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Optical resolution by electrokinetic chromatography using ovomucoid as a pseudo-stationary phase

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ABSTRACT

The utilization of ovomucoid as a chiral pseudo-stationary phase in electrokinetic chromatography was studied. Optical resolution of some drugs was achieved successfully with high efficiency by optimizing the concentrations of both ovomucoid and an organic solvent. The reproducibility was improved by the addition of cellulose derivatives to the separation solutions or by using coated capillaries. This approach is expected to have wide applications.

INTRODUCTION

Recently, it was mentioned that chiral pharmaceutical substances should be evaluated not as racemates but as one of two different compounds or as a mixture with the optimum enantiomeric excess, because optical isomers are often readily distinguished by biological systems and may have different pharmacokinetic properties (absorption, distribution, metabolism and excretion) and quantitatively or qualitatively different pharmacological or toxicological effects [1]. In developing new drugs, high-performance liquid chromatography (HPLC) has been widely employed to meet these needs and various kinds of chiral stationary phases (CSPs) are now commercially available [2–4]. In reversed-phase HPLC, which is suitable for the determination of drug enantiomers in biological fluids, several protein-bonded CSPs have been useful [5–9], *e.g.*, an avidin-bonded CSP could resolve drug enantiomers with direct serum injection [10]. Ovomucoid-bonded CSP is popular because of its wide applications and high column stability [11]. However, relatively lower theoretical plate numbers are generally obtained with the use of protein-bonded CSPs.

On the other hand, capillary electrophoresis (CE) has been developed with higher resolution, shorter analysis times and smaller amounts of samples needed, compared with HPLC [12–14]. Several approaches to optical resolution by CE have been reported. Enantioselective Cu^{2+} complexes [15], cyclodextrins (CDs) [16,17] and chiral micelles such as bile salts [18,19], amino acid derivatives [20] and some saponins [21,22] have been successfully employed in enantiomeric separations of amino acid derivatives and drugs. Although these approaches provided high resolution, the range of their applications is limited and a new approach to optical resolution is needed.

Electrokinetic chromatography (EKC) is a mode of CE in which the separation principle is based on differential distribution between two phases, that is, an aqueous phase and a pseudo-stationary phase. A micelle is a typical pseudo-

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stationary phase used in micellar EKC (MEKC) [23] and the utilization of chiral micelles in MEKC permitted optical resolution. Similarly, other chiral pseudo-stationary phases, such as ionic CD derivatives, have enabled optical resolution to be achieved [24]. In this work, the utilization of ovomucoid as a chiral pseudo-stationary phase was examined. In this approach, the amount of ovomucoid required will be reduced in comparison with an ovomucoid-bonded CSP. Moreover, higher resolution and broader application are expected. Recently, two groups have reported the utilization of bovine serum albumin (BSA) as a chiral selector in capillary gel electrophoresis and EKC, respectively [25,26].

Ovomucoid is one of the most stable proteins purified from egg white [7]. Its molecular mass is about 28 800, the isoelectric point is 3.9–4.5 (chicken) and the UV absorbance of a 1% solution is relatively low among egg proteins ($A_{280} = 4.1–4.6$) [27]. An ovomucoid-bonded CSP has been utilized in HPLC for the optical resolution of many drugs and this CSP exhibited significant stability under various mobile phase conditions at different pH values and with different concentrations of organic modifiers [7,11]. Therefore, its activity will not be lost under EKC conditions.

In this paper, the capability of ovomucoid as a chiral pseudo-stationary phase in EKC is considered and the effects of some modifiers on the separation selectivity are discussed.

EXPERIMENTAL

Apparatus

EKC separations were performed with a Beckman (Palo Alto, CA, USA) P/ACE System 2100. Detection was performed by measuring the UV absorbance at 254 nm. Uncoated eCAP capillary tubing (Beckman) of 75 μm I.D. and capillaries coated with either polyoxyethylene (PEG) (DB-Wax, 100 μm I.D.), (J&W, Folsom, CA, USA) or a monomolecular layer of linear polyacrylamide (75 μm I.D.), which was prepared according to the procedure described by Hjertén [28] were used. All capillaries were thermostated at 25°C by a liquid coolant. All chromatograms shown here were redrawn using Microsoft Excel.

