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Study on alanine aminotransferase kinetics by microchip electrophoresis

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ABSTRACT

Alanine aminotransferase (ALT), which catalyzes the reversible conversion between L-glutamic acid (L-Glu) and L-alanine (L-Ala), is one of the most active aminotransferases in the clinical diagnosis of liver diseases. This work displays a microanalytical method for evaluating ALT enzyme kinetics using a microchip electrophoresis laser-induced fluorescence system. Four groups of amino acid (AA) mixtures, including the substrates of ALT (L-Glu and L-Ala), were effectively separated. Under the optimized conditions, the quantitative analysis of L-Glu and L-Ala was conducted and limits of detection (signal/noise = 3) for L-Glu and L-Ala were 4.0×10^{-7} and 2.0×10^{-7} M, respectively. In the reaction catalyzed by ALT, enzyme kinetic constants were determined for both the forward and reverse reactions by monitoring the concentration decrease of substrate AAs (L-Ala and L-Glu), and the K_m and V_{max} values were 10.12 mM and 0.48 mM/min for forward reaction and 3.22 mM and 0.22 mM/min for reverse reaction, respectively. Furthermore, the applicability of this assay was assessed by analysis of real serum samples. The results demonstrated that the proposed method could be used for kinetic study of ALT and shows great potential in the real application.

As one of the most active organs and the largest solid gland in the human body [1], the liver plays a key role in regulating the overall organism energy balance by controlling the metabolism. The liver has many significant physiological functions, including detoxification, plasma protein synthesis, and glycogen storage [2,3]. Consequently, hepatic diseases such as hepatocellular carcinoma and hepatitis B and C cause much persecution among humans [4]. Alanine aminotransferase (ALT),[1] which is a sensitive indicator of liver cell injury and one of the most significant parameters in the routine clinical liver test, has been used to identify patients with liver diseases for nearly 60 years [5–9]. ALT catalyzes the reversible conversion of L-alanine (L-Ala) and α-ketoglutarate into L-glutamic acid (L-Glu) and pyruvate [10,11] and is a key cytosolic enzyme strongly associated with amino acid (AA) metabolism [12,13]. Therefore, both the study about ALT kinetics and the determination of ALT are of great significance.

Over past decades, many methods for ALT kinetic study and accurate assay have been reported, including colorimetry [14], ultraviolet spectrometry [15], and electrochemical sensor [8,16], but most of them have limitations and face challenges. The colorimetric method is mainly focused on the investigation of the absorbance of derived pyruvate. Unfortunately, poor sensitivity and unsatisfied accuracy limit extensive application. The ultraviolet spectrometric assay is founded on decreased absorbance of NADH at 340 nm, and the rate of absorbance change can be directly proportional to ALT activity; thus, it has been successfully applied in ALT kinetic analysis. Meanwhile, the electrochemical method is based on the determination of electroactive species such as NAD^+ and H_2O_2. Although the ultraviolet spectrometric and electrochemical methods are widely used in the clinical laboratory, researchers are usually faced with challenges resulting from coupled reactions [17]. As the number of coupled reactions is increased, the overall specificity of the protocols decreases, whereas the possible interferences and the required reagents' purity generally increase [17]. Therefore, it is meaningful to propose a sensitive, direct, and simple method to conduct kinetic research and accurate analysis for ALT.

Various kinds of micro total analysis systems have been rapidly developed over the past few decades. Among these strategies, microchip electrophoresis (MCE) has emerged as a powerful

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[1] *Abbreviations used:* ALT, alanine aminotransferase; L-Ala, L-alanine; L-Glu, L-glutamic acid; AA, amino acid; MCE, microchip electrophoresis; LIF, laser-induced fluorescence; FITC, fluorescein isothiocyanate; ACA, 2-amino-2-(4-chlorophenyl)-propanamide; Brij-35, polyoxyethylene lauryl ether; Pluronic F127, ethylene oxide/propylene oxide block copolymer; BS-12, dodecyl dimethyl betaine; SDS, sodium dodecyl sulfate; reservoir B, buffer reservoir; reservoir BW, buffer waste reservoir; reservoir S, sample reservoir; reservoir SW, sample waste reservoir; MEKC, micellar electrokinetic capillary electrophoresis; CE, capillary electrophoresis; EOF, electroosmotic flow; LOD, limit of detection; RSD, relative standard deviation.

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technique that can lead the next revolution in chemical and biological analysis because of its transcendent advantages, including high speed, easy manipulation, nontoxicity, and low consumption of samples and reagents [18,19]. In addition, laser-induced fluorescence (LIF) technique is commonly employed in MCE because it is acknowledged as one of the most sensitive detection techniques [20]. Although it has been demonstrated that MCE devices were developed for a number of important assays, including DNA determination [21], proteomic research [22], and enzyme analysis [23,24], so far the analysis of ALT by the MCE–LIF system has not been investigated to our knowledge.

In this work, a new MCE–LIF method based on the determination of substrate AAs was developed to study the kinetics of ALT directly for both forward and reverse reactions. Furthermore, it was applied to investigate normal and abnormal human serum samples, which could help to establish a deeper understanding of liver diseases.

