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Capillary electrophoretic analysis of cyclodextrins with dynamic fluorescence labeling and detection

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Abstract

The use of 8-anilinonaphthalene-1-sulfonic acid (8,1-ANS) as buffer additive in the capillary electrophoretic separation of cyclodextrins (CDs) was investigated. Better detection sensitivity was obtained for α- and γ-CDs than with previously reported capillary electrophoretic methods. Increasing the concentration of 8,1-ANS improved resolution and sensitivities for α-, β- and γ-CDs, while decreasing the pH of the background electrolyte can improve sensitivities. Detection limits for α-, β- and γ-CDs were determined to be 60, 20 and 7 μ*M*, respectively. The formation constants of CD–8,1-ANS complexes at pH 6 were also measured by capillary electrophoresis. Finally the specificity of amyloglucosidase to CDs was analyzed as a practical application of this method. © 1998 Elsevier Science B.V.

Keywords: Complexation; Cyclodextrins; Aminonaphthalenesulfonic acid

1. Introduction

Cyclodextrins (CDs) have found widespread applications in separation sciences and in many industries [1–5]. Analysis of CDs is normally considered to be difficult by capillary electrophoresis (CE) as they are uncharged in neutral aqueous solution and demonstrate no appreciable UV–Vis absorbance. High-performance liquid chromatography (HPLC) methods were often used to analyze them [6–9]. However, some CE methods have been developed recently. After dynamic labeling with UV chromophores, such as benzoate ion [10], benzylamine and 1-naphthylacetic acids [11], mixtures of native CDs can be separated by CE. The quantity of analyte required for detection (ca. 0.1–1 m*M*) was relatively high. Better sensitivities for CDs have been achieved using 2-anilinonaphthalene-6-sulfonic acid (2,6-ANS) to dynamically label and detect via the direct fluorescence detection method [12]. The detection limit for β-CD was ca. 2.4 μ*M*, while the detection limits for α- and γ-CDs were slightly higher, ca. 62 μ*M* and 24 μ*M*, respectively. In the present research, we exploit the properties of the complexes of CD–8-anilinonaphthalene-1-sulfonic acid (8,1-ANS) to separate and observe mixtures of CDs by CE. As a

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practical application of this method, the specificity of amyloglucosidase to CDs was analyzed.

2. Experimental

2.1. Reagents and solutions

All solutions were prepared using deionized water (18 MΩ) generated by a D4700 Nanopure water-purification system (Barnstead/Thermolyne, IA, USA). 8,1-ANS, hydroxypropyl-β-CD (HP-β-CD), β- and γ-CD were purchased from Sigma (St. Louis, MO, USA). Amyloglucosidase (69.7 units/mg, EC 3.2.1.3), inorganic chemicals and α-CD were obtained from Fluka (Buchs, Switzerland). The background electrolytes (BGEs) for electrophoresis were prepared by dissolving 8,1-ANS into indicated buffers and filtering through a 0.45-μm nylon membrane. When needed, the pH of the BGEs were adjusted by 1 *M* acetic acid or 1 *M* sodium hydroxide to the required pH.

2.2. Apparatus

A RF5000 spectrofluorophotometer (Shimadzu, Japan) was used to investigate the steady-state fluorescence emission of 8,1-ANS in deionized water and solutions saturated with CDs. All CE experiments were carried out on a laboratory-made CE system composed of a RF551 spectrofluorometric detector (Shimadzu) and a high-voltage power supplier (High Voltage Technology, NY, USA). Untreated fused-silica capillaries purchased from Polymicro Technologies (Phoenix, AZ, USA) were 63 cm (50 cm effective length)×50 μm I.D. Injection was performed by hydrodynamic method at the anode. Direct fluorometric detection was performed at excitation wavelength λ_{ex}=366 nm and emission wavelength λ_{em}=495 nm through the capillary at a distance of 51 cm from the inlet. Electropherograms were recorded on a HP3390A integrator (Hewlett-Packard, USA). A laboratory-made waterbath was used to control the temperature during enzymatic hydrolysis of CDs.

