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A chiral ligand exchange CE essay with zinc(II)–L-valine complex for determining enzyme kinetic constant of L-amino acid oxidase

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ABSTRACT

A new strategy for the enantioseparation of D,L-amino acids employing the principle of ligand exchange capillary electrophoresis with Zn(II)–L-valine complex as a chiral selecting system in the presence of β-cyclodextrin has been designed. Successful enantioseparation of label free and labeled amino acids have been achieved with a buffer of 100.0 mM boric acid, 5.0 mM ammonium acetate, 4.0 mM β-cyclodextrin, 4.0 mM $ZnSO_4$ and 8.0 mM L-valine at pH 8.1. This new method was shown to be applicable to the quantitative analysis of label free D- and L-aromatic amino acids. Furthermore, the expanding enzymatic use of L-amino acid oxidase to incubate with different L-amino acids has allowed understanding of the substrate's specificity. An on-column incubation assay has been developed to study the L-amino acid oxidase's catalytic efficiency. It was demonstrated that the enzyme kinetic constant could be determined by using this new method.

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1. Introduction

Although various methods, such as HPLC, GC–MS and capillary electrophoresis (CE), have been developed for chiral separation [1–8], chiral ligand exchange CE (CLE-CE) methods in enantioseparation are valuable due to their specificity and simplicity [9,10]. Since the dansylated D,L-amino acids (Dns-D,L-AAs) were firstly performed by Zare and colleagues [11] using the principle of CLE-CE with Cu(II)–L-histidine complex, several methods of CLE-CE with different ligands and central ions [12–15] have been developed in recent years. Meanwhile, different kinds of cyclodextrins (CDs) also have been used in CLE-CE because CDs can form inclusion complexes and to enhance the regiospecificity and sterospecificity with respect to D,L-AAs [8]. For example, Cucinotta et al. [16] have used β-CD substituted by an imidazole-bound histamine to separate label free tryptophan enantiomers in the presence of Cu(II) ion by CLE-CE.

Meanwhile, some studies have recently been concerned with ligand exchange and enzymic reaction [17–19]. However, rare investigations have been developed for inspecting the enzymatic activity of L-amino acid oxidase (LAAO) by using the CLE-CE method. As we all known that LAAO is a homo-dimeric flavoprotein with a molecular mass around 110–150 kDa and with the isoelectric point around 4.4–8.12 [20], which is usually purified from the venoms of a variety of snake species and is thought to contribute to the toxicity of the venoms. The interesting thing is LAAO contains a non-covalent bond that the flavin adenine dinucleotide (FAD) as cofactor requires for its enzymatic activity [21], which can lead to extensive interaction with proteins [22]. On the one hand, the effects of LAAO on platelet aggregation, haemorrhagic and antibacterial effects vary widely. On the other hand, *via* an imino acid intermediate, L-AAs can be oxidatively deaminated by LAAO to its α-ketoisocaproic acid (KIC) along with the production of ammonia and hydrogen peroxide [20,23]. And then KIC is finally transaminated by transaminase to D-AAs (Scheme 1). Therefore, LAAO has not only become an attractive object for the studies of enzymology and pharmacology, but also for the biotransformation method study of D-AAs in recent years [24–26].

To determine specific LAAO activity, a modified enzyme-linked immunosorbent assay (ELISA) [23] using 96-well plate and micro-plate spectrophotometer was used for testing the products produced in the reaction between horseradish peroxidase and hydrogen peroxide. In general, after the AA substrates have

Abbreviations: LAAO, L-amino acid oxidase; CLE-CE, chiral ligand exchange-capillary electrophoresis; AA, amino acid; Dns-AAs, labeled dansyl amino acids; β-CD, β-cyclodextrin; KIC, α-ketoisocaproic acid.

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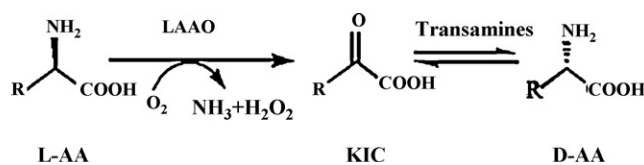

Scheme 1. Enzyme reaction.

been incubated with LAAO for 2 h at 37 °C, the value of OD_{490} can be converted to the concentration of hydrogen peroxide by using the hydrogen peroxide stand curve, then equivalent to the concentration of AAs oxidized based on the enzyme reaction. The process for measuring LAAO activity on the enzyme reaction was a bit complicated. Therefore, a simple and effective CE method should be explored. In addition, the LAAO enzyme kinetics in the chiral inversion and the possibility that L-AAs are unidirectionally converted to D-AAs has not yet been adequately clarified. To boost such research, a new sensitive and selective separation method of D,L-AA enantiomers should first be developed.