Materials and methods

Chemicals and reagents

All AAs and fluorescein isothiocyanate (FITC) used in this experiment were purchased from Sigma Chemical (St. Louis, MO, USA). 2-Amino-2-(4-chloropheny)-propanamide (ACA) was synthesized in our institute [25]. Polyoxyethylene lauryl ether (Brij-35) was obtained from Acros Organics (Morris Plains, NJ, USA). Ethylene oxide/propylene oxide block copolymer (Pluronic F127), dodecyl dimethyl betaine (BS-12), and sodium dodecyl sulfate (SDS) were the products of Beijing Chemical Reagents (Beijing, China). ALT was obtained from AppliChem (Gatersleben, Germany). All chemicals used in this work were of analytical grade.

The normal and abnormal human serum samples were provided by Peking University Third Hospital, and the abnormal samples were obtained from patients infected with hepatitis B virus.

Preparation of sample solution

Triple distilled water was purified by a Yarong Biochemical Instrument model SZ-97 (Shanghai, China). All solutions were filtered through a 0.45-μm pore size membrane filter (Tianjin, China) and stored at 4 °C. Standard stock solutions of 10.0 mM L-AAs were prepared in water and diluted with 80.0 mM boric acid solution for desired folds. ALT (0.12 mg/ml, stored at −18 °C), pyruvate (25.0 mM, stored at 4 °C), and α-ketoglutarate (25.0 mM, stored at 4 °C) all were prepared in 0.3 M sodium phosphate buffer solution (pH 7.4). All of the experiments were performed at room temperature (25 ± 2 °C) except incubation experiments (37 °C).

Enzymolysis process

To determine the kinetic constants of ALT, enzyme incubation experiments were performed. The enzyme reaction is shown as follows:

$$\text{L-alanine} + \alpha\text{-ketoglutarate} \overset{\text{ALT}}{\rightleftharpoons} \text{pyruvate} + \text{L-glutamate},$$

For the reverse reaction, L-Glu and pyruvate solutions were added as the substrate for the kinetic study of ALT. The effect of incubation time on enzyme activity was investigated (from 15 to 60 min), and then 15 min was selected for further analysis. In this study, 20 μl of L-Glu, 20 μl of pyruvate, and 20 μl of ALT solutions were mixed in a 0.5-ml vial and incubated for 15 min at 37 °C. Subsequently, the vial was placed in a boiling water bath for 10 min and centrifuged (10,000 rpm, 10 min) to avoid the interference of proteins. Then, the supernatant was derived by FITC for MCE analysis; the detailed steps for derivatization are given in the next section. All labeled samples were diluted for 10 folds before injection. In addition, the concentration of substrate L-Glu was varied between 166.7 and 833.3 μM.

For the forward reaction, the incubation experiments were the same as that mentioned above except that L-Ala and α-ketoglutarate were used as the substrates for the kinetic study of ALT and the concentration of substrate L-Ala was varied from 333.3 to 833.3 μM.

For the application study, the incubation experiments of serum samples were the same as that of standard enzyme except that ALT solution was displaced by the serum sample. Briefly, equal volumes of L-Glu, pyruvate, and serum samples were mixed and incubated at 37 °C for 15 min. The mixture was then heated in a boiling water bath and centrifuged for removal of proteins. Then, the supernatant was collected and labeled by FITC for MCE analysis. The control experiments of normal and abnormal human serum samples were conducted with an equal volume of 0.3 M sodium phosphate buffer solution (pH 7.4) instead of the substrates L-Glu and pyruvate.

Derivatization of AAs

In this work, FITC was chosen as the precolumn derivatization reagent because it is the most popular amine-reactive fluorescent probe and is able to enhance both separation efficiency and detection sensitivity [26,27]. It should be noted that although excess FITC should be used in the AA derivatization reaction to obtain a better detection limit [27], in this study, to avoid the influence of the excess FITC and its degradation products on the effective separation of AA mixtures in MCE, we decreased the concentration of FITC to achieve well separation and get fine chromatograms. Thus, 2.0 mM FITC was prepared in acetone, and then 1 μl of FITC solution, 20 μl of AA solution, and 19 μl of boric acid buffer (80.0 mM, pH 10.0) were mixed in a 0.5-ml vial and reacted in a Galanz microwave oven (Guangdong, China) at 560 W for 6 min according to the literature [28]. After incubation, heat inactivation, and centrifugation steps, enzymolysis samples were derived through the same process as that of standard AAs. After derivatization, samples were diluted with 80.0 mM boric acid buffer (pH 10.0) as required. All of the AA samples were labeled just before injection to avoid the decomposition of FITC.

MCE

The glass chips used in this work were cross-shaped and purchased from the Dalian Institute of Chemical Physics, Chinese Academy of Sciences (Dalian, China). Both the sampling channel and the sample waste channel were 5 mm in length, and the separation channel was 40 mm in length. All of the channels were 25 μm in depth and 70 μm in width. The reservoirs were 2.0 mm in diameter and 1.5 mm in depth. There were four reservoirs at the end of channels in the chip: buffer (B), buffer waste (BW), sample (S), and sample waste (SW).

The microfluidic channels were rinsed sequentially with 1.0 M NaOH, purified water, and running buffer for 7 min. Potentials for the injection and separation were based on a previous publication with minor modification [27]. Briefly, voltages for reservoirs B, BW, S, and SW were 200, 220, 350, and 0 V, respectively. Thus, the sample solution was transported from reservoir S to reservoir SW in pinched mode. After 30 s, potentials were switched to 2000, 800, and 800 V for reservoirs B, S, and SW, respectively, whereas reservoir BW was grounded for separation. Separations were performed on an intelligent six-path high-voltage electric device and a confocal mode MCE–LIF detection system with a 473-nm semiconductor