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Cyclodextrin aided separation of peptides and proteins by capillary zone electrophoresis

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Abstract

Carboxymethylated-β-cyclodextrin (CMBCD) in the electrophoretic medium (aqueous 50 m*M* sodium phosphate, pH 2.5) enhanced the separation using raw fused-silica capillaries in CZE of the four standard proteins: α-chymotrypsinogen A, cytochrome *c*, lysozyme and ribonuclease A. Furthermore, with 20 m*M* CMBCD in the electrophoretic medium, the *cis–trans* isomers of angiotensin could be separated at room temperature, whereas the separation of the conformers required subambient temperatures as low as −20°C without CMBCD in the electrophoretic medium [50 m*M* sodium phosphate (pH 2.5), containing 10% (v/v) methanol]. Addition of heptakis(2,6-di-O-methyl)-β-cyclodextrin (DMBCD) had no effect on the separation of the above proteins and peptides. The results suggest that in microcolumn separation techniques, certain cyclodextrin additives can be useful selectivity enhancers. © 1998 Elsevier Science B.V.

Keywords: Peptide conformers; Buffer composition; Selectivity; Carboxymethylated-β-cyclodextrin; Angiotensin I

1. Introduction

Separation of proteins at neutral pH by CZE with fused-silica capillaries is encumbered by interactions of the proteins with the capillary wall resulting in poor plate efficiency, peak asymmetry and poor reproducibility of the migration times [1,2]. At acidic or alkaline pH, on the other hand, the proteins are denatured and the selectivity of the separation method towards proteins in random coil form is generally poor [1,3,4]. The most common strategies to circumvent these problems include using coated capillaries [5–8] or masking the silanol groups by agents such as amines, zwitterions, cationic surfactants, amino sugars etc. [9–13]. All of these strategies suffer from some disadvantages, however. The lifetime of the coated capillaries is short and most additives increase the ionic strength of the background electrolyte with a concomitant increase of the current.

Separation of the readily interconverting *cis–trans* isomers of various peptides containing peptidyl–proline bonds is of growing importance in studying the chemical and physiological properties of the conformers and the kinetics of the interconversion. Results obtained by HPLC and CZE in our laboratory show that in most cases subambient and often subzero temperatures are required to bring about the separation of the *cis–trans* isomers [14–20].

The goal of the present study was to examine the effect of addition of cyclodextrins to the electro-

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phoretic medium on the separation of proteins and peptide conformers at acidic pH by CZE.

2. Experimental

2.1. Chemicals

Angiotensin I (DRVYIHPFHL) and the four proteins, cytochrome c, α-chymotrypsinogen A, lysozyme and ribonuclease A, were purchased from Sigma (St. Louis, MO, USA). Highly purified heptakis(2,6-di-O-methyl)-β-cyclodextrin (DMBCD; catalogue No. CY-A-2004.1) and the sodium salt of carboxymethylated-β-cyclodextrin (CMBCD; catalogue No. CY-E-2006) were obtained from Cyclolab (Budapest, Hungary). Reagent-grade phosphoric acid, sodium hydroxide, hydrochloric acid and HPLC-grade methanol were supplied by Fisher (Pittsburgh, PA, USA). Potassium phosphate and chloride buffer standards, pH 7.00 and 2.00, were from Baker and Fisher (Pittsburgh, PA, USA), respectively. Deionized water was prepared by the NanoPure purification system from Barnstead (Boston, MA, USA) and used throughout the experiments.

