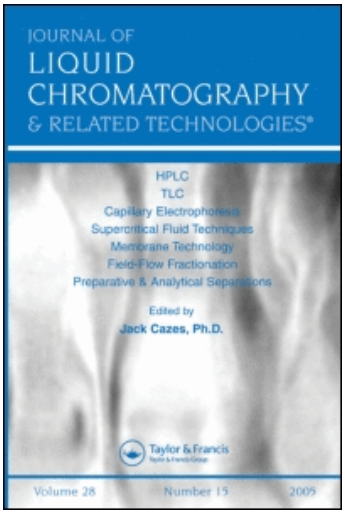

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Chiral Separation of Enantiomers of a Plant Growth Regulator, Abscisic Acid, by Capillary Electrophoresis with Cyclodextrin Additives

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ABSTRACT

A capillary electrophoresis method for the chiral separation of enantiomers of a plant growth regulator—abscisic acid (ABA) was developed. Five cyclodextrins (CDs) were applied as chiral selectors and it was found that dimethyl-β-cyclodextrin (DM-β-CD), hydroxypropyl-β-cyclodextrin (HP-β-CD), and γ-cyclodextrin (γ-CD) could give satisfactory enantioselectivity. The effects of concentration of the CD, pH value of the buffer, the voltage applied, and the addition of methanol on the chiral separation, were investigated. The best resolution obtained was 3.0, with a 100 mM Tris-phosphate buffer (pH 5.90) containing 30 mM HP-β-CD, and an

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applied voltage at 15 kV. The reproducibilities of migration times and peak areas were better than 0.6% and 3.2%, respectively. The detection limit of each enantiomer of ABA was 0.30 μg/mL.

Key Words: Abscisic acid; Capillary electrophoresis; Cyclodextrin; Chiral separation.

INTRODUCTION

Abscisic acid (ABA) is a naturally occurring plant growth regulator. Since 1963 when it was found for the first time from the young fruits of cotton, many studies on its physiological properties have shown that ABA has important effects on controlling the physiological processes of plant, such as: the acceleration of abscission, the induction of seed dormancy, and the defense against environmental stress, etc.[1] Natural ABA is optically active and exists only in the form of *S*-(+)-ABA, however, synthetic ABA is the raceme of *S*-(+) and *R*-(−) isomer. The biological activities of ABA enantiomers are greatly different. The activity of *S*-(+)-ABA is 3–5 fold, or even more in some cases, greater than that of the *R*-(−) enatiomer.[2] And the two enantiomers are degraded in different routes and at substantially different rates in plant tissue.[3] Consequently, chiral separation and testing of ABA enantiomers are becoming necessary to ensure accurate biological studies and applications of this compound.

So far the chiral resolution of racemic ABA has been described in a few reports. The HPLC chiral resolution of ABA on a Nuleodex *β*-PM column was investigated by Kramell et al.[4] Not long before, capillary electrophoresis (CE) enantioseparation of ABA, with macrocyclic antibiotics as chiral selectors, was reported by Hui et al.[5] Cyclodextrins (CDs) are the most popular chiral discrimination agents in CE because of their demonstrated success in many applications, such as chiral drugs,[6,7] pesticides,[8] amino acids,[9] and neurotransmitters,[10] etc., their low absorption in UV region, and their ease of handling, i.e., by simply adding to the buffer electrolyte directly. So, we assumed that compounds of this class be potential chiral selectors for the separation of ABA optical isomers by CE.

In this work, CE with five CDs, namely *β*-cyclodextrin (*β*-CD), *γ*-cyclodextrin (*γ*-CD) and their derivatives, heptakis (2,6-di-*o*-methyl)-*β*-cyclodextrin (DM-*β*-CD), hydroxylpropyl-*β*-cyclodextrin (HP-*β*-CD), and hydroxylpropyl-*γ*-cyclodextrin (HP-*γ*-CD) were applied to the chiral resolution of ABA. The effects of parameters such as CD concentration, buffer pH, and applied voltage were investigated in order to obtain optimum chiral resolution conditions.