Although Cu(II) is the frequently used central ion in CLE-CE, other metal ions, such as Mn(II) [27], have been tried in place of the Cu(II). In our previous study, using Zn(II)–L-lysine, Zn(II)–L-arginine and Zn(II)–L-ornithine [28–30] as the chiral complexes in CLE-CE have been explored, so far to our knowledge, assaying the D,L-AAs and measuring the LAAO enzyme kinetic constant in CLE-CE by using Zn(II)–L-valine (L-Val) as chiral complex selector in the presence of β-CD have not yet been explored.

In this paper, CLE-CE for 20 pairs of D,L-AAs were investigated as a trial and positive results were produced. Furthermore, the study of enzyme reaction at different incubation temperature was well displayed. The measurement exploring the LAAO enzyme reaction kinetic constant has been successfully achieved, implying that the method can be adapted to the study of enzyme reaction mechanism.

2. Experimental

2.1. Chemicals

LAAO (from crotalus atrox venom), dansyl chloride (Dns-Cl), D-AAs and L-AAs were purchased from Sigma Chemical Co. (St. Louis, USA). Tris(hydroxymethyl)aminomethane (Tris), lithium carbonate, zinc sulfate, boric acid, β-CD and other chemicals were all of analytical reagent grade from Beijing Chemical Factory (Beijing, China).

2.2. Preparation of buffer and sample solutions

All solutions were prepared in triply distilled water produced by the distillation apparatus Model SZ-93 (Yarong Biochemical Instrument Co., Shanghai, China) and stored at 4 °C. CE running buffers, unless stated otherwise, were composed of 5.0 mM ammonium acetate, 100.0 mM boric acid, 4.0 mM β-CD, 4.0 mM $ZnSO_4{\cdot}7H_2O$ and 8.0 mM L-Val, adjusted to pH 8.1 with Tris. Before use, all the running buffers were filtered through a membrane filter with 0.45 μm pores and degassed by sonication for 2 min.

Standard stock solutions of D-AAs and L-AAs were prepared at a final concentration of 1.0 mg mL^{-1}. Working solutions were diluted from the stock solutions by 10–10^4-fold.

The solution for AAs labeling was freshly prepared by dissolving 3.0 mg Dns-Cl in 2.0 mL acetone. The LAAO solution was prepared by dissolving 10.0 mg LAAO in 0.4 mL water.

2.3. Dansylation of AAs

AAs were dansylated according to the procedure in Ref. [31]. Briefly, an aliquot of 100 μL AAs in a 0.50-mL vial, with 200 μL 40 mM lithium carbonate buffer and 100 μL labeling solution of dansyl chloride. The mixed solution was allowed to react at room temperature for 35 min. After addition of 5 μL 2% ethylamine to terminate the reaction, the reacted solution was either directly injected for CE separation or kept at 4 °C for future analysis.

2.4. D,L-AAs incubation with LAAO

The solutions of D,L-AAs (20 μL, 80.0 μg mL^{-1}) in 0.50-mL vials were incubated with LAAO (20 μL, 5.0 units mL^{-1} or 10.0 units mL^{-1}) in a water bath at 0 °C or 20 °C or 37 °C for 10–60 min. The samples were depreoteinized by heating in a boiling-water bath for 10 min and then separately centrifuged in TGL-16C (from Shanghai Anting Scientific Instruments Factory, Shanghai, China) at 10,000 rpm for 10 min. Then the supernatants were sucked or dansylated and applied to CE.

In some on-column LAAO incubation experiments, the capillary was filled with running buffer and a sandwich injection in the order of LAAO, D,L-AAs and LAAO for 5 s, 15 s, 5 s each section. To avoid contamination, the inlet tip of the capillary was cleaned by dipping it into water for 2 s in between the injections. The injected sample sandwich was reacted at the end of the capillary inlet for 5 min at 20 °C. Separation was then started at +21.0 kV for label free D,L-AAs and −21.0 kV for labeled D,L-AAs.

