml) was added to the mixture, and kept at 0 °C for 2 h. The precipitate (500 μg) was dissolved in 20 mM Tris–HCl buffer (pH 8.0, 100 μl), and incubated with chondroitinase ABC (4 μl) at 37 °C overnight. After keeping the mixture in a boiling water bath for 5 min followed by centrifugation, the supernatant was lyophilized and labeled with AMAC as described above.

3. Results and discussion

3.1. Selection of fluorescent-labeling reagent

Most commercially available equipments for microchip electrophoresis are designed for the analysis of nucleic acids, and fluorescence due to intercalation with ethidium bromide is monitored using an LED light source (Ex = 518 nm; Em = 605 nm). In the present study, we aimed at rapid analysis of oligosaccharides derived from glycosaminoglycans obtained after digestion with enzymes. Therefore, we selected AMAC as the derivatization reagent [17], because AMAC can be easily removed by extraction with chloroform after derivatization by reductive amination in the presence of $NaBH_3CN$ as described in Section 2. Other reagents such as 8-aminopyrene-1,3,6-trisulfonate (APTS) and 3-aminobenzoic acid requires purification of the labeled oligosaccharides by gel chromatography [18,19], and did not show good sensitivity at this wavelength.

3.2. Analysis of oligosaccharides derived from hyaluronic acid, chondroitin sulfate A and chondroitin sulfate C

Fig. 1 shows the separation of oligosaccharides derived from hyaluronic acid, chondroitin sulfate A and C using ME and CE after digestion of each glycosaminoglycan with hyaluronidase (sheep testis).

In ME, larger oligomers of hyaluronic acids were observed earlier, and all oligomer peaks were observed between 100 and 150 s (Fig. 1A(a)). Hexa- and tetra-saccharides derived from chondroitin sulfate A were observed within 100 s (Fig. 1A(b)). Oligosaccharide mixture derived from chondroitin sulfate C was also observed within 100 s. However, CE showed better resolutions for these oligosaccharides, although longer analysis times were required. In the analysis of oligosaccharides derived hyaluronic acid by CE, we easily confirmed larger oligosaccharides composed of 14 monosaccharide units (Fig. 1B(a)). In ME, we employed a chip made of polymethylmetacrylate. Hydrophobic interaction between AMAC and surface of the chip was one of the reasons for worse resolution among each oligosaccharide peaks than that observed in CE. Polyethyleneglycol was also added at 1.0% concentration to prevent non-specific interactions. At this concentration, molecular sieving effect is negligible, and larger oligomers were observed earlier based on charge/molecular sizes.

3.3. Digestion of hyaluronic acid 20 mer (HA_{20}) with hyaluronidase

Hyaluronidase (sheep testis) is an *endo*-type enzyme, and cleaves the glycosidic bonds to form a mixture of oligosaccharides. In detailed examination of an *endo*-type enzyme reaction, we have to analyze the reaction products during the course of enzyme reaction. We digested AMAC-labeled HA_{20} with hyaluronidase, and monitored the course of enzyme reaction. The results are shown in Fig. 2.

After 0.5 h, HA_8, HA_{10} and larger oligomers were clearly observed (Fig. 2(b)). After 1 h, HA_8, HA_{10} and HA_{12} were observed as the major products. Finally, HA_4 was observed abundantly, and HA_6 was also observed as the minor product after 2 h (Fig. 2(e)). These data indicate that hyaluronidase did not cleave HA_8 to HA_6 and HA_2 but produced two molecules of HA_4. These characteristic patterns of enzyme action were in good agreement with those as reported previously [20].