Reagents

Ovomucoid was purified from chicken egg white. Tolperisone hydrogen chloride (TP) and benzoin (BZ) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Eperisone hydrogen chloride (EP) and chlorpheniramine malate (CP) were of pharmaceutical grade. The structures of these compounds are shown in Fig. 1. *o*-Phosphorylethanolamine (PEA) was obtained from Sigma (St. Louis, MO, USA), 2-(cyclohexylamino)ethanesulphonic acid (CHES) from Dojin (Kumamoto, Japan), hydroxypropylcellulose (1000–4000 cP) (HPC) from Wako (Osaka, Japan) and SCAT 20-X, which is a weak alkaline solution consisting of some detergents for laboratory use, from Dai-ichi Kogyo Seiyaku (Kyoto, Japan). The samples were dissolved in water. The separation solutions were prepared by the addition of ovomucoid to buffer solutions at various concentrations (assuming the molecular mass of ovomucoid to be 28 000) followed by filtration through a membrane filter of 0.45-μm pore size.

Procedure

Uncoated capillaries were rinsed under pressure after every run with water (1 min), SCAT 20-X (7 min, but without pressure for 5 min), water (2 min), 1 *M* sodium hydroxide solution (1 min) and water (1 min) in that order. Coated capillaries were also rinsed in the same way, except that the sodium hydroxide washing was omitted. All injections of samples were per-

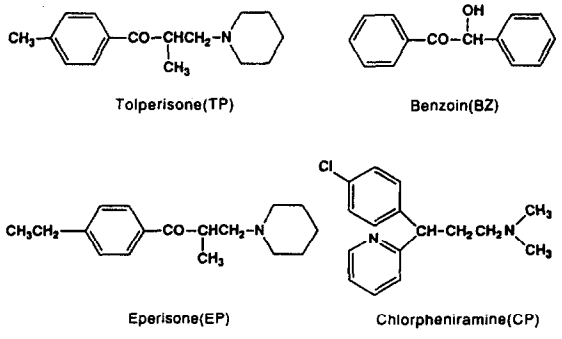

Fig. 1. Structures of compounds employed.

formed by pressure (0.5 p.s.i.; 1 p.s.i. = 6894.76 Pa) for 1.0 or 1.5 s.

RESULTS AND DISCUSSION

Enantiomers of BZ, TP and EP were successfully separated in phosphate buffer (pH 7.0) containing 50 μM ovomucoid. At this pH, ovomucoid had a negative charge and the solutes were neutral or protonated. Under these conditions, the electroosmotic flow toward the cathode was greater than the electrophoretic migration of ovomucoid toward the anode. Therefore, both ovomucoid and solutes migrated toward the cathode and the enantiomer that was bound more strongly to ovomucoid migrated more slowly than the other.

In analogy with MEKC [29,30], the capacity factor, k', defined by

$$k' = n_{ov}/n_{aq} \quad (1)$$

where n_{ov} and n_{aq} are the numbers of solute molecules bound to ovomucoid and those free in the aqueous phase, respectively, is more suitable for the evaluation of separation selectivity than the migration times of the solutes, because the electroosmotic flow and the electrophoretic migration of ovomucoid are different under various conditions. The capacity factor is related to the migration mobility of the ionized solute, $\mu(s)$, which is equal to the migration velocity divided by the electric field strength, by

$$\mu(s) = \mu_{eo} + \frac{1}{1+k'} \cdot \mu_{ep}(s) + \frac{k'}{1+k'} \cdot \mu_{ep}(ov) \quad (2)$$