2.5. Apparatus and electrophoretic procedures

The 1229 HPCE Analyzer (Beijing Institute of New Technology and Application, Beijing, China) was used for conducting the electrophoretic experiments. Unless stated otherwise, separations were performed at 20 °C in an uncoated fused-silica capillary (Yongnian Optical Fiber Factory, Hebei, China) of 50 μm I.D. × 65 cm (50 cm effective). Prior to injection, the bare fused-silica capillary was sequentially rinsed with 0.1 M HNO_3, water, 0.1 M NaOH, water and running buffer for 2 min each. A sample was siphoned for 5 s in 15 cm height and separated at ±21 kV. The separated bands were detected by UV absorption at 254 nm and acquired at 4 Hz. Peaks were identified by spiking relative standard AAs in sample solutions. The peaks with increased height were considered to be the targets.

3. Results and discussion

3.1. Method development

According to our previous study [28–30], Zn(II)-based CLE-CE was adopted for the direct analysis of D,L-AAs. In spite of the fact that most of the research was on the basic AA-based ligands, there were a few experiments using different ligands, such as quinic acid, tartaric acid [11,12] and amino acylamide [14]. Those results suggest that the better enantioresolution (R_s) could be obtained or the migration order of enantiomers might be changed if the ligand was changed. This led us to consider the exploration of some not-yet-well-studied nature AA ligands, such as L-Val. The exploration has lead to a positive result, as shown in Table 1. The most interesting thing is that, comparing with Zn (II)–L-Lys, Zn (II)–L-Arg or Zn (II)–L-Orn complex, the migration orders of these AA enantiomers have been changed when Zn (II)–L-Val complex was used as the chiral selector. It definitely indicated that selecting different kinds of ligands could adjust or control the migration order of D,L-AA enantiomers. Thus L-Val was chosen as the working ligand.

Table 1
Migration time and R_s of labeled and label free D-, and L-AAs[a].

	No.	AAs	t_L (min)	t_D (min)	R_s[b]
Label free AAs	1	Phe	33.98	35.33	1.57
	2	Trp	33.28	35.39	2.24
	3	Tyr	44.12	43.44	1.05
Dns-AAs	4	Ala	41.77	42.33	0.88
	5	Arg	33.33	33.45	0.60
	6	Asn	41.53	42.04	1.51
	7	Asp	23.71	23.71	0
	8	Cys	44.33	46.25	1.68
	9	Glu	15.64	15.64	0
	10	His	28.39	28.39	0
	11	Ile	34.08	35.11	1.51
	12	Leu	38.96	40.52	1.91
	13	Lys	30.04	30.04	0
	14	Met	42.23	43.63	1.56
	15	Phe	58.49	59.63	1.33
	16	Pro	40.53	41.56	0.85
	17	Thr	38.09	39.37	2.16
	18	Trp	46.38	46.92	0.80
	19	Tyr	32.46	33.31	1.53
	20	Val	40.22	42.18	1.50

[a] Running buffer: 5 mM ammonium acetate, 100 mM boric acid, 4 mM β-CD, 4 mM Zn(II) and 8 mM L-Val at pH 8.1.
[b] $R_s = 2(t_D - t_L)/(W_D + W_L)$; t: migration time.

In addition to selecting the suitable ligand, other critical parameters including buffering reagent, its concentration and pH, the concentration of complexes, applied electric field strength and temperature were checked. After a systematic search, the running buffer of 5.0 mM ammonium acetate, 100.0 mM boric acid with Zn(II) and L-Val complex in the presence of β-CD was shown the best choice for the test D,L-AAs when the ratio of Zn(II):L-Val was kept in 1:2. As expected, the buffer pH and the concentration of complexes were critical and had a major impact on the chiral separation resolution and migration time.

Fig. 1 reveals that the R_s of D,L-Trp and Dns-D,L-Tyr increased with pH increasing from 7.5 to 8.1, then decreased at pH 8.3. Although the R_s of Dns-D,L-Asn kept increasing when pH was increased from 7.5 to 8.3, the migration time of Dns-D,L-Asn was prolonged, even longer than 70 min. It indicates that the changing capability between the ligand and the D,L-AAs strongly depends on pH. Thus, for getting the higher enantioseparation in shorter time, the buffer pH should be selected by combining the R_s and the migration time. In this study, pH at 8.1 was adopted finally. However, the pH window usable for those D,L-AAs was clearly quite narrow, which may explain why zinc(II)–L-Val complexes was not used before this investigation.