2.2. Instrumentation

A Model P/ACE 2210 capillary electrophoresis unit (Beckman, Fullerton, CA, USA) was modified for low-temperature CZE as described earlier [18–22]. A PowerMate SX/20 from NEC Technologies (Boxborough, MA, USA) with P/ACE 2000 Series Microsoft for Windows version 3.0 from Beckman was used for the control of the instrument and for data processing. The P/ACE UV–Vis detector was used in all experiments with a setting at 200 and 214 for protein and peptide analysis, respectively. Raw fused-silica capillaries of 50 μm I.D. were obtained from Quadrex (New Haven, CT, USA). The capillary length and the pertinent migration distances were 57–50 cm and 27–20 cm for protein and peptide analysis, respectively. The protein and peptide samples were dissolved in deionized water at a concentration of ~1 mg/ml and injected for about 5–15 s at 0.5 p.s.i. (1 p.s.i.=6894.76 Pa). Between runs the capillary was flushed with at least five column volumes of the electrolyte at an inlet pressure of 20 p.s.i. Pressure injected acrylamide pulse did not emerge from a 27/20 cm×50 mm capillary at pH 2.5 and 25 kV applied voltage within 60 min, indicating that the mobility of the acrylamide peak, μ_{eof}, of less than $6 \cdot 10^{-10}$ $m^2 s^{-1} V^{-1}$, so that the electrosmotic flow (EOF) was negligible under the conditions of the experiment. In order to evaluate the viscosity, an acrylamide pulse was injected in a 27/20 cm×50 mm capillary and a low pressure (0.5 p.s.i.) rinse was applied to the column. From measurements of the time taken by the pulse to traverse the column, the viscosities of the electrophoretic medium with and without 20 m*M* CMBCD and DMBCD were calculated using the Hagen–Poiseuille equation [23,24] and the corresponding viscosity values at 27°C were found to be 0.91 and 0.84 cP, respectively.

2.3. Electrophoretic media

Neat aqueous 50 m*M* sodium phosphate (pH 2.5), which was used in the temperature range 1–40°C, was prepared by adjusting the pH of a 50 m*M* aqueous phosphoric acid solution with 1 *M* NaOH. For CZE at subzero temperatures the 50 m*M* sodium phosphate solution was mixed with methanol (90:10, v/v) and the apparent pH* of the solution was adjusted to 2.5, as measured by the glass electrode by addition of 1 *M* HCl. The freezing point of this hydro–organic medium is about −20°C [25]. CMBCD and DMBCD were dissolved in aqueous 50 m*M* sodium phosphate (pH 2.5), to obtain 5, 10 or 20 m*M* concentrations of the cyclodextrins. The apparent pH* of the electrophoretic medium increased from 2.5 to 2.8 and from 2.5 to 3.1 upon addition of 10 m*M* and 20 m*M* CMBCD, respectively. For low-temperature CZE, a cool off period of 10 min was used before sample injection to reach thermal equilibrium and thus to eliminate possible temperature bias due to a difference in temperature of the coolant and the inside of the capillary [22].

3. Results and discussion

3.1. Protein separation

The effect of adding the readily soluble sodium salt of CMBCD to the electrophoretic medium is

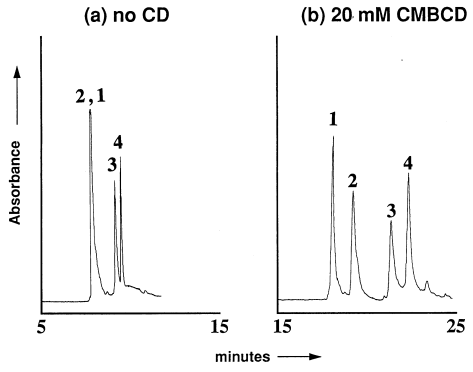

Fig. 1. Electropherograms illustrating the effect of CMBCD on the separation of four standard proteins by CZE with a raw fused-silica capillary, 57/50 cm×50 μm; 50 m*M* sodium phosphate; applied voltage, 20 kV; temperature, 26°C; detection, 200 nm; pressure injection, 3 s; (a) without CMBCD, pH 2.5, current 25 μA; (b) with 20 m*M* CMBCD, pH 3.1, current 67 μA. Proteins: (1) Lys, (2) Cyt C, (3) Rnase A, (4) α-Chy A.

seen by comparing the two electropherograms in Fig. 1a and b that show separation with and without 20 m*M* CMBCD of the four standard proteins: α-chymotrypsinogen A (α-Chy A), cytochrome *c* (Cyt C), lysozyme (Lys) and ribonuclease A (Rnase A). It is seen that in the absence of the carboxymethylated cyclodextrin, proteins α-Chy A and Rnase A are only partially separated, whereas, Cyt C and Lys comigrate. The separation could not be improved by lowering the applied voltage. This is expected in CZE at strongly acidic pH, where the selectivity is very poor for proteins that have similar charge to size ratios and are in random coil form [1,3,4].