where μ_{eo} is the electroosmotic mobility and $\mu_{ep}(s)$ and $\mu_{ep}(ov)$ are the electrophoretic mobilities of the solute free from ovomucoid and ovomucoid itself, respectively. It should be noted that μ_{eo}, $\mu_{ep}(s)$ and $\mu_{ep}(ov)$ are the mobilities in the separation solution containing ovomucoid. $\mu_{ep}(s)$ cannot be calculated from the migration data for the solute, because the solute will interact with ovomucoid in the presence of the latter, and $\mu_{ep}(ov)$ (toward the anode) also cannot be found because no suitable tracer of ovomucoid is available, whereas μ_{eo} could be obtained from the migration time of the bulk solution (t_0), which was indicated by a dip in the UV absorbance. Therefore, free zone electrophoresis of the solute and ovomucoid in the phosphate buffer was carried out separately to find $\mu_{ep}(s)$ and $\mu_{ep}(ov)$ using the following equation:

$$\mu_{ep} = L \cdot \frac{l}{V}\left(\frac{1}{t_m} - \frac{1}{t_0}\right) \quad (3)$$

where L and l are the total length of the capillary and the effective length to the detector respectively, V is the applied voltage and t_m is the migration time of an injected sample. If it is assumed that μ^*_{ep}, which is the mobility calculated according to eqn. 3, is equal to $\mu_{ep}(s)$ or $\mu_{ep}(ov)$ in eqn. 2, then k' of the solute can be obtained using eqn. 2. In practice, however, $k^{*\prime}$, which is the capacity factor obtained using μ^*_{ep} instead of μ_{ep}, sometimes became negative. The reason was not obvious but it may be caused by the slight differences between μ_{ep} and μ^*_{ep} due to the experimental error or the difference in the properties of the separation solutions, such as the permittivity, the viscosity and zeta potential of the solute. Although $k^{*\prime}$ may not be the same as the true k', it is still useful for evaluating the separation selectivity when $\mu_{ep}(s)$ and $\mu_{ep}(ov)$ are constant.

Effect of pH

Fig. 2 shows the separation efficiency of EP under the different pH conditions. Below the p*I* of ovomucoid (3.9–4.5), no peak was detected even when the sample was injected at either the cathode or anode end. On the other hand, under alkaline conditions electroosmosis was strong and no enantiomeric separation was achieved. Enantiomeric separation of EP was successful in the pH range *ca.* 4–8 and the enantioselectivity of EP was improved as the pH was lowered from 8 to 4. However, the theoretical plate numbers of EP were low in the low pH region. All peaks obtained in these runs tailed seriously and the later-eluted enantiomer always gave a broader and more tailed peak shape than the first-eluted enantiomer. It was considered that these poor peak shapes were due to several causes, such as adsorption of ovomucoid on the capillary wall,

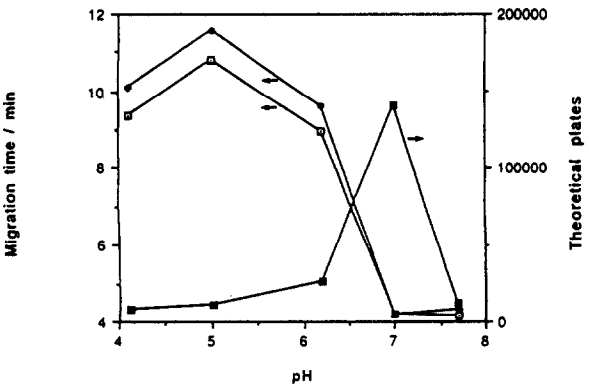

Fig. 2. Effect of pH on separation efficiency of EP. Conditions: uncoated capillary, 57 cm × 75 μm I.D. (50 cm to the detector); separation solution, 60 μ*M* ovomucoid in 50 m*M* phosphate buffer; sample concentration, 50 μ*M*; applied voltage, 18 kV. ■ = N_1; □ = t_1; ◆ = t_2.

slow kinetics of the interaction between EP and ovomucoid and the heterogenity of the ovomucoid employed.

It is known that many proteins adsorb on the inside wall of the capillary in HPCE [28,31]. The migration time of the bulk solution gradually became long without rinsing the capillary after every run. This was because adsorption of ovomucoid on the wall reduced the effective negative charge on the wall. As electroosmosis became weaker, that is, as ovomucoid adsorbed on the wall more, the peak shape became poorer. It was suggested that the lower plate numbers might be caused mainly by the interaction of the solute with ovomucoid adsorbed on the wall.