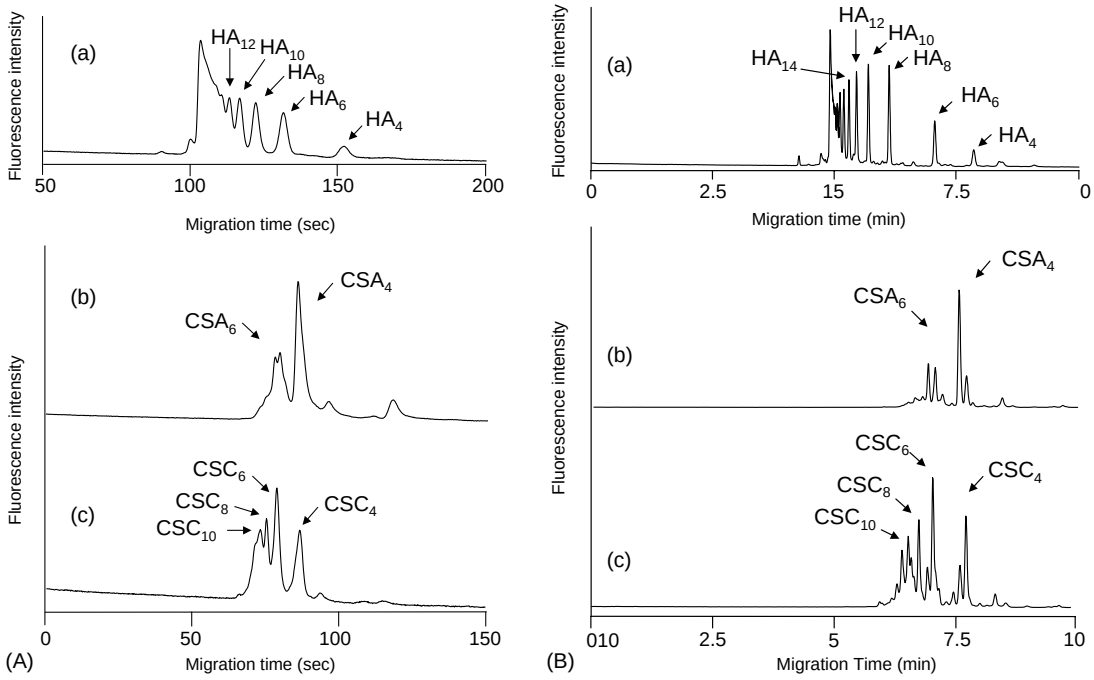

Fig. 1. Microchip electrophoresis (A) and capillary electrophoresis (B) of oligosaccharides derived from hyaluronic acid (a), chondroitin sulfate A (b) and chondroitin sulfate C (c). The samples were obtained by digestion with hyaluronidase followed by labeling with AMAC. The numbers in the figure indicate the degree of polymerization. Analytical conditions for microchip electrophoresis: buffer, 0.1 M Tris–acetate (pH 7.5) containing 1% polyethyleneglycol 70,000; applied voltage for sample injection: 300 V; for analysis: 750 V. Analytical conditions for capillary electrophoresis: capillary, fused silica capillary coated with dimethylpolysiloxane (60 cm, 50 μm i.d., effective length of 50 cm); buffer, 0.1 M Tris–acetate (pH 7.5) containing 1% polyethyleneglycol 70,000; applied voltage, 20 kV; temperature, 25 °C. Fluorescence detection was performed with a 520 nm light filter for emission by irradiating with an argon laser (488 nm). The sample solution was introduced by pressure method (1 psi) for 10 s. The numbers indicate the degree of polymerization.

It should be noticed that the time required for each run was only 3 min, and ME made it possible to monitor the reaction course using a portion of the reaction mixture. This is quite important for monitoring the enzyme reaction course, because other chromatographic and electrophoretic methods require longer operation times.

3.4. Analysis of unsaturated disaccharides derived from chondroitin sulfates

Glycosaminoglycans such as chondroitin/dermatan sulfate and hyaluronic acid are usually converted to unsaturated disaccharides through the action of chondroitin sulfate lyases, and these disaccharides are analyzed by HPLC or CE [21,22]. In the analysis of these unsaturated disaccharides by ME, it is important to find appropriate conditions for resolving ΔDi-4*S* and ΔDi-6*S* (positional isomers of sulfate groups). Fig. 3 shows pH effect on the resolution of ΔDi-4*S* and ΔDi-6*S* derived from chondroitin 4-sulfate and 6-sulfate, respectively.

We examined Tris–acetate buffer and Tris–borate buffer at some pHs that showed good resolution in the CE analysis of sialic acid-containing oligosaccharides [18]. When 0.1 M Tris–acetate buffer was used as the running buffer, resolution was poor as shown in Fig. 3A(a–d). However, both unsaturated disaccharides were successfully resolved in 0.1 M Tris–borate buffer at pH 8.0 (Fig. 3A(f)), although baseline resolution was not achieved. In the carbohydrate analyses, Tris–borate buffer often shows better separation efficiency than Tris–acetate buffer probably due to competitive complex formation between carbohydrates/Tris and borate ions. Low-operation voltage in Tris–borate buffer is also preferable for electrophoresis [8].