Table 2 clearly displays that the ligand exchange became impossible if the complex was not sufficient (≤9.0 mM). In contrast, when the complex was sufficient (≥12.0 mM), the ligand exchange could be carried out efficiently, and the baseline separation of the test D,L-AAs could be achieved. Somewhat unexpectedly, when the concentration of the complex was increased to 15.0 mM, the migration time of the test Dns-D,L-AAs was prolonged obviously, even longer than 100 min. In other words, enough complex was the prerequisite to conduct ligand exchange and chiral separation in short time. For obtaining higher R_s, the concentration of complex, 4.0 mM Zn (II) and 8.0 mM L-Val, was finally adopted in this study.

Table 2
Influence of complex concentration on the performance of CLE-CE[a].

Complex concentration (mM)	Dns-Tyr			Dns-Asn		
	t_L (min)	t_D (min)	R_s	t_L (min)	t_D (min)	R_s
6.0	55.25	55.25	0	58.20	58.20	0
9.0	38.03	39.30	1.19	51.66	52.45	0.76
12.0	32.46	33.31	1.53	41.53	42.04	1.51
15.0	63.04	65.02	1.94	96.88	105.47	2.39

[a] Running buffer: 5 mM ammonium acetate, 100 mM boric acid, 4 mM β-CD, the ratio of Zn(II) and L-Val was kept in 1:2 at pH 8.1.

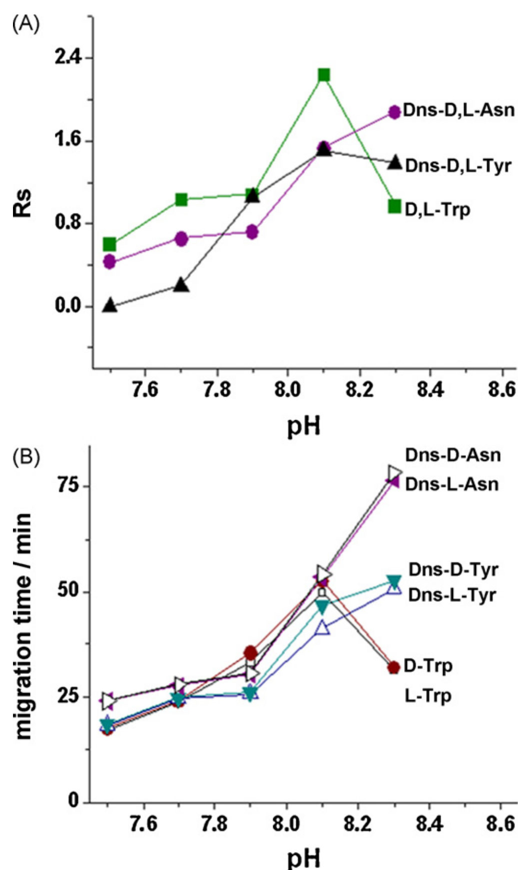

Fig. 1. Dependence of pH on the enantioresolution. Running buffer of 100 mM boric acid, 5 mM ammonium acetate, 4 mM β-CD, 4 mM Zn(II) and 8 mM L-Val, adjusted pH with solid Tris; capillary: 50 μm I.D. × 65 cm (50 cm effective); temperature: 20 °C; UV detection: 254 nm.

CDs are commonly used in chiral separation because of their intrinsic chirality. It was reported [8,27] that the CDs could act as an additional ligand toward the central metal ions, their coordinating ability was able to be used for CLE-CE. The assisting possibility of β-CD in the enantioseparation was thus investigated. As shown in Fig. 2, by only using β-CD (Fig. 2A) or the Zn(II)–L-Val complex (Fig. 2B) as the chiral selector, the baseline enantioseparation could not be obtained. But β-CD indeed played an important assisting role in the CLE-CE (Fig. 2C and D). The baseline enantioseparation of Dns-D,L-Leu could be achieved when 4.0 mM β-CD combining with Zn(II)–L-Val complex (Fig. 2D) was used as the chiral selector. It should be noted that although β-CD had exhibited the well effect in assisting chiral separation, there were still some pairs of Dns-D,L-AAs could not be chiral separated (Table 1). The reason was not very clear, but the reasonable explanation was that the selector and the analytes might not formed complex, or might formed too strong chelate-inclusion complex to be enantioseparated.

