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A chiral ligand exchange CE system for monitoring inhibitory effect of kojic acid on tyrosinase

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ABSTRACT

A facile chiral ligand exchange capillary electrophoresis (CLE-CE) system with Zn(II)-L-alanine as the chiral selector in the presence of β-cyclodextrin has been developed for enantioseparation of dansyl amino acids. The influence of the key factors, such as buffer pH, the ratio of Zn (II) to ligand, the concentration of β-cyclodextrin and the concentration of the complex, were investigated in detail when D, L-Tyr and D, L-Thr were selected as the model analytes. The proposed method showed favorable quantitative analysis property of dansyl D, L-tyrosine with good linearity ($r^2 \geq 0.999$) and reproducibility (RSD $\leq 3.8\%$), then, it was applied in studying the activity of tyrosinase through the determination of L-tyrosine concentration variation after being incubated with the enzyme. Further, the inhibitory efficiency of kojic acid and soy sauce on the tyrosinase was investigated. The IC_{50} of kojic acid obtained from the sigmoidal inhibitory curve was 21.35 μM. The results imply that the proposed CLE-CE system has the potential in exploring the activity of enzyme and screening the inhibitors of enzyme.

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1. Introduction

Pigmentation is one of the most obvious phenotypical characteristics in the natural world. Enzymatic browning in plants, animals and microorganisms is a typical pigmentation that exists in vivo [1,2]. As a multifunctional copper-containing enzyme, tyrosinase can catalyze both the hydroxylation of monophenols to *o*-diphenols and the oxidation of *o*-diphenols to *o*-quinones [3,4]. The produced *o*-quinones in this reaction is highly reactive compound and can spontaneously polymerize to form melanin, which widely spreads in skin, hair and eyes of mammals. Moreover, this highly active *o*-quinone also can react with amino acids (AAs) and proteins in vegetables and fruits which enhance the development of melanin-related browning. Thus browning reaction becomes a big problem in the food industry and one of the main causes of quality loss during post-harvest handling and processing [5,6]. The inhibitors of tyrosinase are effective in preventing browning of food products and inhibiting the produce of melanin in medicinal and cosmetic products. Thus, the exploration of new and available tyrosinase inhibitors is of great significance.

Various tyrosinase inhibitors, such as chalcones, benzoic acid, ascorbic acid, trifluoroethanol have been reported in literatures [7–10]. As a typical non-competitive inhibitor of tyrosinase, kojic acid has been widely used as depigmenting agent in cosmetics and as food additive in food machining industry [11–13]. Moreover, soy sauce is a familiar condiment which contains a certain amount of kojic acid [14]. Study of the inhibitory efficiency of kojic acid and soy sauce on tyrosinase is meaningful for exploring the inhibition of browning reaction.

So far, UV-spectrophotometry has been widely used to estimate the inhibitory of kojic acid for tyrosinase [15,16]. As a fundamental detection method, UV-spectrophotometry exhibits a lot of favorable advantages, such as easy operation, high reproducibility and high precision [17]. However, this method presents several limitations and drawbacks. For example, it demands consecutive solvent extractions of analytes, consumes a large amount of analytes and exhibits high susceptibility to interference [18]. Therefore, developing a simple and sensitive method to evaluate the tyrosinase inhibitors is of great urgency.

Capillary electrophoresis (CE) as a method which is highly efficient, fast and low cost has attracted much interest and has been widely used to analyze AAs, proteins, DNA and drugs [19–22]. AAs are one of the foremost types of chiral molecules for life sciences. As one kind of chiral separation mode used in CE, chiral ligand exchange

Abbreviations: AAs, amino acids; CE, capillary electrophoresis; CLE, chiral ligand exchange; Tyr, tyrosine; L-Ala, L-alanine; Dns-AA, dansylated-AA; Dns-Cl, dansyl choride; Tris, Tris(hydroxymethyl)aminomethane; β-CD, β-cyclodextrin; *Rs*, Resolution; Thr, threonine; LOQ, limit of quantitation; LOD, limit of detection; RSD, relative standard deviation