2.3. Procedure for analyzing specificity of amyloglucosidase to CDs

The stock solution of amyloglucosidase was prepared by dissolving 11.0 mg enzyme in 100 μl acetate buffer (pH 5.1) and was kept in a refrigerator (4°C) until use. Before the enzymatic reaction, 1.0 ml solution of HP-β-CD or 1.0 ml mixture solution of α-, β- and γ-CD in a plastic vial was put in the water bath (55°C) for 20 min in order to warm the solutions to 55°C. Then 20 μl of enzyme solution was added into the vial. After a certain time, the vial was taken out from the waterbath and the solution in the vial was injected into the CE instrument. The vial was put back into the water bath to continue the hydrolysis after injection.

3. Results and discussion

3.1. Anilinonaphthalenesulfate fluorescence determination

Fig. 1 shows the steady-state emission spectra for 8,1-ANS in distilled water and in different solutions saturated with α, β and γ-CD. It is clear that γ-CD gave the greatest enhancement and α-CD gave the smallest among the three CD solutions. The comparison of fluorescence emission enhancement between CD–2,6-ANS and CD–8,1-ANS is summarized in Table 1. We can see that the formation of α- and γ-CD–8,1-ANS gave a greater fluorescence enhancement than that of α- and γ-CD–2,6-ANS, while 2,6-ANS shows a high selectivity to form an inclusion complex with β-CD. The greater enhancement means that better detection limits for α- and γ-CD are possible.

The difference in fluorescence enhancement between 8,1-ANS and 2,6-ANS system may be due to the difference in their structures. The structure of 8,1-ANS is most favored among anilinonaphthalenesulfates to form CD–ANS complexes by a mechanism called ''axial approach'' [13], illustrated in Fig. 2A. This complexation scheme may strain the CD backbone [13]. The larger the dimension of CD, the less the effect. Therefore, the magnitude of the fluorescence enhancement (FE) of CD is α-CD–8,1-ANS<β-CD–8,1-ANS<γ-CD–

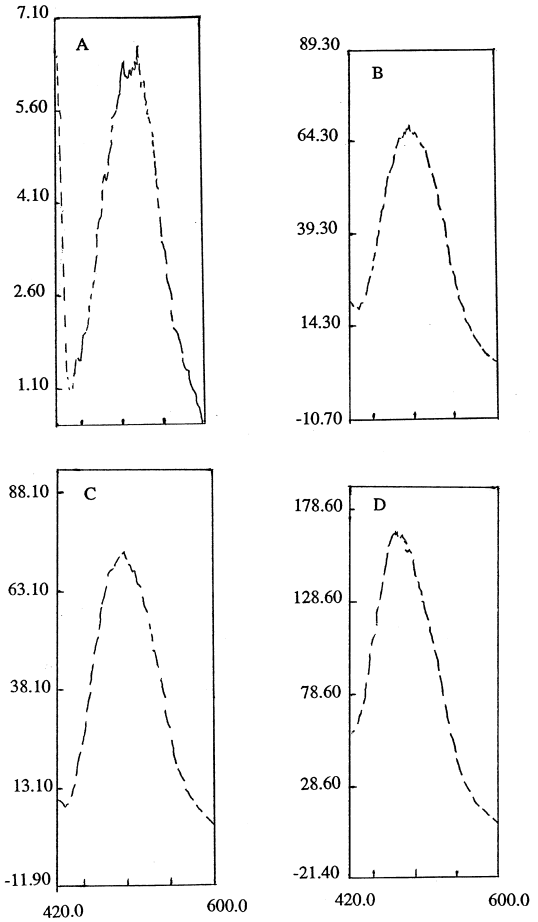

Fig. 1. Fluorescence emission spectra for $6.7 \cdot 10^{-6}$ *M* 8,1-ANS. (A) Spectrum in 18 MΩ water. (B), (C) and (D) correspond to α-CD (0.15 *M*), β-CD (0.016 *M*) and γ-CD (0.18 *M*), respectively. All emission spectra were excited at 366 nm.