Upon addition of 20 m*M* CMBCD to the background electrolyte, the resolution of Rnase A and α-Chy A does not improve significantly, whereas there is a large increase in the resolution of Cyt C and Lys as shown in Fig. 1b. It has been postulated [26–29] that cyclodextrin molecules interact with accessible hydrophobic moieties of proteins and such interactions have been taken advantage of in protein folding as well as in solubilizing and/or stabilizing of a variety of therapeutical proteins such as interleukin-2, ovine growth hormone, bovine insulin, tumor necrosis factor, human growth hormone, human insulin and macrophage colony stimulating factor [30,31]. The charges and hydrodynamic radii of such adducts and consequently their electrophoretic migration velocities [32] are likely to be different from those of the uncomplexed proteins and may result even in a reversal in the migration order of Lys and Cyt C as seen in Fig. 1a and b. Furthermore, CMBCD may reduce protein adsorption at the inner wall of the raw fused-silica capillary and this may be in part responsible for the increase in the peak areas of the four proteins upon addition of CMBCD to the background electrolyte (Fig. 1a and b).

In some experiments the heptakis(2,6-di-O-methyl)-β-cyclodextrin, DMBCD, was added to the electrophoretic medium. As shown in Table 1, the migration times increase only slightly on addition of 20 m*M* of DMBCD, and suggest a very low level of interaction between DMBCD and the proteins with the exception of cytochrome *c*. The failure of DMBCD in affecting the CZE separation is under investigation in our laboratory. The migration times of all the four proteins rise sharply with increasing CMBCD concentration indicating strong interactions between protein and CMBCD molecules (Table 1). The large increase in migration times is observed with CMBCD but not with DMBCD. This demonstrates that the functional groups that are intro-

Table 1
Migration times of the four basic proteins: lysozyme; cytochrome *c*; ribonuclease A and α-chymotrypsinogen A, at various concentrations of DMBCD and CMBCD

Protein	Migration time (min)				
	No CD	DMBCD	CMBCD		
		20 m*M*	5 m*M*	10 m*M*	20 m*M*
α-Chymotrypsinogen A	10.11	10.36	12.95	16.77	23.29
Ribonuclease A	9.71	9.93	12.16	15.53	21.88
Cytochrome *c*	8.10	9.97	11.21	13.40	19.86
Lysozyme	8.21	8.37	10.83	12.80	18.45

Conditions as in Fig. 1a and b.

duced as a result of derivatization of the native β-cyclodextrin have a profound effect on the ability of the cyclodextrin derivative to act as a host for hydrophobic molecular moieties of the guest molecule or to interact otherwise with the migrants.

The migration times of the four proteins are also plotted against the CMBCD concentration in the electrophoretic medium in Fig. 2. The results show that without added CMBCD, Cyt C and Lys essentially comigrate and α-Chy A and Rnase A are poorly separated. However, upon addition of some CMBCD to the electrolyte, the selectivity increases with the CMBCD concentration and the four proteins become very well separated at a concentration of 20 m*M* CMBCD. This is further shown in Fig. 3 where the intrinsic selectivity of the system, defined as the ratio of the mobility of the faster moving migrant to that of the slower one [33], is plotted against the CMBCD concentration for the two protein pairs, Cyt C–Lys and α-Chy A–Rnase A. In both cases the selectivities increase with the CMBCD concentration in the range considered and the plots are almost linear above 5 m*M* CMBCD. Since the solutions of both the cyclodextrins had the same viscosity and the viscosity increased by about 8% upon addition of 20 m*M* cyclodextrin to the medium, the strong increases in both the migration times and the selectivities are attributed to complex formation. The above findings suggest, somewhat unexpectedly, that the hydrophobic cavity may not, or only in concert with another binding mechanics, be responsible for the complex formation.

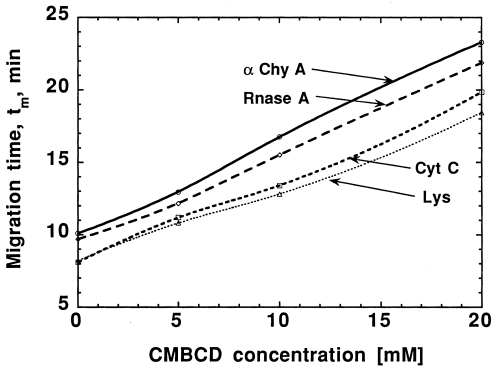

Fig. 2. Plots of the migration time, t_m, against the CMBCD concentration for the four basic proteins: (○) α-Chy A; (◇) Rnase A; (□) Cyt C; (△) Lys. Without CMBCD: pH 2.5, current 25 μA; with 5 m*M* CMBCD: pH 2.6, current 30 μA; with 10 m*M* CMBCD: pH 2.8, current 42 μA; with 20 m*M* CMBCD: pH 3.1, current 67 μA. Other conditions as in Fig. 1.