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(CLE) is widely used in the enantioseparation of D, L-AAs because of its advantages of high convenience, low cost, environment friendly and controllable enantiomer migration order [23,24]. Using D, L-AA as substrate to develop a simple and sensitive enzyme kinetic method with a useful and fast chiral separation technique is deserved to be researched. Moreover, it has been reported that L-tyrosine (L-Tyr) is the specific substrate for tyrosinase [25]. Therefore, building a CLE-CE system to investigate the activity of tyrosinase through monitoring the concentration variation of L-Tyr after incubation, inhibitors and kinetics of tyrosinase is available. More importantly, so far to our knowledge, the analysis of kojic acid as an inhibitor of tyrosinase by CLE-CE method has not been explored. Herein, exploration of the new, simple and potential method for determining and assessing kinetics and inhibitor of tyrosinase is very meaningful.

In this work, a new CLE-CE system which employs Zn(II)-L-alanine (L-Ala) complex as the chiral selector has been developed for quantitative analysis of dansylated D, L-AAs (Dns-D, L-AAs). After quantification of D, L-Tyr by the proposed CE-CLE system, the kinetics of tyrosinase and the inhibitory efficiency of kojic acid have been investigated. Furthermore, we have studied the inhibitory efficiency of soy sauce which consists of kojic acid. The system has been testified to be powerful in measuring of tyrosinase enzyme kinetic constant, indicating that the method can be potentially adaptable to study the enzyme mechanism and inhibitors.

2. Materials and methods

2.1. Chemical reagents and instruments

Tyrosinase, dansyl choride (Dns-Cl) and all D, L-AA standards were obtained from Sigma-Aldrich Chemical Company (St. Louis, USA). Tris(hydroxymethyl)aminomethane (Tris), lithium carbonate, zinc sulfate, boric acid, ammonium acetate, β-cyclodextrin (β-CD) and other chemicals were all of analytical reagent grade from Beijing Chemical Factory (Beijing, China). Kojic acid was purchased from Aladdin Chemistry Company (Shanghai, China). Soy sauce was obtained from local supermarket.

All CE experiments were conducted with the 1229 HPCE Analyzer (Beijing Institute of New Technology and Application, Beijing, China). The separations were carried out in fused silica capillaries (75 μm i.d.) with a total length of 65 cm and an effective length of 50 cm obtained from Yongnian Optical Fiber Factory (Hebei, China). A distillation apparatus model SZ-97 (Yarong Biochemical Instrument Co., Shanghai, China) was used for manufacturing triple distilled water.

2.2. Sample preparation and dansylation of D, L-AAs

All aqueous solutions were prepared with triply distilled water and stored at 4 °C. Standard sample solutions were prepared by dissolving 2.0 mg/mL D, L-AAs in 40.0 mM lithium carbonate buffer (adjusted to pH 9.5 with 0.1 M HCl), and diluted by 10–10^4 fold to get degassed work solutions with 40.0 mM lithium carbonate. Derivative solution was freshly prepared by dissolving 6.0 mg Dns-Cl in 4.0 mL acetone. The dansylation of D, L-AAs was conducted according to literature [26]. In brief, 20 μL 40.0 mM lithium carbonate buffer, 20 μL D, L-AA solution (2.0 mg/mL) and 20 μL Dns-Cl solution were mixed in a 200 μL vial and kept at room temperature in the dark for 30 min, and then 5 μL 2.0% ethylamine was added to terminate the reaction. The reacted solution was either directly injected for CE separation or kept at 4 °C.

2.3. Determination of kinetics and inhibitor of tyrosinase

The D, L-Tyr and tyrosinase were dissolved in buffer (0.05 M boric acid 0.025 M phosphate acid adjusted to pH 7.4 with 1.0 M potassium hydroxide). All enzymatic reactions were performed at 25 °C. The concentration of tyrosinase is 146 U/mL. Being added to 0.2 mL polypropylene tubes, 40 μL D, L-Tyr and 40 μL tyrosinase were mixed and incubated for 20 min. After the incubation, the solutions were centrifuged at 10,000 rpm for 10 min. Then the supernatants were sucked, dansylated and applied to CE. The kinetic study of tyrosinase was initiated by adding D, L-Tyr solution of various concentrations (0.41 mM, 0.50 mM, 0.62 mM, 0.83 mM and 1.24 mM) into the tyrosinase solution.

