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Sequential micellar electrokinetic chromatography analysis of racemization reaction of alanine enantiomers

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ABSTRACT

A novel method for online monitoring racemization reaction of alanine (Ala) enantiomers was developed, by combining sequential sample injection and micellar electrokinetic chromatography (MEKC) technique. Various conditions were investigated to optimize the sequential injection, Ala derivatization and MEKC chiral separation of D-/L-Ala. High reproducibility of the sequential MEKC analysis was demonstrated by analyzing the standard Ala samples, with relative standard deviation values ($n = 20$) of 1.35%, 1.98%, and 1.09% for peak height, peak area and migration time, respectively. Ala racemization was automatically monitored every 40 s from the beginning to the end of the reaction, by simultaneous detection of the consumption of the substrate enantiomer and the formation of the product enantiomer. The Michaelis constants of the racemization reaction were obtained by the sequential MEKC method, and were in good agreement with those obtained by traditional off-line enzyme assay. Our study indicated that the present sequential MEKC method can perform fast, efficient, accurate and reproducible analysis of racemization reaction of amino acids, which is of great importance for the determination of the activity of racemase and thus understanding its metabolic functions.

1. Introduction

Alanine racemase (AlaR), which is widely distributed in bacteria, belongs to the fold-type III group of pyridoxal 5′-phosphate (PLP) dependent enzymes that catalyzes the interconversion between alanine (Ala) enantiomers, D-Ala and L-Ala. It is well known that D-Ala is an indispensable component of the peptidoglycan layer of bacterial cell wall which can only be provided by AlaR. Thus, AlaR has attracted much research attention due to its pivotal role in cell wall biosynthesis and unique distribution to prokaryotens [1–5]. It has been recognized that AlaR could be a possible therapeutic target for antibacterial drugs for various diseases, such as tuberculosis (TB), anthrax, tympanitis, etc. [6–8]. Development of a fast, user-friendly, sensitive and reliable enzyme assay is therefore of great importance for determination of the activity of AlaR, thus identification of an inhibitor of a novel pharmaceutical target [9–13].

Due to its unique advantages such as high efficiency and sensitivity, rapid analysis, extremely low sample volume requirements, and the ability to utilize several detection methods, capillary electrophoresis (CE) is used not only for high performance separation but also as a powerful platform for online quantitative study of enzyme-catalyzed reactions [14–19]. CE has been widely applied in nearly all aspects of enzyme assays, including the evaluation of enzymatic activity, enzyme kinetics, enzyme inhibition and activation, and the investigation of enzyme-mediated metabolic pathways. Although having not been reported yet in the literature, it is expected that online CE analysis of the D-/L-Ala racemization reaction could have valuable potential for understanding the AlaR activity. Particularly, online monitoring of the racemization reaction from the beginning to the end should be of vital importance to fully understand the metabolic functions of the AlaR and to determine their uses for clinical diagnostics.

However, it is a rather challenging task to accomplish online monitoring of the racemization reaction. The bottleneck lies in the difficulties to achieve reproducible sequential injection and efficient chiral separation and sensitive detection of the pair of the enantiomers during the racemization reaction. Due to the absence of a strong chromophore, fluorophore, or electroactive moiety in the L-Ala and D-Ala molecule, derivatization with chromogenic or fluorogenic compounds is required to increase the sensitivity of detection. The derivatization process is required to be in-capillary and very fast for online monitoring of D-/L-Ala of the racemization reaction. Although CE enantioseparation of amino acids has been attracted research interest during the past several decades, in case of the racemization reaction that the pair of enantiomers are

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the substrate and the product, it is still a difficult task for efficient chiral separation to be compatible with the AlaR activity and the sequential enzyme assay.

In our previous study, we have developed an easy-to-operate and effective method for sequential online CE enzyme assay using two-section capillaries and power cycling of a high-voltage power supply [20]. The method can provide reproducible sequential injection and fast in-capillary derivatization of amino acids. Here, we extended the study to online monitor the racemization reaction, in which efficient chiral separation is required in addition to accurate sequential enzyme assay. The approach was achieved by combining the sequential injection method with micellar electrokinetic chromatography (MEKC) technique, which has been successfully employed for chiral separation of amino acids by several studies [21–25]. Various experimental conditions were investigated and optimized, which are essential for sequential injection, Ala derivatization as well as MEKC chiral separation of D-/L-Ala. With the application of the present sequential MEKC method, we investigated the AlaR catalyzed reactions by automatic and simultaneous monitoring of the substrate consumption and the product formation every 40 s from the beginning to the end of the reaction. Kinetic constants of the racemization reaction were obtained, and were compared with the results of traditional off-line enzyme assay. The study indicates that the sequential MEKC method could be applied for determination of the activity of racemase thus has great potential for identification of possible inhibitors of a novel pharmaceutical target.

