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Application of capillary enzyme micro-reactor in enzyme activity and inhibitors studies of glucose-6-phosphate dehydrogenase

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ABSTRACT

In this study, we present an on-line measurement of enzyme activity and inhibition of Glucose-6-phosphate dehydrogenase (G6PDH) enzyme using capillary electrophoresis based immobilized enzyme micro-reactor (CE-based IMER). The IMER was prepared using a two-step protocol based on electrostatic assembly. The micro-reactor exhibited good stability and reproducibility for on-line assay of G6PDH enzyme. Both the activity as well as the inhibition of the G6PDH enzyme by six inhibitors, including three metals (Cu^{2+}, Pb^{2+}, Cd^{2+}), vancomycin, urea and $KMnO_4$, were investigated using on-line assay of the CE-based IMERs. The enzyme activity and inhibition kinetic constants were measured using the IMERs which were found to be consistent with those using traditional off-line enzyme assays. The kinetic mechanism of each inhibitor was also determined. The present study demonstrates the feasibility of using CE-based IMERs for rapid and efficient on-line assay of G6PDH, an important enzyme in the pentosephosphate pathway of human metabolism.

1. Introduction

Glucose-6-phosphate dehydrogenase (G6PDH) (EC.1.1.1.49) is an enzyme that catalyzes the first step in the pentosephosphate pathway. It is expressed ubiquitously and highly conserved in most mammalian species [1,2]. G6PDH is implicated in various cell functions, including cell growth, survival, and redox regulation. However, its deficiency causes hemolytic anemia and neonatal jaundice [3,4]. Recent studies have elucidated novel roles of adipose tissue G6PDH in the etiology of metabolic disorders and show G6PDH as an effectual therapeutic target for metabolic disorders, including obesity and diabetes [5–9]. New and novel potential inhibitors of G6PDH are required to probe the causative mechanisms, or treat the pathology. An efficient method to perform enzyme activity and inhibition assay for G6PDH not only improves the understanding of the regulation for G6PDH in human metabolism, but also its beneficial effects as a drug therapy for related diseases [8,10–12].

Since enzyme immobilization has been revealed as a powerful tool for improving almost all enzyme properties, such as stability, activity, selectivity and reusability, immobilized enzyme reactors (IMERs) have also been applied widely in chemical and biological assays [13–15] for similar purposes. Several G6PDH-IMERs, which were immobilized on the surface of suitable materials, have been reported to be useful [16–19]. Studies have also demonstrated that immobilization is a promising method to achieve efficient enzyme assay of G6PDH with the advantages of easy reloading and enhanced enzyme stability in comparison to free enzymes. However, all the reported G6PDH-IMERs performed the enzymatic assays off-line, in which plenty of sample preparation work as well as large amount of enzyme and chemicals were required. This is a time-consuming, laborious work and more importantly, reduces the repeatability and accuracy of enzyme assays. On-line enzyme assays that integrate IMERs with a separation and identification system for G6PDH enzyme assay are now on high demand.

Among a variety of separation techniques, capillary electrophoresis (CE) offers several advantages, such as high efficiency, sensitivity, fast analysis, low sample volume requirements and so on. By combining with IMERs, CE can be applied not only as a separation tool with high performance but also as a versatile platform for on-line enzyme studies [13,20]. Over the past few decades, CE-based IMERs, in which IMERs are fabricated on capillaries, have attracted intense research interest, representing a promising miniature approach over a wide range of application of enzyme assays, including enzyme activity, peptide mapping in proteomics, inhibition screening and diagnostics [21–26].

Here, we describe a simple but reliable strategy for the production of CE-based G6PDH-IMERs for rapid enzyme activity

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assay and inhibition studies, which is accomplished based on electrostatic assembly. The performance of the CE-based G6PDH-IMERs is investigated to show the feasibility and accuracy of the method for on-line enzyme assay of G6PDH. The activity of the G6PDH enzyme as well as the enzyme inhibition by six inhibitors was investigated on-line using CE-based IMERs. The kinetic constants of the G6PDH enzyme reaction and inhibition were determined and were found to be consistent with those obtained using off-line assay. The inhibition mechanism of each inhibitor of the G6PDH enzyme was also investigated.

2. Materials and methods

2.1. Chemicals

Poly (diallyldimethylammonium chloride) (PDDA) (20%, w/w in water, M_w = 200,000–350,000) was purchased from Jing Chun Reagent Inc. (Shanghai, China). G6PDH from leuconostoc mesenteroides, β-Nicotinamide adenine dinucleotide (NAD^+), D-glucose-6-phosphate (G6P) and Nicotinamide adenine dinucleotide (NADH) were purchased from Sigma Chemical Co. (Mt. Louis, MO, U.S.A.). Vancomycin was purchased from J&K Scientific Co., Ltd. (Beijing, China). Urea, potassium permanganate ($KMnO_4$), pentahydrate copper sulfate ($CuSO_4 \cdot 5H_2O$), lead nitrate ($Pb(NO_3)_2$) and cadmium nitrate ($Cd(NO_3)_2$) were purchased from Tianjin Fine Chemicals Co., Ltd. (Tianjin, China). All other reagents were of analytical grade and were used without further purification. All solvents and solutions were filtered using a 0.22-μm membrane filter prior to use.