8,1-ANS, as shown in Fig. 1. For 2,6-ANS, it is most favored to form complexes through ''equatorial approach'', illustrated in Fig. 2B. Hydrogen bonding from the tapered rim of the CD to the deprotonated sulfonate group will help to stabilize the complex more beside the hydrophobic interaction between the naphthalene moiety and CD cavity [1,13]. The dimension of β-CD may be quite suitable for the above two interactions, while the dimensions of α- and γ-CDs may be either too small or large for the interactions. This results in the largest fluorescence enhancement for β-CD–2,6-ANS and quite small enhancement for α-CD–2,6-ANS and γ-CD–2,6-ANS.

Table 1
Comparison of fluorescence emmission enhancement between 8,1-ANS and 2,6-ANS in solutions saturated with α-, β- and γ-CD

	α-CD	β-CD	γ-CD
8,1-ANS[a]	12	13.5	28.5
2,6-ANS[b]	1.8	55	2.3

[a] All values were measured at $\lambda_{ex}=366$ nm and $\lambda_{em}=495$ nm.
[b] Data were cited from Ref. [12].

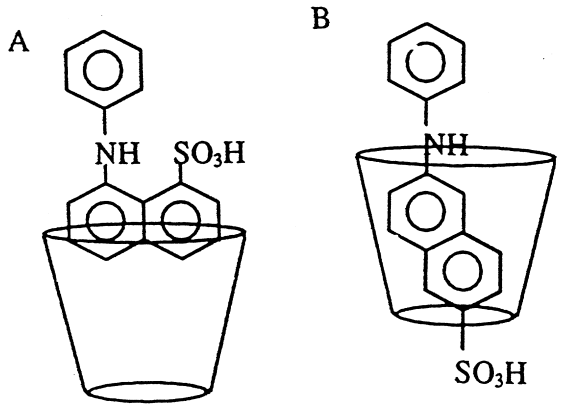

Fig. 2. (A) 'Axial' approach of an incoming CD molecule to a 8,1-ANS molecule. (B) 'Equatorial' approach of an incoming CD molecule to a 2,6-ANS molecule.

3.2. Separation of native CDs

The following equilibria exist in the separation zone during electrophoresis,

$$\begin{array}{ccc} \mathrm{CD}+8,1-\mathrm{ANS} & \overset{K_1}{=} & \mathrm{CD}-8,1-\mathrm{ANS} \\ \| \kappa_a & & \| \kappa_a' \\ \mathrm{H}^+ + \mathrm{CD}+8,1-\mathrm{ANS}^- & \overset{K_2}{=} & \mathrm{CD}-8,1-\mathrm{ANS}^- + \mathrm{H}^+ \end{array}$$

Where κ_a and κ_a' are the acid dissociation constants of 8,1-ANS and CD–8,1-ANS; and K_1, K_2 are the complex formation constants of the unionized and the ionized CD–8,1-ANS. Based on the above equilibria, we can obtain the following equation [11,14],

$$1/\mu_{\mathrm{eff}} = 1/\mu_{\mathrm{CD}-8,1-\mathrm{ANS}^-} + 1/K_2\mu_{\mathrm{CD}-8,1-\mathrm{ANS}^-} \cdot \left([\mathrm{H}^+]+\kappa_a\right)/\kappa_a \cdot 1/C_{8,1-\mathrm{ANS}} \quad (1)$$

From Eq. (1), we can see that both the pH of the background electrolyte and the concentration of 8,1-ANS can affect the effective mobility of CDs. Generally, we can determine the conditional formation constants through Eq. (1).