For studying the inhibitory efficiency of kojic acid and soy sauce, different concentrations of 40 μL kojic acid or 5.0 mg/mL soy sauce, 40 μL D, L-Tyr (0.83 mM each) and 40 μL tyrosinase (48.67 U/mL) solution were mixed together. Then the mixture was incubated for 20 min at 25 °C and disposed by the same procedures as described above. Soy sauce was filtrated and vaporized. Then it was dried at 50 °C for 12 h and then the residual solid was dissolved by water and diluted to the desired concentrations.

2.4. CE analysis

Dns-D, L-AAs samples were siphoned to the capillary for 8.0 s at 15.0 cm height and separated at −21 kV [23]. Prior to injection, the bare fused-silica capillary was sequentially rinsed with 1.0 M NaOH, water and running buffer for 2 min. UV absorption at 254 nm and acquired at 4 Hz were selected to detect the separated bands.

Resolution (*Rs*) of Dns-D, L-AA enantiomers was calculated by using the following equation:

$$R_S = 2(t_2 - t_1)/(w_1 + w_2)$$

where t_1, t_2 represent the migration times of D and L-analytes, respectively. The w_1, w_2 are the base line peak width of corresponding enantiomers.

The velocity of tyrosinase-catalyzed reaction (V) was calculated from the decreased amount of L-Tyr in the enzymatic reaction. We defined the velocity by the following formula:

$$V = C_0 - C_E/t$$

where C_0 is the initial concentration of L-Tyr, C_E stands for the residual concentration of L-Tyr after being incubated with tyrosinase, t is the incubation time.

The following formula was used for calculating the inhibition efficiency:

$$I\% = C_I - C_E/C_0 - C_E \times 100\%$$

where $I\%$ stands for the inhibition efficiency and C_I is the residual concentration of L-Tyr in the presence of an inhibitor, while C_E is the residual concentration of L-Tyr without an inhibitor and C_0 represents the concentration of L-Tyr in the absence of tyrosinase and inhibitor.

3. Results

3.1. Optimization of separation conditions

According to previous work [23,27,28], Zn(II)-based CLE-CE was thus adopted for the analysis of Dns-D, L-AAs. In this study, L-Ala was selected as the ligand for chiral separation. By using, Dns-D, L-Tyr and Dns-D, L-threonine (Dns-D, L-Thr) as the model analytes, the effects of the key parameters, such as buffer pH, the ratio of Zn (II) to L-Ala, the concentration of β-CD and the concentration of Zn (II) complex, on the separation efficiency have been investigated in detail.

3.1.1. Effect of pH

Due to its significant influence on the complexation reaction between the central ion, the chiral ligand, the dissociation of analytes and the silanol groups on the inner capillary surface, the pH value of the running buffer plays a crucial role in the CLE-CE separation mode. Therefore, the influence of pH on the migration times and the *Rs* was investigated in the range from 7.8 to 8.6. As displayed in Fig. 1, *Rs* and the migration times both increased with the increase of pH. Taking the CE requirements of high resolution and fast speed into consideration, pH at 8.2 was finally selected for further analysis.

3.1.2. Effect of the ratio of Zn(II) to L-Ala

As the complex of Zn(II) and L-Ala functions as the chiral selector, thus the concentration ratio of Zn(II) to L-Ala has a great impact on *Rs* and the migration time. Its influence was investigated by altering the concentration of L-Ala from 1.3 mM to 12.0 mM with Zn(II) at the constant concentration of 4.0 mM. As shown in Fig. S1, it could be found that both *Rs* and the migration times increased with the increase of the ratio of Zn(II) and L-Ala. To get the high *Rs* in a short time, the ratio of 1:2 was finally selected.