3.2. Effect of temperature on LAAO catalytic reaction

Considering that the most of enzyme catalytic reactions are very sensitive to temperature, a range of temperature was selected and the decreased peak area of L-AAs could be used for evaluating the effect of temperature on the enzyme reaction referring to LAAO with the established CLE-CE method. As expected, the temperature

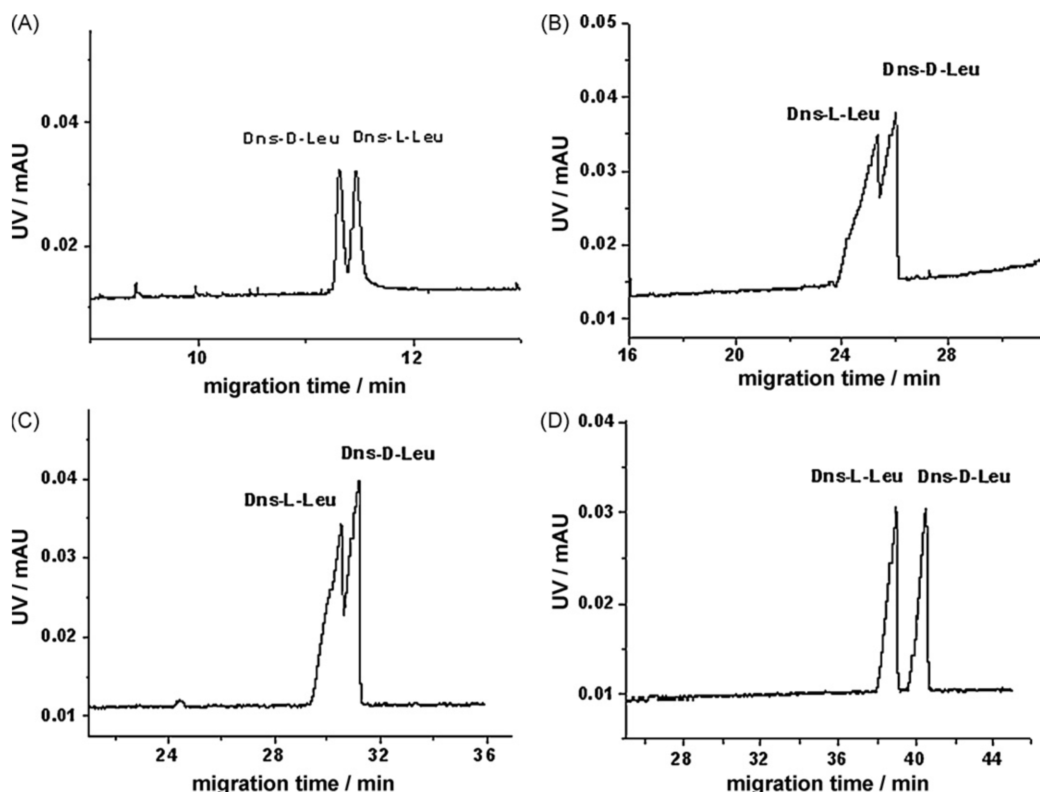

Fig. 2. Dependence of β-CD concentration on the enantioresolution: (A) buffer without Zn(II)–L-Val complex; (B) buffer without β-CD; (C) buffer with 2 mM β-CD; (D) buffer with 4 mM β-CD. Other conditions were the same as that in Fig. 1.

was found to be the key factor in controlling the decreased amount of L-AAs produced in the enzyme reaction when the reaction time was fixed. Fig. 3 shows that the peak area of L-Trp decreased significantly when the enzyme reaction temperature increased from 0 °C to 37 °C. Interestingly, about 20% of L-Trp could be remained when the enzyme reaction time was only 10 min at 0 °C (Fig. 3B), then the peak area of L-Trp decreased to about a tenth of its original peak area (Fig. 3A) at 20 °C (Fig. 3C) and was almost not observed at 37 °C (Fig. 3D). However, for converting more L-Trp into KIC by LAAO in a shorter reaction time, higher temperature was required.

3.3. Substrate specificity

It has been reported that the LAAO protomer consists of a FAD-binding domain, a substrate-binding domain and a helical domain [22]. A special funnel is thus formed by the interface between the helical domain and substrate-binding. Although the funnel can provide access to the active sites for substrates, the direction of the access is bended. In other words, it is not easy for all kinds of substrates to approach the active sites. If the substrates can arrive at the active sites, the innermost molecule might make hydrogen bond by contacting with the active site residues. Meanwhile the aromatic portion of the substrate may be situated in a hydrophobic pocket. Thus, we assume that the oxidizing activity of LAAO should be different for the different types of L-AAs.