Effect of concentration of running buffer

The concentration effect on the phosphate buffer was examined. Table I shows that with an increase in buffer concentration, t_0 and k' decreased, μ^*_{ep}(s) was almost constant, μ^*_{ep}(ov) increased (the absolute mobility decreased) and the R_s values also decreased in spite of an increase in plate number. It can be considered that the decrease in t_0 and the increase in plate number were caused by the decrease in the adsorption of ovomucoid on the wall and the change in μ^*_{ep}(ov) was due to the change in the conformation of ovomucoid by the ionic

TABLE I

EFFECT OF BUFFER CONCENTRATION ON SEPARATION EFFICIENCY OF EP

Separation solution, 60 μ*M* ovomucoid in phosphate (pH 7.0); other conditions as in Fig. 2a.

Parameter	Phosphate concentration (m*M*)		
	10	25	50
t_0 (min)	8.43	8.72	5.69
$k_1^{*\prime}$	5.60	5.69	4.20
$k_2^{*\prime}$	5.74	5.83	4.22
μ^*_{ep}(s) (10^8 m^2 s^{-1} V^{-1})	1.85	1.78	1.84
μ^*_{ep}(ov) (10^8 m^2 s^{-1} V^{-1})	−1.10	−0.95	−0.88
N_1[a]	44 700	40 500	140 800
R_s	1.38	1.31	0.47

[a] Theoretical plate number of the first-eluted enantiomer is calculated by the equation, $N_1 = 5.54(t_m/W_{1/2})^2$, where t_m is the migration time and $W_{1/2}$ is the peak width at half-height.

strength, which might have influenced the zeta potential of the surface of ovomucoid, and the k' of EP was altered by the change in the surface of ovomucoid in addition to the effect of salts on the ion–ion interaction. The R_s values decreased because the separation factor, $k_2^{*\prime}/k_1^{*\prime}$, decreased and the migration time window was reduced by both the decrease in t_0 and the increase in μ^*_{ep}(ov).

Effect of coated capillaries

To eliminate the influence of the protein adsorption on the wall, two kinds of coated capillaries, linear polyacrylamide coated and PEG coated, were employed. In both instances, the peak tailing could not be suppressed although the reproducibility was improved with the PEG-coated capillary (see *Reproducibility*), while separations using a polyacrylamide-coated capillary could be performed for only a few runs. This result suggested that the peak tailing was caused by not only the ovomucoid adsorbed on the wall but also other factors such as the slow kinetics of solute–ovomucoid interaction and the heterogeneity of the ovomucoid. In fact, hetero-

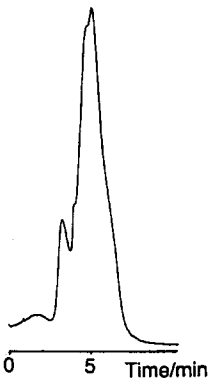

Fig. 3. Separation of ovomucoid by capillary zone electrophoresis. Conditions: PEG-coated capillary, 27 cm × 100 μm I.D. (20 cm to the detector); separation solution, 10 m*M* phosphate buffer (pH 7.0); applied voltage, 12 kV; detection wavelength, 214 nm.

geneity of the ovomucoid was observed in free zone electrophoretic separation using a PEG-coated capillary at pH 7.0 (Fig. 3). However, it is not evident whether the heterogeneity affected the peak shape or not.

Effect of buffer constituents including zwitterions

Table II shows the effect of buffer constituents on the separation efficiency at pH 5 and 7. At both pHs, the phosphate buffer was preferable for optical resolution although phosphate ions gave a higher current, which might cause a temperature rise of the solution in the capillary resulting from Joule heating [29,30]. Zwitterions are known as buffer constituents having low conductivities and preventing proteins from adsorbing on the capillary wall [32]. Moreover, it is expected that protein–protein interactions will be suppressed and protein–solute interactions promoted. When zwitterions play these roles, the peak shape may be improved. PEA and CHES, which led to the improvement of the peak shapes in protein analysis [33,34], were employed in the separation solutions adjusted by phosphate to pH 5 and 7, respectively. However, the addition of CHES to the separation solution at lower concentration did not lead to a significant improvement in the peak shape although the current was diminished. On the other hand, the

TABLE II

EFFECT OF BUFFER CONSTITUENTS ON SEPARATION EFFICIENCY OF EP

Separation solution, 50 μ*M* ovomucoid; applied voltage, 25 kV (pH 5.0), 18 kV (pH 7.0). Other conditions as in Fig. 2a.