3.1.3. Effect of the concentration of Zn(II)

In the CLE-CE system, the ligand concentration is key effect to regulate *Rs*. As a result, the influence of concentration of Zn(II) was studied from 0 to 6.0 mM while the ratio of Zn(II) to L-Ala was kept at 1:2. Table 1 showed that the *Rs* of D, L-AAs were less than 1.0 without the addition of ligand complex in the running buffer. While when the concentration of Zn(II) increased from 2.0 to 6.0 mM, the *Rs* increased at first until achieved the highest value of 3.0 mM, and then decreased. However, the migration times decreased. Finally, 4.0 mM Zn(II) was selected as the optimum condition for the chiral separation in CLE-CE.

3.1.4. Effect of the concentration of β-CD

Different kinds of CDs and its derivatives are commonly used in chiral separation because of their intrinsic chirality [29–32]. As reported in the literatures that the CDs could act as an additional ligand toward the central metal ions for CLE-CE [28,33], thus the impact of β-CD concentration on *Rs* was investigated. Table 1 displayed that the presence of β-CD in the electrolyte buffer could improve the separation efficiency. As shown in Fig. S2, it could be observed that with the increase of β-CD concentration, both *Rs* and the migration times increased. For achieving better separation in a short time, 4.0 mM β-CD was finally chosen as the optimum condition for further study.

Based on the experimental results, it could be easily found that the optimal buffer was consisted of 100.0 mM boric acid, 5.0 mM ammonium acetate, 4.0 mM Zn(II) and 8.0 mM L-Ala and 4.0 mM β-CD at pH 8.2. Under the optimal condition, the well-separated electrophoretogram of the two model analytes was shown in Fig. 2. Meanwhile, the separation of other AA enantiomers was investigated, 12 pairs of D, L-AA enantiomers could be effectively separated with 5 pairs baseline separated and 7 pairs partially resolved with β-CD acting the role as an additional ligand [28] (Table S1 and Fig. S3). Since Dns-D, L-Tyr could be baseline separated, thus the CLE-CE method could be used to explore the enzyme kinetics and the inhibitors of tyrosinase in the following study.

3.2. Quantitation feature

Since L-Tyr was the efficient substrate of tyrosinase for investigating the enzyme inhibitor, thus the developed CLE-CE method for quantitative determination of Dns-D-Tyr and Dns-L-Tyr was validated by linearity, detection limit, and reproducibility. To obtain the linear calibration curve, the standard solutions containing Dns-D-Tyr and Dns-L-Tyr were sampled and the resulting peak areas were analyzed. The dynamic linear ranges for both Dns-D-Tyr and Dns-L-Tyr were from 31.04 to 2483 μM, and the typical regression equations for peak areas versus concentrations were as follows: $Y=448.8x+2298.3$ ($r^2=0.999$) for Dns-L-Tyr; $y=447.24+3825.4$ ($r^2=0.999$) for Dns-D-Tyr. The limit of quantitation (LOQ) for both Dns-D- and Dns-L-Tyr was 31.04 μM and the limit of detection (LOD) was 15.52 μM. The run-to-run relative standard deviation (RSD) of migration times was less than 2.4% and that of peak areas was less than 3.8%.

3.3. Kinetics study of tyrosinase

In this work, monitoring of L-Tyr concentration in tyrosinase mediated catalytic reaction and determining of the kinetic constants were investigated by the proposed CLE-CE method. For

Table 1
Influence of Zn(II) concentration on the performance of CLE-CE.[a]

Zn(II) concentration (mM)	Dns-D, L-Thr			Dns-D, L-Tyr		
	t_L (min)	t_D (min)	*Rs*	t_L (min)	t_D (min)	*Rs*[b]
0	4.70	4.73	0.60	5.36	5.39	0.54
2.0	52.33	55.36	1.80	47.56	47.56	1.75
3.0	39.70	41.57	2.17	37.25	37.25	2.47
4.0	29.22	30.03	1.83	30.60	31.33	1.89
5.0	30.92	32.12	1.35	33.62	33.62	1.60
6.0	24.89	25.68	1.10	24.34	24.34	1.00

[a] Running buffer: pH 8.2, 100.0 mM boric acid, 5.0 mM ammonium acetate, 4.0 mM β-CD, the ratio of Zn(II) and L-Ala is 1:2 with different concentration of Zn (II) from 0 to 6.0 mM. Other conditions are the same as indicated in Fig. 1.