While incubated with the LAAO at 20 °C, three kinds of D,L-aliphatic-AAs (D,L-Met, D,L-Ile and D,L-Thr) were chosen as substrates for testing the substrate specificity. As displayed in Fig. 4, both the L-Met and L-Ile were shown to have higher substrate specificity for the LAAO after incubating 10 min (Fig. 4A) and 30 min (Fig. 4B) respectively. Meanwhile, L-Thr did not show the substrate specificity for the LAAO even though the incubation time was 60 min (Fig. 4C). Noticeably, among the test three aliphatic L-AAs, the substrate specificity of LAAO is in an order of L-Met (containing methylmercapto group) > L-Ile (containing methyl group) ≫ L-Thr (containing hydroxyl group), which is more or less parallel to the structure of alkyl at the α or β position of the L-AAs. The result is the same as mentioned in Refs. [20,23].

Although it has been [32] reported that most of the LAAOs prefer hydrophobic AAs as the substrate, binding of the substrate with different electrostatic and hydrophobic properties can provide useful insights into the environment of the catalytic sites in LAAO, the complete reasons are not very clear until now. The interesting thing we found in this work is that if L-Met and L-Ile were first dansylated and then incubated with LAAO, Dns-L-Met and Dns-L-Ile could not be oxidizied by the LAAO. The possible reason is that the naphthyl group of Dns-Cl might be the obstacle for Dns-L-Met or Dns-L-Ile

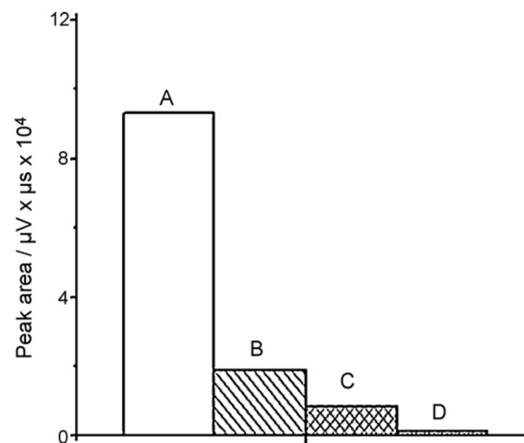

Fig. 3. Peak area measured from L-Trp incubated without LAAO at 20 °C (A) or incubated with LAAO for 10 min in water bath at: (B) 0 °C; (C) 20 °C; (D) 37 °C. Other conditions were the same as that in Fig. 1.