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EXPERIMENTAL

Apparatus

The CE instrument used in this study is a Binda 1229 system (Beijing Institute of New Technology, Beijing, China) equipped with a fixed wavelength detector (254 nm) and an integrator of model 9423. The separation was performed in a fused silica capillary column (70 cm × 50 μm i.d., effective length 55 cm) (Supelco, USA).

Chemicals

(±)Abscisic acid (>99% purity) was purchased from Fluka (Buchs, Switzerland). β-CD and heptakis (2,6-di-*o*-methyl)-β-cyclodextrin was from Sigma (St. Louis, MO), and γ-cyclodextrin was from Fluka (Buchs, Switzerland). Hydroxylpropyl-β-cyclodextrin and HP-γ-CD were obtained from Aldrich (Milwaukee, MI). Tris (hydroxymethyl) aminomethane (Tris) was purchased from Bio-Rad laboratories (Richmond, CA). Phosphoric acid (85%) was obtained from Beijing Chemical Factory (Beijing, China). Methanol of HPLC grade was obtained from J. T. Baker (Phillipsburg, NJ). Water was of Milli-Q quality (Millipore Corporation, Bedford, MA).

Sample and Buffer Preparation

(±)Abscisic acid standard was dissolved in water to make a concentration of 100 μg/mL. Tris-H_3PO_4 buffers were prepared by titrating 100 mM Tris solution with phosphoric acid to the desired pH. The selected CD was dissolved in the above buffer under sonication. The buffers were filtered through 0.45 μm membrane filters prior to use.

Analytical Conditions

The capillary was preconditioned for 15 min with 1 M NaOH before the first run, and was purged for 5 min with 0.1 M NaOH, 5 min with water and 30 min with run buffer at the beginning of each working day. Between each run the capillary was flushed with buffer for 3 min. Sample was injected at a height of 10 cm for 20 sec and the detection was on the cathode side. All separations were carried out at ambient temperature of 18°C.

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RESULTS AND DISCUSSION

Selection of Buffer System and pH

Initially, two buffer systems, 100 mM phosphate and 100 mM Tris-phosphate, were investigated with 10 mM of different CDs as buffer additives. These CDs included β-CD, DM-β-CD, HP-β-CD, γ-CD, and HP-γ-CD. Generally, there was no essential difference on separation between the two buffer systems. However, Tris-phosphate provides a lower current since Tris is a low mobility cation compared to sodium, and the peak shapes were better in some cases when using Tris-phosphate buffer. Hence, 100 mM Tris-phosphate buffer was chosen to carry out subsequent experiments.

Buffer pH is an important parameter in CZE, since alternations in pH can affect the solute charge and change the electroosmotic flow (EOF), thus influencing the resolution. Abscisic acid (Fig. 1) is a weak acid and its pK_a value is not available from reference. In this study, the pH effect on separation was tested over the range of 4.8–9.0. When the pH of buffer was below 4.8, the enantiomers could not be resolved at all. Separation was not possible at low pH, since the solutes were neutral with only neutral cyclodextrins used. As indicated in Fig. 2, the separation increased fast with the increase of buffer pH and reached its maximum around pH 5.90 with 30 mM HP-β-CD in the buffer. The observed migration time decreased with increasing buffer pH and the further increase of the pH above 5.90 resulted in a slowly decrease of resolution. This can be explained by the faster EOF at higher pH and the consequent shortened time for the interaction of the analyte with the CD. Thus, the pH of 5.90 was thought to be most favorable to the separation, for it could ensure the ABA ionized and provided a moderate EOF velocity. The following studies were conducted by keeping the pH of running buffer at 5.90.

Effect of Cyclodextrin Type and Concentration

The enantioselectivities of five selected CDs as chiral additives were evaluated over the concentration range of 5–15 mM for β-CD and 5–30 mM for DM-β-CD, HP-β-CD, γ-CD, and HP-γ-CD. For calculation of resolution (Rs),

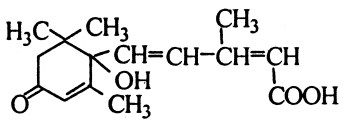

Figure 1. The molecular structure of ABA.