[b] $Rs=2(t_D-t_L)/(W_D+W_L)$; t: migration time.

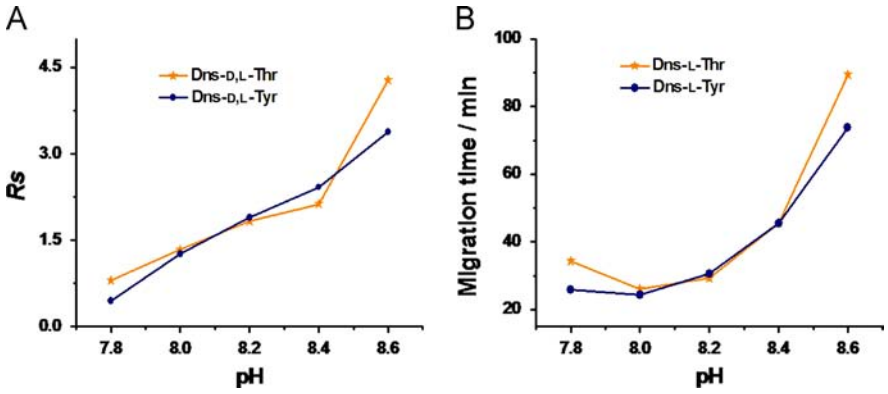

Fig. 1. Influence of pH on *Rs*. Running buffer: 100.0 mM boric acid, 5.0 mM ammonium acetate, 4.0 mM Zn(II) and 8.0 mM L-Ala, 4.0 mM β-CD, adjusted pH from 7.8 to 8.6 with Tris. Capillary: 75 μm i.d. × 65 cm (50 cm effective); voltage: −21 kV; 25 °C; UV detection: 254 nm.

kinetic study, the apparent kinetic parameters, Michaelis–Menten constant (K_m) and the maximum velocity (V_{max}), were estimated by varying substrate concentrations [34,35]. According to the Michaelis–Menten equation, the velocity of tyrosinase-catalyzed reaction was plotted as a function of the L-Tyr concentration. As shown in Fig. 3, the corresponding K_m and V_{max} for the oxidation of L-Tyr catalyzed by tyrosinase were confirmed to be 374.0 μM and 172.0 μM min^{-1}, respectively.

3.4. Tyrosinase inhibition assay

As reported in the previous literatures [15,16], as a good inhibitor for tyrosinase, kojic acid has sufficient inhibitory activity and stability. In Fig. 4, the inhibitory activity of kojic acid against tyrosinase has been investigated. Compared with the blank, the increased peak area of L-Tyr was obtained with the concentration of kojic acid increased. The reacted concentration of L-Tyr could be calculated according to the deviation between the concentration of D-Tyr and L-Tyr after incubation, then the inhibitory effect of kojic acid on tyrosinase was obtained [27]. Fig. 5 displayed that the value of the coefficient of determination (R^2) is 0.959, which means that the selected variable can explain about 95.9% of the variability of the inhibitory effect of kojic acid on tyrosinase mediated reaction. Consequently, the IC_{50} value of kojic acid determined here by using the CLE-CE method with L-Tyr as the substrate, was calculated to be 21.35 μM, which was close to that reported by the previous literature [16,36]. This result indicated that the proposed CLE-CE method could be applied in studying tyrosinase activity by the exact determination of L-Tyr as the substrate.