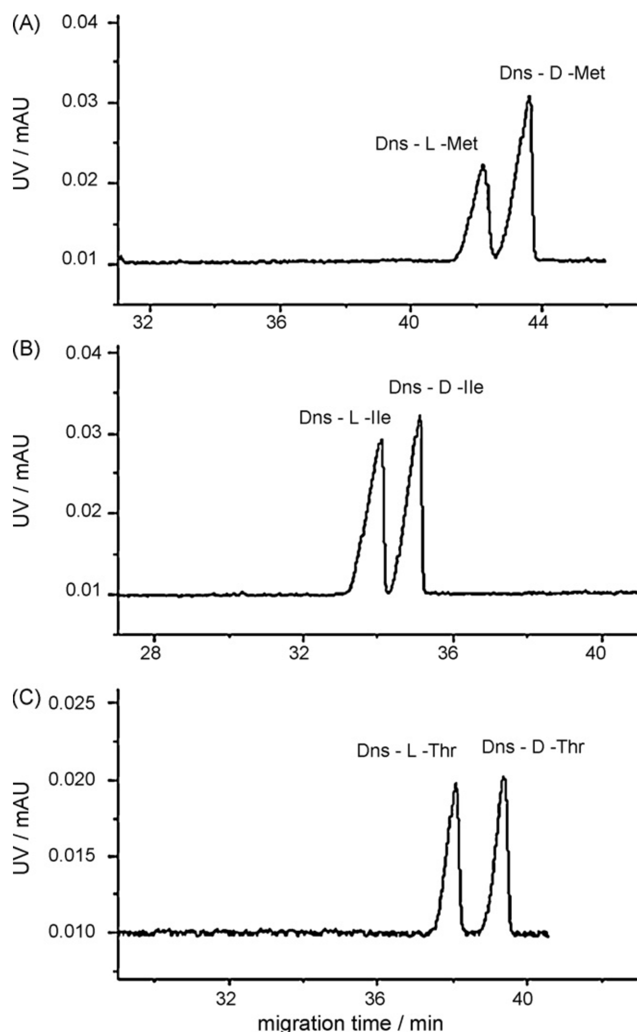

Fig. 4. Effect of enzyme reaction on substrate specificity. After D,L-AAs had been incubated with LAAO at 20 °C for different on-column enzyme reaction time: (A) 10 min; (B) 30 min; (C) 60 min, they were dansylated. Other conditions were the same as that in Fig. 1.

approaching the access and the bonding sites of the LAAO. The LAAO catalytic differences for different substrates may be explained by the bend of the access and the differences of side chain binding sites, such as hydrophobic site and hydrogen bonding site existing in the LAAO enzyme, which should be responsible for the substrate specificity.

3.4. Quantitation feature

Six injections of standard mixed D- and L-Trp solution (200.0 μg mL^{-1}) were used to determine the repeatablity of the peak area and migration time in this work. The run-to-run RSD (relative standard deviations) of migration time was less than 1.0% and that of peak area less than 3.0%.

A series of standard mixed solutions of D- and L-Trp ranging from 15.0 μg mL^{-1} to 800.0 μg mL^{-1} were tested to determine the linearity of detection. Standard working equations for D-Trp and L-Trp were constructed between peak area (y) and concentration (x) and summarized in Table 3. The recovery of the method was determined from the two samples (contained 50.0 μg mL^{-1} of D- and L-AAs respectively) as background added with 50.0 μg mL^{-1} of standard D- and L-AAs respectively. The measured content was averaged over five measurements, giving values between 96.7% and 112.5% (Table 3, the last line). The results demonstrated that the method was suitable for detection of D- and L-Trp, enabling repeatable and sensitive quantification of samples containing D- and L-Trp. Then the samples of D,L-Trp incubated with LAAO were analyzed.

Table 3
Quantitation features of CLE-CE measured from D,L-Trp.

Sample	Working equation[a]	Range (μg mL^{-1})	r^2 [b]	LOD[c] (μg mL^{-1})	Mean recovery (%) [d]
L-Trp	$y = 6493.7 + 668.5x$	15–400.0	0.997	8.0	96.7 ± 4.7
D-Trp	$y = 4408.5 + 704.2x$	15–400.0	0.998	8.0	112.5 ± 1.4

[a] y is the peak area and x the concentration of amino acids.
[b] r^2 the linear correlation coefficient.
[c] LOD the limit of detection.
[d] The recovery was averaged over five measurements with the target samples as a background.

3.5. On-column LAAO catalytic reaction

Fig. 5 displays the general principle of the on-column enzyme reaction using the CLE-CE method, and enantioresolution of AAs carring out within a single capillary during electromigration. In order to obtain a better on-column incubating performance, the injection sequence of sample, injection time and the incubation time have been optimized. The injection sequence was found to be the important factor. The "sandwich" injection sequence could produce the best incubating performance, therefore, it had been finally chosen in this work.

The condition for the enzyme reaction was very mild so that the enzyme activity could be maximally preserved [33]. Thus the effect of the on-column LAAO catalytic reaction with D,L-Trp was studied. The results reveal that the LAAO definitely catalyzed the enzyme reaction and the peak height of L-Trp decreased when the on-column incubating time increased. It clearly shows that the on-column LAAO enzyme reaction can be identified because the CLE-CE method is very simple, implying that the method may have potential for studying the LAAO enzyme kinetics.