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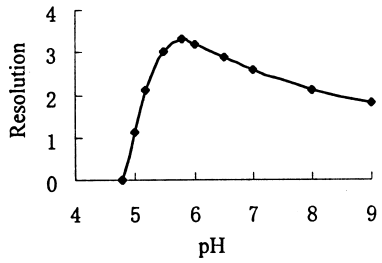

Figure 2. Effect of pH value of the buffer on the resolution. Buffer: 100 mM Tris-phosphate with 30 mM HP-β-CD as additive; Column: 50 μm i.d., 55.0 cm effective, and 70 cm overall length; Detection wavelength: 254 nm; Applied voltage: 15 kV.

the following equation was used: $Rs = 1.18(t_2 - t_1)/(W_{1/2\ (1)} + W_{1/2\ (2)})$. No chiral recognition was observed for ABA enantiomers when β-CD was used. Separations of different degrees (Rs = 0.6–3.0) were obtained when DM-β-CD, HP-β-CD, γ-CD, and HP-γ-CD were used. The effect of CD type and concentration was shown in Table 1. As can be seen, the concentration of DM-β-CD and HP-γ-CD had little effect on the separation of ABA enantiomers. But, the resolution significantly increased with increasing the concentration of HP-β-CD, and slightly decreased with increasing the concentration of γ-CD. Figure 3 depicts the results obtained from experiments with the selected CDs (except β-CD). Hydroxypropyl-γ-Cyclodextrin could cause peak splitting but could not give baseline separation. Successful chiral resolutions were achieved with DM-β-CD, HP-β-CD, and γ-CD. The best concentration for these three CDs was 10 mM, 30 mM, and 5 mM, respectively.

Table 1. Effect of CD type and concentration on resolution of ABA enantiomers.

Concentration of CD (mM)	Resolution				
	β-CD	DM-β-CD	HP-β-CD	γ-CD	HP-γ-CD
5	0	1.9	1.0	2.2	0.6
10	0	2.0	1.9	2.0	0.6
15	0	2.0	2.3	1.9	0.6
20		1.9	2.7	1.9	0.6
30		1.9	3.0	1.8	0.6

Note: Buffer: 100 mM Tris-phosphate of pH 5.90. Other conditions as described in Fig. 2.

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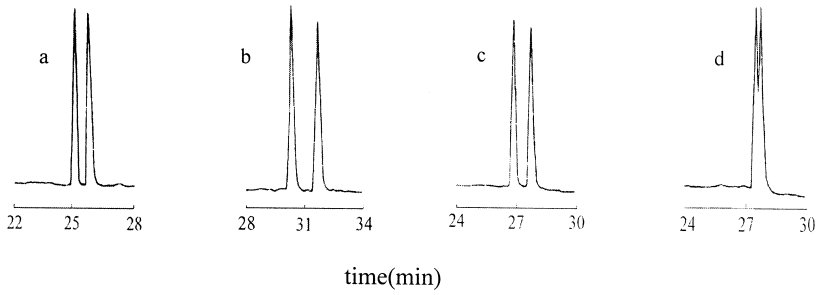

Figure 3. Separation of ABA Enantiomers. (a) DM-β-CD, 5 mM; (b) HP-β-CD, 30 mM; (c) γ-CD, 5 mM; (d) HP-γ-CD, 10 mM. Buffer: 100 mM Tris-phosphate of pH 5.90. Other conditions as described in Fig. 2.