Under the conditions described above, we analyzed several commercially available standard samples of unsaturated disaccharides (Fig. 3B). ΔDi-4*S* and ΔDi-6*S* were observed at 95 s and 90 s, respectively, and ΔDi-UA2*S* was observed at 95 s (Fig. 3B(a–c)). ΔDi-diS_B containing two sulfate groups was observed earliest at 70 s. From these results, we could determine all the sulfate-containing unsaturated disaccharides within 100 s.

We digested chondroitin sulfate A, C and D and dermatan sulfate with chondroitinase ABC, and analyzed the unsaturated disaccharides after labeling with AMAC (Fig. 4).

In digestion of chondroitin sulfate A (i.e. chondroitin 4-sulfate), ΔDi-4*S* was observed abundantly, and a small peak of ΔDi-6*S* was also observed (Fig. 4(a)). Chondroitin sulfate C (i.e. chondroitin 6-sulfate) gave ΔDi-6*S* with a small peak of ΔDi-4*S* as shown in Fig. 4(b). In digestion of dermatan sulfate, ΔDi-4*S* was observed at the same migration time with that of ΔDi-4*S* derived from

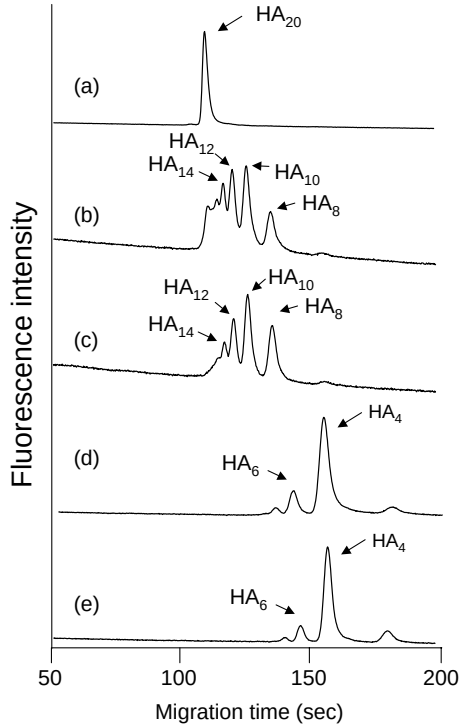

Fig. 2. Digestion of 20 mer of hyaluronic acid (HA_{20}) with hyaluronidase. HA_{20} was incubated with hyaluronidase at 37 °C. After a specified intervals from 0 h to 3 h, a portion was analyzed by ME. Reaction time: (a) 0 h, (b) 0.5 h, (c) 1.0 h, (d) 2.0 h and (e) 3.0 h. Analytical conditions were the same as in Fig. 1. The numbers indicate the degree of polymerization.

chondroitin sulfate A. On the contrary, chondroitin sulfate D gave almost equal amount of ΔDi-6*S* and ΔDi-4*S* at around 100 s. All electropherograms also showed a peak due to unsaturated disaccharide containing disulfate groups (ΔDi-diS_D and ΔDi-diS_B) around 70 s. Especially, chondroitin sulfate D gave a large amount of ΔDi-2*S*6*S* (ΔDi-diS_D) on digestion with chondroitinase ABC (Fig. 4(d)).

3.5. Detection limit and calibration curves

Fig. 5 shows the electropherograms of unsaturated disaccharides derived from chondroitin sulfate A at three different concentration levels.

At 10 ng/ml as the amount of chondroitin sulfate A, we could confirm ΔDi-4*S* at the signal to noise ratio of ca. 3 (Fig. 5(c)).

The apparatus used in the present study is customized for the analysis of nucleic acids, and the peak responses are normalized using specific software. Therefore, we observed linearity for quite narrow range from 10 μg/ml to 100 μg/ml. Thus, we could not show linearity data in the present work. However, it is often necessary to identify which glycosaminoglycans are present in routine clinical analysis, and the present technique will be useful for such purpose.