Parameter	pH 5			
	50 m*M* acetate	50 m*M* phosphate	50 m*M* phosphate –0.5 *M* PEA	
t_1	8.63	8.97	8.84	
t_2	8.69	9.08	8.96	
N_1	130 800	113 200	210 300	
R_s	0.62	1.07	1.56	
	pH 7			
	50 m*M* phosphate	50 m*M* phosphate –100 m*M* borate	50 m*M* Tris –50 m*M* HCl	50 m*M* phosphate –50 m*M* CHES
t_1	4.75	6.78	7.27	4.52
t_2	4.97	–	–	4.65
N_1	10 800	183 400	4600	14 000
R_s	1.18	0	0	0.87

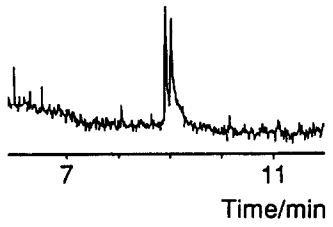

Fig. 4. Optical resolution of EP by EKC. Conditions: separation solution, 50 μM ovomucoid in 0.5 M PEA–50 mM phosphate buffer (pH 5.0); applied voltage, 25 kV. Other conditions as in Fig. 2.

addition of PEA at a higher concentration clearly reduced the peak tailing (Fig. 4, $N_1 >$ 200 000). This result suggested that the zwitterion at higher concentrations suppressed the peak tailing considerably. Thus, the addition of PEA enhanced the separation efficiency, but the day-to-day reproducibility of the optical resolution with PEA was poor, because PEA was unstable at room temperature.

Reproducibility

As mentioned above, the electroosmotic flow decreased gradually unless the capillary was washed with a detergent after each run with uncoated capillaries. Leaving the detergent solution for 5 min in the capillary was important to improve the reproducibility in the washing procedure described under Experimental. Nevertheless, it was insufficient to obtain constant results (R.S.D. > 5%). In protein analysis by CE, many strategies for preventing adsorption of proteins have been developed [31]. Among them, two approaches were selected by considering that the pH variation of the separation solutions was needed. One was the use of capillaries coated covalently with hydrophilic polymers and the other was the use of dynamically coated capillaries. In the former approach, a commercially available PEG-coated capillary was employed, and in the latter, hydroxypropylcellulose (HPC) was added to the separation solutions. The PEG-coated capillary has been employed in EKC with bovine serum albumin (BSA) and significantly improved the R.S.D. values for an optical isomer [26]. This also gave better reproducibility in this instance, as shown in Table III. Better reproducibility (Table III) was obtained in the separations with HPC, which is known as a suppressor of electroosmosis. In enantiomeric separations with BSA, uncoated capillaries allowed only 10–15 injections [26], whereas the separation with ovomucoid allowed more than 200 runs with the use of uncoated capillary without any additives. Therefore, it was suggested that the adsorption of ovomucoid on the wall was not so strong as that of BSA. This might be a consequence of the higher carbohydrate content of ovomucoid [11].

Effect of concentration of ovomucoid

Fig. 5 shows the dependence of separation selectivity on the concentration of ovomucoid. An increase in the concentration of ovomucoid resulted in a decrease in the apparent mobilities of the isomers and enhanced resolution. In this instance, it was also observed that the peak tailing became serious with an increase in $k^{*\prime}$, that is, an increase in the fraction of the solutes interacting with ovomucoid. Table IV shows the dependence of the relative capacity factor on the concentration of ovomucoid. A negative value of $k_1^{*\prime}$ was obtained according to eqn. (2) at 25 μM ovomucoid. This was because μ_{ep}(s) in the presence of ovomucoid might be higher than μ_{ep}^{*}(s) and this might be caused by the increase in the permittivity of the separation solutions.