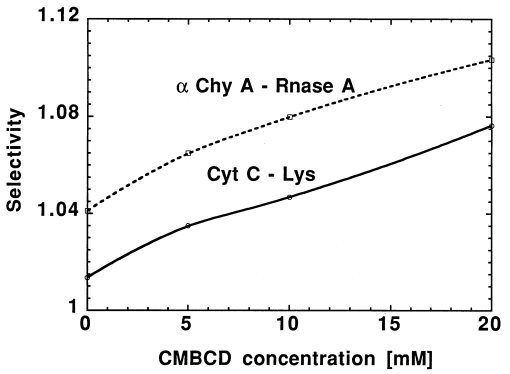

Fig. 3. Plots of the selectivity for the two protein pairs: (□) α-Chy A and Rnase A; (○) Cyt C and Lys, against the CMBCD concentration. Conditions as in Fig. 2.

It should be noted that the employment of cyclodextrins in a microcolumn separation system offers a major advantage over several other techniques like CZE with surfactants added to the electrophoretic medium, MEKC and SDS-capillary gel electrophoresis. This is because of the low interference of cyclodextrins that allows usage of other popular detection methods like ion spray and matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry [34].

3.2. Separation of peptides

3.2.1. At low temperature

CZE at low temperatures has emerged as the method of choice for the analysis of interconverting or otherwise reacting species such as the *cis–trans* conformers of various peptides having one or more peptidyl–proline bonds [18–20]. The decapeptide angiotensin I (DRVYIHPFHL) has one peptidyl–proline bond. It is present in the form of *cis–trans* isomers that have only very small differences in their electrostatic charge and hydrodynamic radii. Fig. 4 illustrates electropherograms of the *cis* and *trans* angiotensin I by CZE at different temperatures. At room temperature, the two conformers interconvert so fast that they appear as a single peak as seen in Fig. 4a. Upon lowering the temperature to −3°C and finally to −20°C, the separation improved as shown

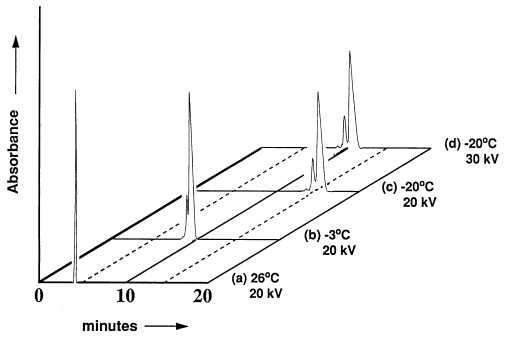

Fig. 4. Electropherograms of the *cis–trans* isomers of angiotensin I obtained at different coolant temperatures. Raw fused-silica capillaries 27/20 cm×50 μm I.D.; electrophoretic medium, 50 m*M* sodium phosphate in water–methanol (90:10, v/v), pH* 2.5, detection, 214 nm. Temperatures and applied voltages are shown in the legends. Injection times and currents (a) 5 s, 30 μA; (b) 8 s, 15 μA; (c) 10 s, 13 μA; (d) 10 s, 20 μA.

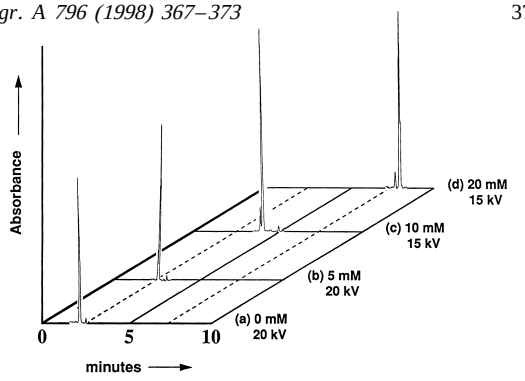

Fig. 5. Electropherograms of the *cis–trans* isomers of angiotensin I obtained at different CMBCD concentrations in the electrophoretic medium, 50 m*M* sodium phosphate; raw fused-silica capillaries, 27/20 cm×50 μm I.D.; detection, 214 nm; injection, 5 s; temperature 26°C. pH and currents (a) pH 2.5, 37 μA; (b) pH 2.6, 64 μA; (c) pH 2.8, 77 μA; (d) pH 3.1, 110 μA. CMBCD concentrations and separation voltages are shown in the legend.

in Fig. 4b and c. Complete separation of the two conformers at −20°C still required an increase of the applied voltage from 20 to 30 kV as shown in Fig. 4d. The peaks of the *cis* and *trans* conformers have to be identified by NMR. On the basis of earlier studies with a number of interconverting peptide isomers, we may assume that the *cis* isomer is the faster migrant in the present case [18].