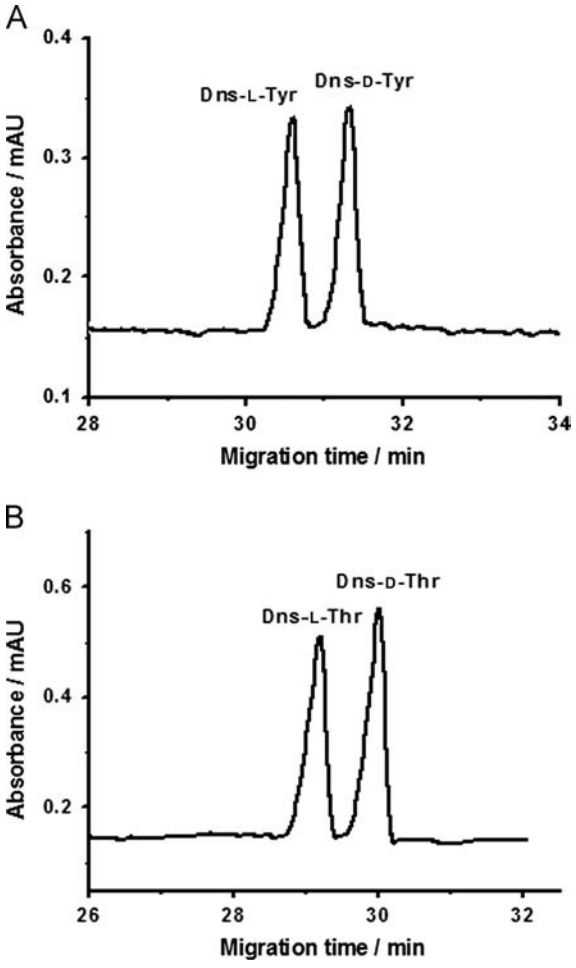

Fig. 2. Electropherograms of (A) Dns-D, L-Tyr, (B) Dns-D, L-Thr. The CLE-CE separation conditions were the same as those in Fig. 1 except at pH 8.2.

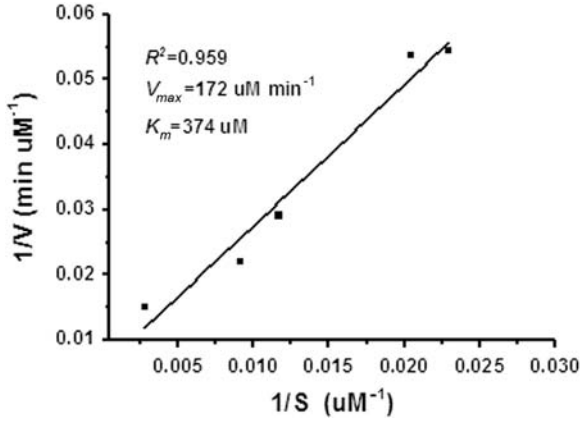

Fig. 3. Lineweaver–Burk plot for the oxidation of L-Tyr catalyzed by tyrosinase (48.67 U/mL) with the concentrations of L-Tyr varied from 0.03 to 1.24 mM. The Michaelis–Menten equation was described as: $v=V_{max}[S]/(K_m+[S])$, where $[S]$ is the concentration of the substrate.

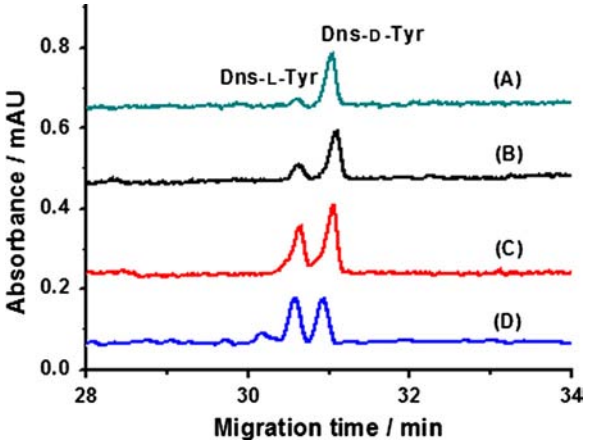

Fig. 4. Electropherogram of Dns-D, L-Tyr in the presence of different amounts of tyrosinase and kojic acid. (A) D, L-Tyr (0.83 mM)+tyrosinase (48.67 U/mL); (B) D, L-Tyr (0.83 mM)+tyrosinase (48.67 U/mL)+kojic acid (5 μM); (C) D, L-Tyr (0.83 mM)+(48.67 U/mL)+kojic acid (35 μM); and (D) D, L-Tyr (0.83 mM).