TABLE III

REPRODUCIBILITY OF MIGRATION TIMES OF TP ENANTIOMERS

Separation solution, 50 μM ovomucoid in 10 mM phosphate (pH 5.7); applied voltage, 25 kV. Other conditions as in Fig. 2a.

Parameter	R.S.D. (%) ($n = 5$)	
	PEG-coated capillary	Uncoated capillary with 0.25% HPC
t_1	2.85	0.25
t_2	4.41	0.23
t_0	–	0.80

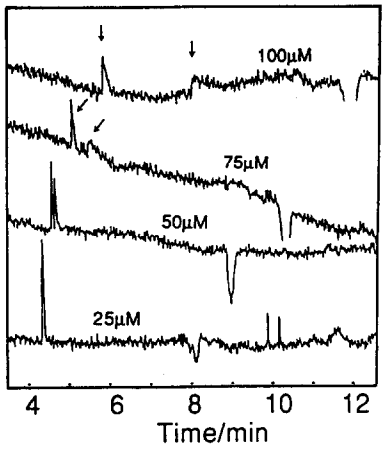

Fig. 5. Effect of concentration of ovomucoid on the separation selectivity of TP. Conditions: separation solution, 60 μM ovomucoid in 10 mM phosphate buffer (pH 5.5) containing 0.1% HPC; applied voltage, 25 kV. Other conditions as in Fig. 2.

Effect of organic modifiers

With an ovomucoid-bonded column in HPLC, a fairly strong hydrophobic interaction with basic solutes has been observed and addition of some organic modifiers has been found to be effective in controlling retention times [7]. In analogy with HPLC, 2-propanol was added to the separation solution. The peak shape was improved significantly, while the separation factor was diminished. This result suggested a possibility of improving the resolution, that is, if the concentrations of ovomucoid and organic modifiers are properly adjusted, baseline separations of optical isomers could be achieved with higher plate numbers.

TABLE IV

DEPENDENCE OF CAPACITY FACTOR ON CONCENTRATION OF OVOMUCOID

Conditions as in Fig. 6.

Ovomucoid concentration (μM)	$\Delta k_1^{*\prime}$ [a]	$\Delta k_2^{*\prime}$ [a]
25	0	0
50	0.006	0.028
75	0.044	0.155
100	0.176	0.758

[a] $\Delta k_i^{*\prime} = k_i^{*\prime}$ (x μM) $- k_i^{*\prime}$ (25 μM) ($i = 1$ or 2, $x = 25$, 50, 75, 100), $k_1^{*\prime}$ (25 μM) $= k_2^{*\prime}$ (25 μM) $= -0.025$.

TABLE V

EFFECT OF ORGANIC MODIFIERS ON SEPARATION EFFICIENCY OF EP

Separation solution, 250 μM ovomucoid in 10 mM phosphate (pH 5.0); sample solution, 0.5 mM. Other conditions as in Fig. 2a.

Parameter	No modifier	5% ethanol	5% acetonitrile	5% 2-propanol
t_1	9.84	8.81	7.99	8.35
t_2	12.13	9.54	8.86	8.58
N_1	1500	16400	19600	83700
R_s	2.28	2.64	3.78	1.96

The effects of different organic solvents added to the separation solution at a high concentration of ovomucoid were examined. Table V shows that 2-propanol was most effective in increasing the plate number with baseline separation (Fig. 6). As was expected, an increase in 2-propanol concentration led to a higher plate number with a slight decrease in the enantioselectivity (Figs. 7 and 8). Then EKC separation with 2-propanol and a high concentration of ovomucoid was employed to separate CP enantiomers, which could not be detected without organic modifiers

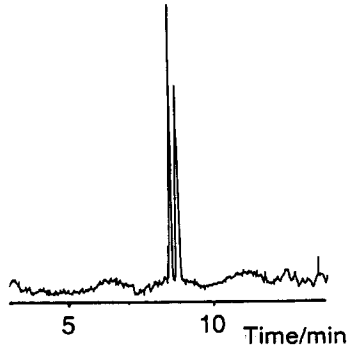

Fig. 6. Optical resolution of EP by EKC. Conditions: separation solution, 250 μM ovomucoid in 10 mM phosphate buffer (pH 5.0) containing 5% 2-propanol; sample concentration, 0.5 mM. Other conditions as in Fig. 2.