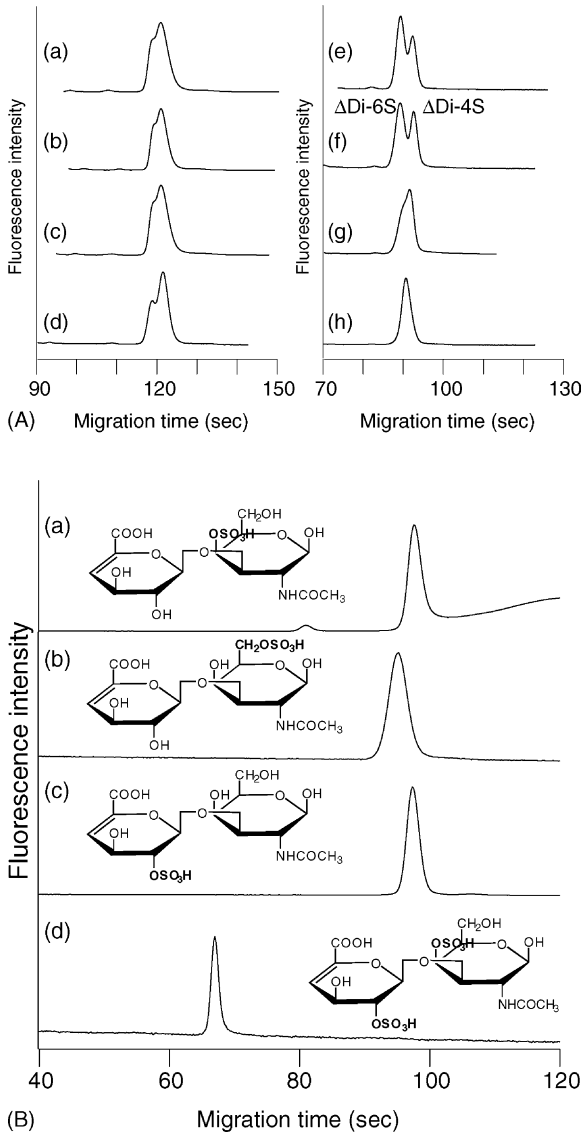

Fig. 3. Optimization for the analysis of unsaturated disaccharides (ΔDi-4*S* and ΔDi-6*S*) derived from chondroitin sulfates and analysis of some unsaturated sulfated disaccharides. (A) Mixture of ΔDi-4*S* and ΔDi-6*S* (unsaturated disaccharides derived from chondroitin sulfate A and C, respectively) was analyzed in 0.1 M Tris–acetate buffer containing 1% polyethyleneglycol 70,000 at pH 6.0 (a), pH 7.0 (b), pH 8.0 (c) and pH 9.0 (d), and also analyzed in 0.1 M Tris–borate buffer containing 1% polyethyleneglycol 70,000 at pH 7.5 (e), pH 8.0 (f), pH 8.5 (g) and pH 9.0 (h). (B) Analysis of ΔDi-4*S* (a), ΔDi-6*S* (b), ΔDi-UA2*S* (c) and ΔDi-diS_B (ΔDi-di2*S*4*S*) (d). Analytical conditions for Fig. 3B: running buffer, 0.1 M Tris–borate buffer containing 1% polyethyleneglycol 70,000 at pH 8.0.

3.6. Application

We applied the present method to the analysis of glycosaminoglycans derived from HeLa cells. The results are shown in Fig. 6.

Proteoglycan fractions derived from HeLa cells gave six peaks after digestion with chondroitinase ABC followed by labeling with AMAC. By comparison with standard samples

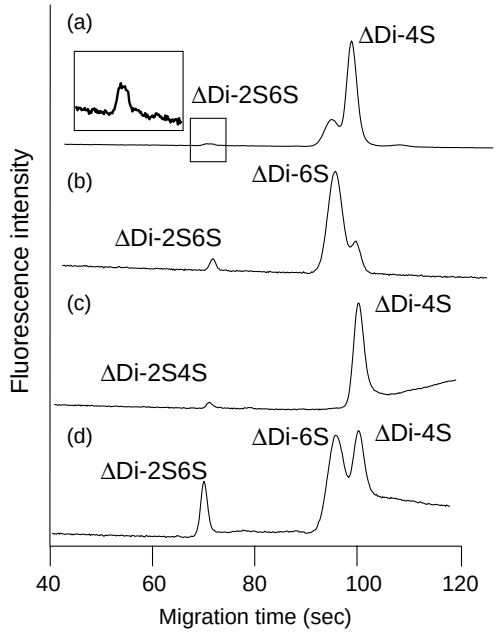

Fig. 4. Analysis of unsaturated disaccharides after digestion of chondroitin sulfates followed by labeling with AMAC. (a) Chondroitin sulfate A, (b) chondroitin sulfate C, (c) dermatan sulfate and (d) chondroitin sulfate D. Analytical conditions were the same as described in Fig. 3B.