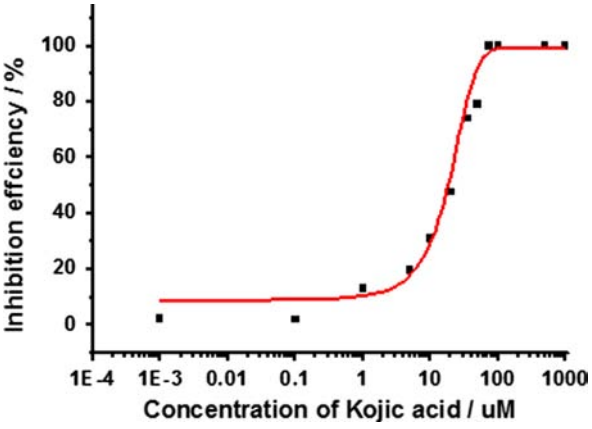

Fig. 5. Inhibitory efficiency of kojic acid for tyrosinase. Dose-dependent inhibition of tyrosinase activity by kojic acid. Incubation condition: D, L-Tyr (0.83 mM) and kojic acid varying from 0.001 to 1000 μM incubated with tyrosinase (48.67 U/mL) for 20 min at 25 °C.

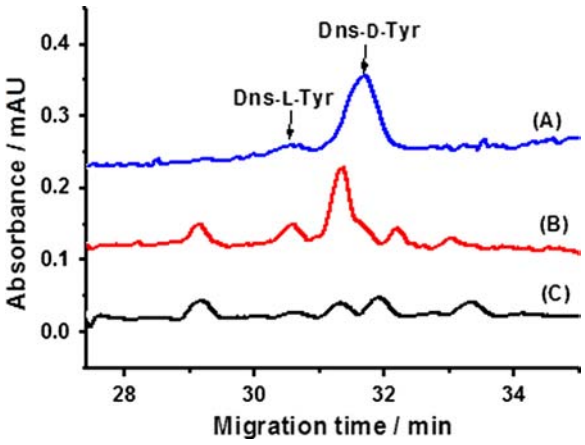

Fig. 6. Inhibitory efficiency of soy sauce for tyrosinase. Incubation condition: (A) D, L-Tyr (0.83 mM)+tyrosinase (48.67 U/mL)+triple distilled water; (B) D, L-Tyr (0.83 mM)+tyrosinase (48.67 U/mL)+soy sauce (1.67 mg/mL); and (C) the blank soy sauce (1.67 mg/mL) sample. Other conditions are the same as those in Fig. 5.

The effectual inhibitor of kojic acid on tyrosinase has been proved in this study. Additionally, it has been reported that soy sauce as a familiar condiment contains a certain amount of kojic acid [14]. For investigating the potential application in real food samples, the proposed CLE-CE method was used to test whether soy sauce had the inhibitory effect on tyrosinase. Compared Fig. 6A with Fig. 6B, it was found that the concentration of L-Tyr in the sample containing soy sauce was higher than that without soy sauce. This phenomenon demonstrated that soy sauce indeed had the effective inhibitory effect on tyrosinase.

4. Conclusion

In this work, Zn(II)-L-Ala complex was used as a novel chiral selector in the presence of β-CD in the CLE-CE system for successful enantioseparation of Dns-D, L-AAs. In efforts to get better enantioresolution, various separation parameters were examined. The method displayed its power in the direct analysis of Dns-D, L-AAs. It was also shown to be applicable to the quantitative analysis of L-Tyr for investigating the inhibitory effect of kojic acid on tyrosinase activity. The ability to determine IC_{50} of kojic acid with this assay made it a useful tool for studying the enzymatic reaction of tyrosinase, investigating the potential enzyme mechanism and substrate specificity. Further, it revealed that this method not only can offer a promising insight into the exploration of novel CLE-CE systems, but also can provide a new strategy for the screening of tyrosinase inhibitors, which might be applied for relevant food manufacturing industry, drug discovery and clinical analysis in the future.

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Appendix A. supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.08.028.

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