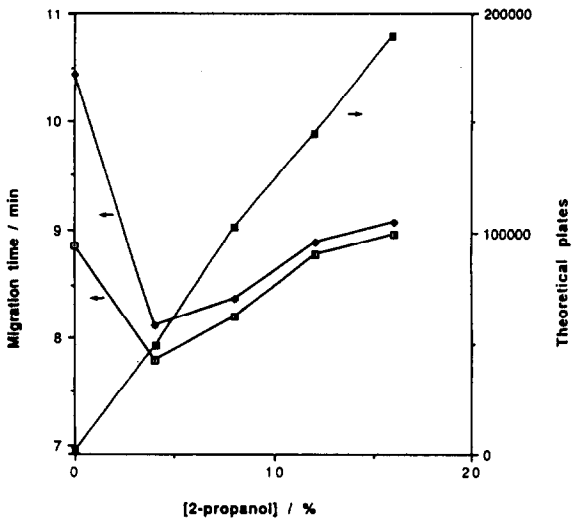

Fig. 7. Effect of 2-propanol on the separation efficiency of EP. Conditions: 250 μM ovomucoid in 10 mM phosphate buffer (pH 5.0) containing 2-propanol. Other conditions as in Fig. 6. ■ = N_1; □ = t_1; ◆ = t_2.

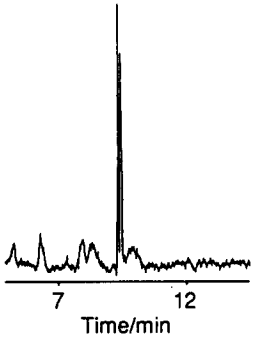

Fig. 8. Optical resolution of EP by EKC. Conditions: separation solution, 250 μM ovomucoid in 10 mM phosphate buffer (pH 5.0) containing 16% 2-propanol. Other conditions as in Fig. 6.

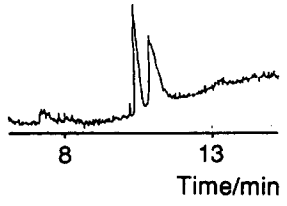

Fig. 9. Optical resolution of chlorpheniramine by EKC. Conditions: separation solution, 250 μM ovomucoid in 10 mM phosphate (pH 5.0) containing 9% 2-propanol; sample concentration, 1 mM. Other conditions as in Fig. 6.

because of seriously broadened peaks. The baseline separation of CP was achieved by the addition of 9% 2-propanol to 250 mM ovomucoid solution (Fig. 9). This result suggested that the organic modifiers reduced the strong hydrophobic interaction between the solute and ovomucoid and enhanced the equilibrium rate of the interaction.

CONCLUSIONS

Ovomucoid was employed as a pseudo-stationary phase in EKC to separate optical isomers. Successful results were obtained when HPC and organic solvents or zwitterions were added to the separation solution. The conditions are easily optimized by adjusting both the concentration of ovomucoid and the amount of of organic modifiers. The general advantages of HPCE, such as a short analysis time, high resolution and lower running costs, are also maintained in this approach. Moreover, this system can be easily prepared for chiral recognition compared with conventional chromatographic systems using CSPs in HPLC, because ovomucoid is dissolved in the running buffers in the CE system. In analogy with ovomucoid, other proteins, polysaccharides and other biopolymers could be used as chiral pseudo-stationary phases. However, ovomucoid is potentially useful for EKC because it is relatively stable to variations in pH, to heating and to organic solvents and may be less adsorptive on the wall than BSA. Judging from the wider applications of ovomucoid-conjugated columns in HPLC [11], the approach described here is expected to be applicable to a wide range of optical isomers.

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