The concentrations of 8,1-ANS in the separation media is critical to achieve a certain resolution for the three CDs. The three CDs could be baseline separated only when the concentration of 8,1-ANS exceeded 4.0 m*M*. From Fig. 3, we can see that the signal-to-noise ratios (*S*/*N*) for the three CDs increased with increases in the concentration of 8,1-ANS and did not change much after the concentration exceeded 6 m*M*. Based on Eq. (1), the conditional formation constants for CD–8,1-ANS at pH 6 were 51.3 M^{-1}, 125 M^{-1} and 166.6 M^{-1} for α-, β- and γ-CD–8,1-ANS, respectively.

Under pH range from 5.2 to 11.0, the CDs can be well separated if the concentration of 8,1-ANS in the separation media is 8 m*M*. For β-CD–8,1-ANS, it was expected that the sensitivity would be higher at pH 11 since its formation constant was highest at pH 11 among the tested pH range [13], nearly twice of that at pH 6. However, the *S*/*N* ratios for the three CDs, especially for α-CD, were higher at low pH than at high pH, as shown in Fig. 4. This result was contrary to the above expectation. The reason for the unexpected behavior may be due to the increase in the ratio of non-ionic form of 8,1-ANS to its ionic form with decrease in the pH of the BGE. The non-ionic form could be more favored to form inclusion complexes with the CDs than the ionic form since the non-ionic form is more hydrophobic.

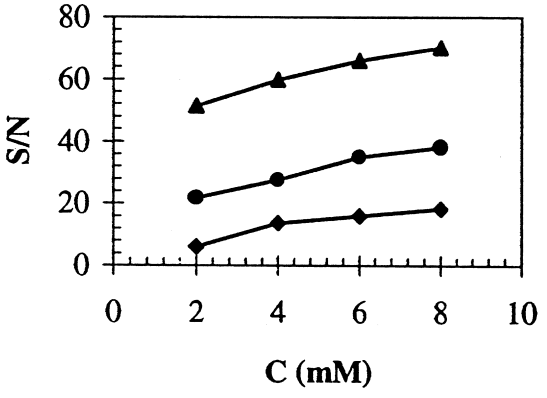

Fig. 3. Effects of concentration of 8,1-ANS on the *S*/*N* ratios for α-CD (♦), β-CD (●) and γ-CD (▲). Separation buffer: 20 m*M* acetate buffer, pH 6.0. Separation voltage, 16.5 kV; The sample was introduced into the capillary by hydrodynamic injection; 12 cm for 45 s from a sample containing α-CD (1.0 m*M*), β-CD (0.25 m*M*) and γ-CD (0.25 m*M*). Detection was made by fluorescence excited at 366 nm and monitored at 495 nm.

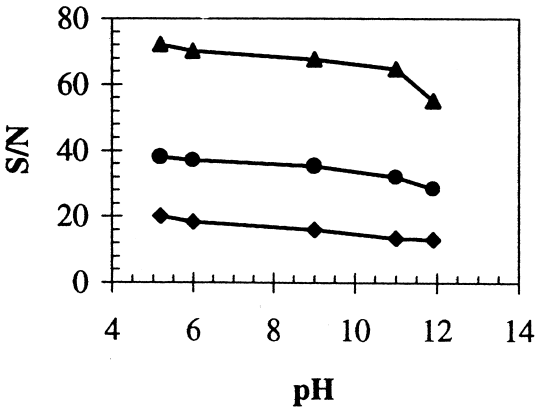

Fig. 4. Effects of pH of background electrolyte on the *S*/*N* ratios for α-CD (♦), β-CD (●) and γ-CD (▲). Separation buffers at pH 11.89, pH 11 and pH 9 are 15 m*M* phosphate buffer. pH 6.0 and pH 5.2 separation buffers are 20 m*M* sodium acetate buffer. The concentration of 8,1-ANS in the background electrolyte is fixed at 8.0 m*M*. Other conditions as in Fig. 3.