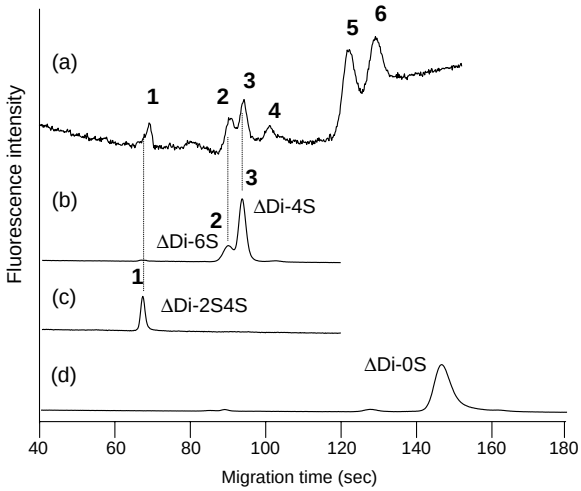

Fig. 6. Analysis of unsaturated disaccharides derived from glycosaminoglycan fractions obtained from HeLa cells. Analytical conditions: buffer; 0.1 M Tris–borate/1% polyethyleneglycol 70,000 (pH 8.0), applied voltage; for sample injection: 300 V; for analysis: 750 V. (a) Unsaturated disaccharides derived from HeLa cells digested with chondroitinase ABC. (b) Unsaturated disaccharides derived from chondroitin sulfate A (whale cartilage). (c) A commercial sample of ΔDi-diS_B (ΔDi-di2*S*4*S*). (d) A commercial sample of ΔDi-0*S*. The peak of ΔDi-HA was observed at the later migration time than ΔDi-0*S* (date not shown).

of unsaturated disaccharides, we identified the earlier observed three peaks (1, 2 and 3). Peak 1 was due to ΔDi-di*S*, and peaks 2 and 3 were due to ΔDi-6*S* and ΔDi-4*S*, respectively. These data indicated the presence of chondroitin sulfate A, and C and dermatan sulfate in HeLa cells. We compared three peaks (4, 5 and 6) with those of ΔDi-0*S* and ΔDi-HA. ΔDi-HA was observed at later migration times and not included in the figure. We found that these peaks were not due to ΔDi-0*S* and ΔDi-HA, and further studies are required.

From these results, microchip electrophoresis is a powerful tool for fast examination of glycosaminoglycans in cultured cells (within 150 s), and will be useful for the studies on cell cultures and tissue matrices.

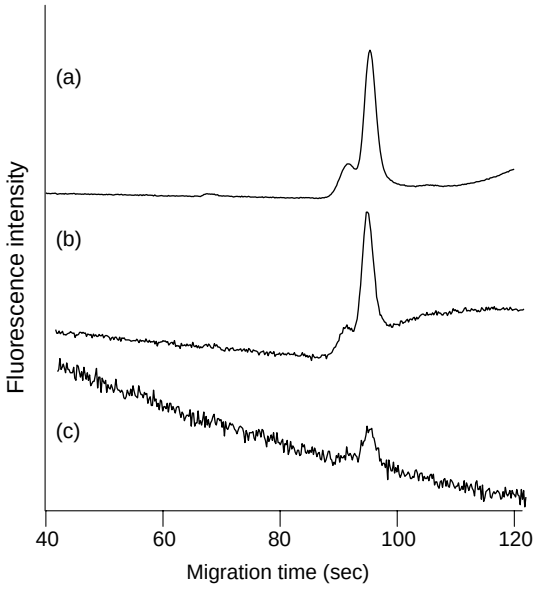

Fig. 5. Analysis of ΔDi-4*S* derived from chondroitin sulfate A at (a) 1.0 μg/ml, (b) 100 ng/ml and (c) 10 ng/ml. Analytical conditions were the same as in Fig. 3B.