Effect of Applied Voltage

The applied voltage mainly influences the electroosmotic flow velocity, the Joule heating, and peak efficiency. It was noted, that the migration time was as long as more than 30 min when the voltage was below 15 kV. The migration time reduced significantly when the voltage was increased. As a result, the resolution was slightly influenced by the voltage in the range of 15–25 kV. The resolution started to decline beyond 15 kV, as shown in Fig. 4. It was unlikely that there was excessive Joule heat, which could lead to the reduction in resolution since the migration current increased from 14 μA at 10 kV to 46 μA at 25 kV, with the relationship between current and voltage approximately running a plot following Ohm's law in this investigation. Although, column separation efficiency is favored by high voltage, an increased EOF is unfavorable for the difference in the apparent electrophoretic

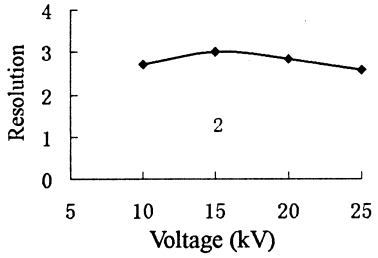

Figure 4. Effect of applied voltage on the resolution. Buffer: 100 mM Tris-phosphate of pH 5.90 containing 30 mM HP-β-CD. Other conditions as described in Fig. 2.

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mobility between two analytes when enantiomer–cyclodextrin complexes are formed.[11] In this experiment, EOF increased with increasing voltage and might play a more dominant role in affecting the enantioseparation. Fifteen Kilo Volt was selected as the optimum value for applied voltage.

Effect of Methanol

Previous studies[12,13] have shown that the use of methanol and other organic solvents could lead to either an increase in enantiomeric resolution or an opposite effect. Bachet et al.[12] observed that when the CD concentration was equal to or below the optimum value for a methanol-free buffer, the addition of methanol should lead to a decrease of the apparent mobility difference between the enantiomers, and hence, of the chiral resolution. On the contrary, at cyclodextrin concentration above the optimum value for a solvent-free buffer, chiral resolution should increase by addition of methanol to the buffer. In our study, the effects of methanol addition were briefly investigated by experiments that were conducted with 5%, 10%, or 20% methanol added to each buffer, which contained 10 mM of one of the selected five CDs, respectively. The addition of methanol caused the increases in migration time, peak interval, and peak width, which correlated with the amount of methanol added. This can be explained by the fact that methanol can increase the electrolyte viscosity causing an increase in frictional drag. However, there was no qualitative change on the chiral separation with addition of methanol. β-Cyclodextrin and HP-γ-CD still could not separate the ABA enantiomers as without methanol. The resolution with γ-CD as an additive was slightly increased and the resolutions with the other two CDs slightly decreased, in accordance with Bachet's explanation. No significant difference was found between the separations with different amounts of methanol added.

Reproducibility and Detection Limit

Experiments on reproducibilities in migration times and peak areas with 30 mM HP-β-CD as additive, and other conditions optimized, were also carried out. For five consecutive runs, RSDs of migration times of both isomers were better than 0.6% at an injection concentration of 10 μg/mL. RSDs of peak areas were below 3.2%. Since none of the standards of the optical isomers was available, the detection limit of each enantiomer was estimated to be half of the minimum injection concentration of ABA raceme, supposing the two isomers existed equally in the synthesized ABA product. By detection at 254 nm, the detection limit of each enantiomer was

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0.30 μg/mL, calculated on the basis of single noise ratio (S/N) of 3. Since the sensitivity of UV detection in CE is limited by the short optical path, a sample pre-capillary concentration, or on column concentration, is often required for identification or quantification purpose. For plant hormone determination by CE, the sample-stacking method could be employed to enhance the sensitivity with the detection limit down to ng/mL level.[14]

CONCLUSIONS

This paper shows, that the enantioseparation of the plant growth regulator abscisic acid can be successfully achieved by CE, with DM-β-CD, HP-β-CD or γ-CD as additives. Optimum separation conditions found were a 100 mM Tris–phosphate buffer of pH 5.90, and an applied voltage of 15 kV. Addition of methanol to the buffer could not essentially affect the separation. The best separation was reached by using 30 mM HP-β-CD with a resolution of 3.0. The reproducibilities of migration times and peak areas of the two enantiomers were good, and detection limits were acceptable.

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