From the above results, we concluded that it would be better to separate and detect CDs at low pH of BGE and relatively high concentration of 8,1-ANS. The electropherogram obtained at pH 5.2 and 8 m*M* 8,1-ANS are shown in Fig. 5A. It can be seen that mobility for α-CD–8,1-ANS < β-CD–8,1-ANS < γ-CD–8,1-ANS, which are consistent with the magnitude of FE and their conditional formation constants. In order to maintain high concentration of 8,1-ANS, further decreasing pH to that lower than pH 5 was not possible because of the low solubility of 8,1-ANS in aqueous solution at low pH. Due to the consideration that high temperature is adverse to the formation of CD–8,1-ANS complexes, the separation voltage was set at 16.5 kV to minimize the effect of Joule heating. Under these conditions, a wide peak for HP-β-CD was obtained due to the different degrees of substitution, as shown in Fig. 5B.

Reproducibility of migration times was also determined. From five independent experiments we obtained a relative standard deviation (R.S.D.) of 0.35%. Both peak height and peak area measure-

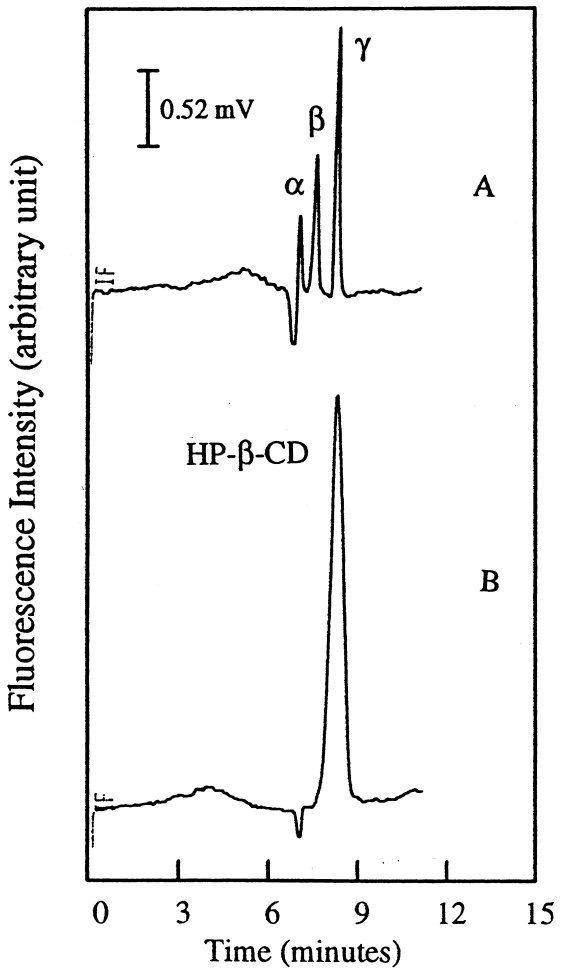

Fig. 5. The electropherograms of cyclodextrins (A) and HP-β-CD (B). Background electrolyte: 20 m*M* acetate buffer, pH 5.2, 8 m*M* 8,1-ANS. Separation voltage, 16.5 kV. In (A), sample contained α-CD (1.0 m*M*), β-CD (0.25 m*M*) and γ-CD (0.25 m*M*). In (B), sample contained HP-β-CD (1.0 m*M*) only. Other conditions as in Fig. 3.

ments provided a linear signal response over an extended range of concentration. The signals for α-, β- and γ-CDs increased linearly with concentration from $1.2\cdot10^{-4}$ *M* to $1.4\cdot10^{-2}$ *M*, $5.0\cdot10^{-5}$ *M* to $1.8\cdot10^{-3}$ *M* and $1.5\cdot10^{-5}$ *M* to $1.0\cdot10^{-2}$ *M*, respectively. Detection limits (based on $S/N=3$) were determined by injection of lower sample concentrations ($8.0\cdot10^{-5}$ *M* each) at injection height of 12 cm for 45 s. The detection limits obtained were 60 μ*M*, 20 μ*M* and 7 μ*M* for α-, β- and γ-CDs, respectively. These results are consistent with the magnitude of FE. Compared to the 2,6-ANS system [12], better detection limits were obtained for α- and γ-CDs. However, the detection limit for β-CD was slightly worse. It is clearly due to the difference in fluorescence enhancement between the two systems as shown in Table 1.