4. Conclusion

Microchip electrophoresis has been used for the analysis of nucleic acids, and the rapidness in the analysis will be useful to clinical analysis. Furthermore, fabrication of the apparatus with multichannel mode will allow the analysis of many samples at a time. In the present paper, we digested some glycosaminoglycan samples with hyaluronidase (sheep testis) and chondroitinase ABC, and the di- and oligosaccharides were labeled with AMAC in the presence of sodium cyanoborohydride. The labeling method using AMAC is quite easy, because the excess amount of the reagent can be removed by extraction with chloroform. Unfortunately, the wavelength of AMAC derivative has the excitation maximum at 425 nm, and is different from that of LED installed in the apparatus.

All unsaturated disaccharides labeled with AMAC were observed within 150 s. ΔDi-4*S* was detected at 10 ng/ml with signal to noise ratio of 3. If more appropriate light source is available, we will be able to detect the enzyme reaction products at higher sensitivity.

We applied the present method to the analysis of the digestion course of HA_{20} with hyaluronidase, and showed that

the rapidness is especially useful for the assay of *endo*-type enzyme such as hyaluronidase.

In conclusion, microchip electrophoresis will be a promising alternative to HPLC or CE in the analysis of unsaturated disaccharides derived from glycosaminoglycans.

References

[1] V. Dolnik, S. Liu, S. Jovanovich, Electrophoresis 21 (2000) 41–54.
[2] N. Chiem, D.J. Harrison, Anal. Chem. 69 (1997) 373–378.
[3] I. Rodriguez, L.J. Jin, S.F. Li, Electrophoresis 21 (2000) 211–219.
[4] F.Q. Dang, L.H. Zhang, H. Higiwara, Y. Mishina, Y. Baba, Electrophoresis 24 (2003) 714–721.
[5] S. Suzuki, S. Honda, Electrophoresis 24 (2003) 3577–3582.
[6] J. Khandurina, T.E. McKnight, S.C. Jacobson, L.C. Waters, R.S. Foote, J.M. Ramsey, Anal. Chem. 72 (2000) 2995–3000.
[7] K. Kakehi, S. Honda, J. Chromatogr. A 720 (1996) 377–393.
[8] K. Kakehi, M. Kinoshita, M. Nakano, Biomed. Chromatogr. 16 (2002) 103–115.
[9] F. Lamari, A. Theocharis, A. Hjerpe, N.K. Karamanos, J. Chromatogr. B Biomed. Sci. Appl. 730 (1999) 129–133.
[10] L. Kjellen, U. Lindahl, Ann. Rev. Biochem. 60 (1991) 443–475.
[11] H. Xu, T. Ito, A. Tawada, H. Maeda, H. Yamanokuchi, K. Isahara, K. Yoshida, Y. Uchiyama, A. Asari, J. Biol. Chem. 277 (2002) 17308–17314.
[12] D.C. West, I.N. Hampson, F. Arnold, S. Kumar, Science 228 (1985) 1324–1326.
[13] K. Kakehi, Y. Maeya, Y. Miki, Y. Oda, S. Hayase, Anal. Biochem. 252 (1997) 56–61.
[14] M. Lyon, J.T. Gallagher, Anal. Biochem. 185 (1990) 63–70.
[15] N.K. Karamanos, S. Axelsson, P. Vanky, G.N. Tzanakakis, A. Hjerpe, J. Chromatogr. A 696 (1995) 295–305.
[16] A. Kinoshita, K. Sugahara, Anal. Biochem. 269 (1999) 367–378.
[17] H. Kitagawa, A. Kinoshita, K. Sugahara, Anal. Biochem. 232 (1995) 114–121.
[18] K. Nakajima, Y. Oda, M. Kinoshita, K. Kakehi, J. Proteome Res. 2 (2003) 81–88.
[19] K. Kakehi, T. Funakubo, S. Suzuki, Y. Oda, Y. Kitada, J. Chromatogr. A 863 (1999) 205–218.
[20] M. Kinoshita, A. Okino, Y. Oda, K. Kakehi, Electrophoresis 22 (2001) 3458–3465.
[21] S. Sakai, J. Onose, H. Nakamura, H. Toyoda, T. Toida, T. Imanari, R.J. Linhardt, Anal. Biochem. 302 (2002) 169–174.
[22] M. Koketsu, R.J. Linhardt, Anal. Biochem. 283 (2000) 136–145.