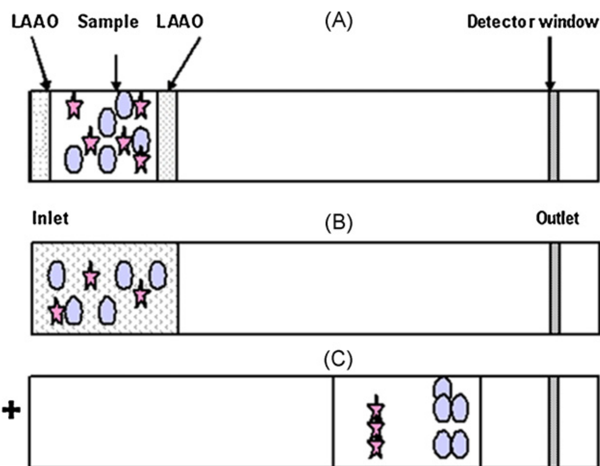

Fig. 5. Schematic procedure for on-column incubating D,L-AAs with LAAO and CLE-CE separation. The samples are injected between LAAO plugs (A); after these injections, the sandwich sections are mixed, and the enzyme reactions occur (B); then the enantioseparation is started and the separated L- and D-AAs move to the detector window (C).

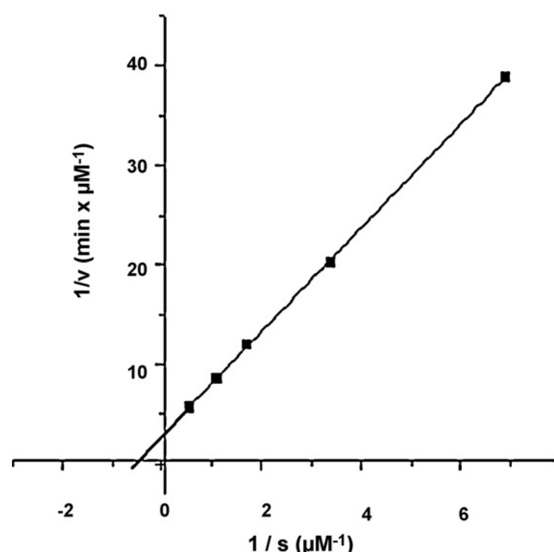

Fig. 6. LAAO enzyme kinetic constants determined with the on-column enzyme reaction and the CLE-CE assay. A Lineweaver–Burk plot is shown. Reactions at each substrate concentration were repeated three times. Points were fit using a linear regression model (r^2 = 0.99). Kinetic constants were $K_m = 1.81 \times 10^{-6}$ M, and $V_{max} = 5.83 \times 10^{-9}$ M s^{-1}(kat). Other conditions were the same as that in Figs. 1 and 5.

3.6. Analysis of enzyme reaction kinetic constants by means of on-column incubation

The advantage of the CLE-CE assay mentioned above allows us to vary D- and L-AAs concentrations in the catalytic reaction and determine kinetic constants for LAAO under the study. The effect of the on-column LAAO catalytic reaction was clearly observed. The kinetic study of the enzyme reaction was completed at various concentrations of D,L-Trp ranging from 0 μM to 400.0 μM. The same amount of the enzyme (5.0 units mL^{-1}) was incubated with those substrates on-column at 20 °C for 5 min. The velocities of LAAO-catalyzed reaction were calculated from the decreased amount of L-Trp in the enzymatic reaction and the velocities were plotted as a function of the L-Trp concentrations. The kinetic constant was analyzed by nonlinearn regression analysis of Michaelis–Menten equation [20,26,34,35]. Michaelis–Menten equation for L-Trp incubating with LAAO could be expressed as follows:

$$\nu = \frac{V_{max} \cdot [S]}{K_m + [S]}$$

where ν and V_{max} are the initial and maximum velocities respectively. K_m is the Michaelis constant, [S] is the concentration of L-Trp.

Initial velocities were calculated from the decreased amount of L-Trp versus time for reactions containing five different L-Trp concentrations. A Lineweaver–Burk plot [36] was constructed as shown in Fig. 6, and LAAO enzyme kinetic constants were determined from this plot. The Michaelis constant (K_m) of LAAO for L-Trp was 1.81×10^{-6} M. The V_{max} was determined to be 5.83×10^{-9} M s^{-1}(kat).

4. Conclusion

We have developed a sensitive and facile CLE-CE assay for the enatioseparation of D,L-AAs based on Zn(II)–L-Val complex in the presence of β-CD. In efforts to optimize the enantioresolution, various separation parameters were examined. The method was used the direct analysis of 20 pairs of AA enantiomers, with 10 pairs baseline separated, 6 pairs partially resolved and 4 pairs no resolved. It was also shown to be applicable to the quantitative analysis of L-AAs in on-column enzyme reaction. The ability to determine enzyme kinetic constants with this assay made it a useful tool for studying LAAO enzyme reaction and investigating the potential enzyme mechanism and substrate specificity.

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