4. Determining the specificity of amyloglucosidase to CDs

Amyloglucosidase can sequentially hydrolyze α-1,4 and α-1,6 glycosidic bonds in starch into glucose units. It is usually necessary to know the specificity of amyloglucosidase to different substrates, such as in biosensor field [15]. CDs could be hydrolyzed by amyloglucosidase. Once one of the α-1,4 bonds in the CD was broken, the cavity of CD will no longer exist and the CD becomes unable to form inclusion complexes with 8,1-ANS. This reaction can be expressed as the decrease in fluorescence intensity. If the ratio of the decrease in fluorescence intensity to time for one CD is greater than those of others, we can conclude that this CD is a better substrate for amyloglucosidase than other CDs and amyloglucosidase is more specific to this CD.

Fig. 6 shows the electropherograms before and after amyloglucosidase was added into the mixture of α-, β- and γ-CDs. γ- CD was nearly completely hydrolyzed in 7 min while the hydrolysis of α- and β-CDs was much slower. However, we can see from Fig. 6C that β-CD is a better substrate than α-CD. Under the same conditions and procedure, HP-β-CD did not show any hydrolysis. This may suggest that amyloglucosidase cannot hydrolyze HP-β-CD. From the above results, we can conclude that amyloglucosidase is most specific to γ-CD among the CDs studied.

5. Conclusions

Better sensitivities for α- and γ-CDs can be obtained using 8,1-ANS to separate and detect CDs by CE than other reported CE methods. Because the 8,1-ANS system gave worst sensitivity for β-CD, the best choice may be using 8,1-ANS to detect α- and

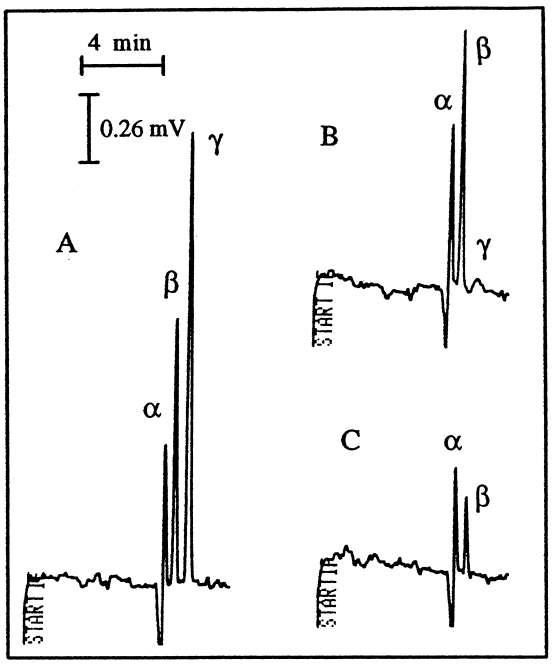

Fig. 6. Electropherograms of cyclodextrins during enzymatic hydrolysis. (A) Before amyloglucosidase was added into the mixture of cyclodextrins; (B) 7 min after amyloglucosidase was added; (C) 140 min after amyloglucosidase was added. Electrophoresis conditions as in Fig. 5.

γ-CDs and 2,6-ANS for β-CD in the practical analysis of CDs by CE. As a practical application, we have demonstrated that the proposed method can be successfully used to analyze the specificity of amyloglucosidase to CDs.

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