2. Materials and methods

2.1. Chemicals

L-Alanine, D-alanine, and 2,3,4,6-tetra-O-acetyl-l-thio-β-glucopyranose (TATG) were purchased from J&K Chemical Ltd. (Shanghai, China), Alanine racemase (EC 5.1.1.1) and O-phthaldialdehyde (OPA) were purchased from Sigma Chemical (St. Louis, China). All other reagents were of analytical grade and were used without further purification. Sodium dodecyl sulfate (SDS) was purchased from Tianda Chemical Reagent Factory (Tianjin, China). Acetonitrile, acetone and methanol were purchased from Beijing Chemical Factory (Beijing, China). Prior to use, all solvents and solutions were filtered using 0.2 μm membrane filters which were purchased from Jinteng Experimental Equipment Co., Ltd. (Tianjin, China).

2.2. Sequential MEKC analysis of alanine enantiomers

Fig. 1(a) presents the picture of our sequential CE analysis system, which has been described in detail in our previous study [20]. The system was simply constructed by coaxially aligning two capillaries with a distance of 5 μm between the smooth ends and passing them through a sample vial. The alignment of the two capillaries was ensured under a microscope, as shown in the photograph of the joint portions of the capillaries in Fig. 1(b). The principle of the present sequential MEKC analysis using the system for chiral determination of D-/L-Ala is schematically shown in Fig. 1(c). The MEKC running buffer was prepared by dissolving specific amounts of SDS in the 40 mM borate buffer. The selected organic modifier, acetonitrile, acetone or methanol, was added with an optimized concentration in the buffer to increase the enantioselectivity. The MEKC running buffer also contained OPA/TATG, the derivatization reagent that has been demonstrated for rapid and stable derivatization of amino acids for UV or LIF detection [22,24,26,27]. A high voltage power supply, which was designed by Yangzhou Shuanghong Electronics Co., Ltd. (Yangzhou, China) and can be automatically turned on and off with controllable periodic time, was applied across the two capillaries. At the interfaces of the two capillaries, Ala enantiomers in the sample vial are rapidly derivatized by the reagent OPA/TATG, which changes the concentration gradient at the interfaces thus enhances the diffusion efficiency of the injection as the high-voltage power supply is turned off. The derivatized Ala enantiomers were then MEKC separated and UV detected when the high-voltage power supply was switched on. By periodically switching the high-voltage power supply off (for sample injection) and on (for MEKC separation), sequential MEKC analysis of the Ala enantiomers was achieved without any physical disturbance of the capillary inlet.

In the experiments to obtain the calibration curves and optimization, a 50 μL standard D-Ala and L-Ala mixture in 50 mM phosphate buffer (pH 7.5) was added in the sample vial (300 μL). In the experiments for analysis of the racemization reaction enzyme assay experiments, 45 μL substrate L-Ala (or D-Ala) with a concentration range of 0–15 mM was initially put into the sample vial. When the AlaR enzyme (2 U/mL, 5 μL) was added to start the racemization reaction, the substrate and the product Ala enantiomers were simultaneously detected as a function of the reaction time to achieve the online enzyme assay.

2.3. CE conditions

All experiments were carried out in a home-built CE apparatus with UV detection at 340 nm by a 6000PVW UV–visible detector (Cometro Technology Ltd., USA). Two fused silica capillaries (365 μm o.d., 50 μm i.d., Hebei Yongnian Optical Fiber Factory, China) were used for the sequential MEKC analysis, with the total length of 12 cm and 7.5 cm, respectively. The 12-cm capillary was used as the MEKC separation channel with an effective length of 6 cm. The detection window was made by removing the polyimide coating at the middle of the capillary. The sample buffer in the sample vial was a 50 mM phosphate buffer (pH 7.5). The MEKC running buffer was a 40 mM borate buffer (pH 9.5) containing specific concentrations of SDS, organic modifier and derivatization reagent OPA/TATG. Before sequential MEKC analysis, the capillaries were pressure-rinsed successively with 0.1 M NaOH for 2 min, distilled water for 3 min, the borate buffer for 5 min, and the sample vial was rinsed with distilled water and the phosphate buffer using a syringe.

For off-line analysis of the racemization reaction, the reaction mixture (200 μL) contained 50 mM phosphate buffer (pH 7.5) and the substrates of different concentrations. Reactions were initiated by the addition of 10 μL of 2 U/mL AlaR enzyme into the mixture. Aliquots of 10 μL were periodically removed from the reaction mixture, and the AlaR enzyme was inactivated by the addition of 2 μL of 0.1 M HCl to each aliquot. The CE running buffer was a 40 mM borate buffer (pH 9.5) containing 12 mM/24 mM OPA/TATG, 30 mM SDS and 6% acetonitrile. The sample was injected at a height of 10 cm for 3 s. The substrate consumption and product formation were measured at a separation electric potential of 400 V/cm. The total length of the separation capillary (50 μm i.d., 365 μm o.d.) was 50 cm with an effective length of 42 cm.

3. Results and discussion

3.1. Sequential MEKC separation of standard Ala enantiomers

We first employed the present method to sequential analyze a standard 1.0 mM D-Ala and L-Ala mixture, in order to optimize the experimental conditions and to obtain the calibration curve for the quantitative measurement of the racemization reaction. Various important experimental conditions were investigated to optimize