3.2.2. With cyclodextrins

The effect of CMBCD on the separation of *cis–trans* isomers of angiotensin I is illustrated by the electropherograms in Fig. 5. Without CMBCD in the medium, the two isomers interconvert very quickly and comigrate as shown in Fig. 5a. Upon increasing the CMBCD concentration to 5, and then to 10 m*M*, the separation improves rapidly (Fig. 5b and c). Finally, baseline separation of the two conformers is accomplished at 20 m*M* CMBCD (Fig. 5d). The observed decrease in the migration velocities of the angiotensin I isomers with increasing CMBCD concentration supports the hypothesis that both forms form complexes with CMBCD with the complexation being more favorable with the second, slower migrating conformer. As a result, both the migration velocities and the isomerization kinetics slow down and the separation of the two isomers is facilitated. Again, the two peaks have to be identified by NMR in order to identify the *cis* and the *trans* isomers. A closer look at the electropherograms in Fig. 4d and Fig. 5d reveals that the peak area of the second peak increases in the presence of CMBCD. This observation suggests that a shift occurs in the equilibrium as a result of the action of CMBCD . The hypothesis that CMBCD forms complexes with the angiotensin I isomers that have different electrophoretic mobilities has strong support in various studies on peptide–cyclodextrin complexes via competitive spectrophotometry [35], crystallography [36] and NMR spectroscopy [37] and by similar observations in chromatography and electrophoresis [38–42]. The shifting of the equilibrium distribution of the conformers in the presence of CMBCD has great implications for cases where the two isomers differ in their bioactivities.

3.2.3. Comparison of the two methods

In order to compare the effect of the temperature and CMBCD on the separation of the peptide isomers, the resolution is plotted against the reciprocal of absolute temperature and the CMBCD concentration as shown in Fig. 6. The effect of decreasing temperature parallels that of increasing the CMBCD concentration. As the temperature decreases, the reaction kinetics becomes slower and the resolution of the two conformers increases from 0 at room temperature to 0.85 at −20°C, and the curve apparently plateaus at −20°C indicating saturation of the beneficial effects of low temperature. On the

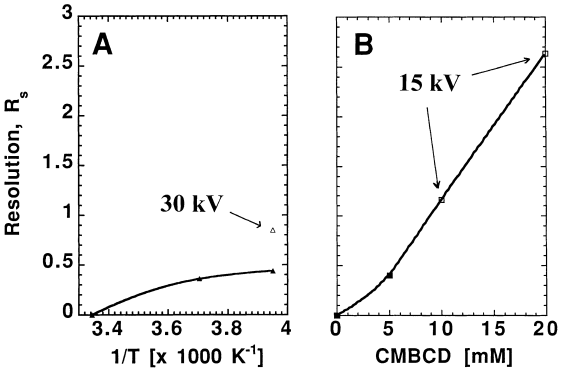

Fig. 6. Plots of the resolution against the reciprocal absolute coolant temperature (A) and the CMBCD concentration (B). Separation voltage, 20 kV, unless indicated otherwise. Conditions as in Figs. 4 and 5.

other hand in Fig. 6, improvement in resolution shows a monotonous increase with the concentration of the CMBCD which is believed to complex with the peptide isomers as discussed above.

4. Conclusion

The results of the present work strongly suggest that separation of peptides and proteins can be enhanced by adding an appropriate cyclodextrin to the electrophoretic medium. Therefore, the role of cyclodextrins in CZE as selectivity enhancers is expected to go far beyond enantiomeric separations.

The emergence of microcolumn liquid phase separation techniques such as CZE, μ-HPLC and CEC, which consume minute amount of reagents per run, offers a sphere of action to explore the potential of this approach in a convenient fashion. Further support for the use of cyclodextrins in microcolumn separation processes comes from their MS compatibility. Cyclodextrins do not interfere (unlike most surfactants) with a successful interfacing of the analytical separation instrument with the mass spectrometer and thus could have wide applications in 'hyphenated' techniques.

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