2.2. Fabrication of CE-based G6PDH-IMERs

Fig. 1 presents our schematic procedure for the production of the CE-based G6PDH-IMERs, which is made using a two-step protocol based on the electrostatic assembly. Because it possesses negative charges at pH 7.8 (pI is ~4.6), the enzyme G6PDH cannot be adsorbed on the inner surface of the bare capillary due to the presence of negatively charged silanol groups. Therefore, the inner surface of the capillary is first modified by treating it with a water-soluble polyelectrolyte possessing strong cation groups as the ion exchanger [27]. In this study, PDDA was used to modify the inner surface of the capillary to create a positively charged coating [21,26]. Prior to this modification, an untreated capillary was successively rinsed with 0.1 M NaOH for 30 min and deionized water for 10 min. Once preconditioned, PDDA solution was injected into the capillary by pressure at 50 mbar for 20 s, resulting in a ~2 cm plug of PDDA solution. The plug was then kept in the capillary for 1 h to create a positive charged coating on the inner wall. The positive PDDA coating layer produced reversed EOF on the inner surface of capillary and sped up the migration of negatively charged analytes (in this study, the product NADH of G6PDH enzyme reaction) thereby reducing the analysis time. The G6PDH enzyme solution (1.0 mg/mL in 50 mM phosphate buffer at pH 7.8) was then injected into the capillary and kept for 30 min to allow G6PDH to be absorbed on the positive-charged PDDA layer by electrostatic assembly coating. In this way, a 2-cm long CE-based G6PDH-IMER was produced at the inlet end of the capillary. Between the steps, deionized water was flushed through the capillary for 5 min to wash out any unreacted reagent.

The resultant IMERs were easily regenerated, by flushing the capillary with 1 M NaCl, 0.1 M HCl, and 0.1 M NaOH, consecutively, to elute the immobilized enzyme (desorption), then repeating the two-step protocol for enzyme immobilization.

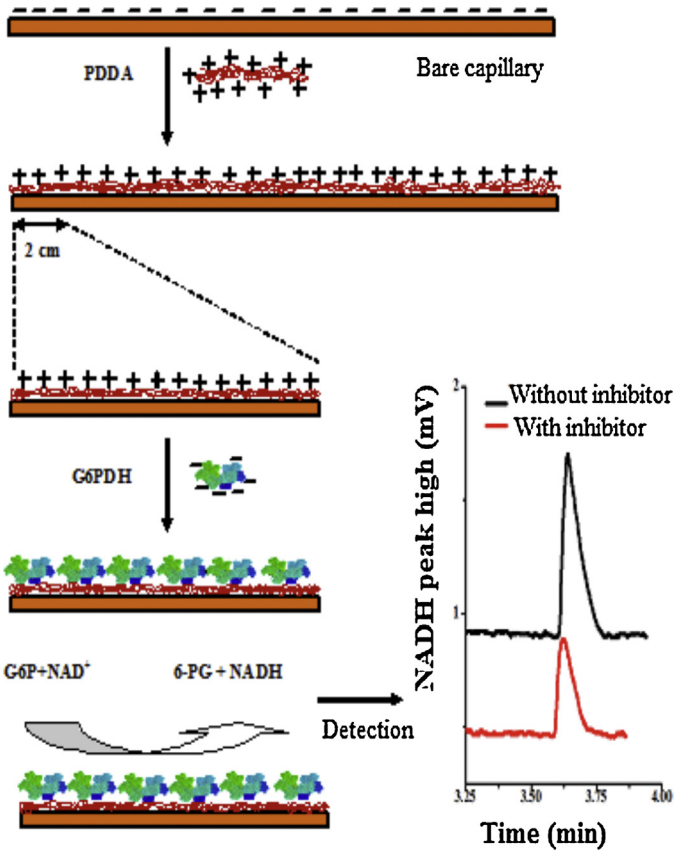

Fig. 1. Schematic diagram of the procedure for fabrication of the CE-based G6PDH-IMERs by electrostatic assembly via PDDA layer.

2.3. On-line enzyme and inhibition assay

All experiments were carried out in a CE apparatus (CL1020Beijing Cailu Science Apparatus, China) under 22 °C cooling air. Fused silica capillaries (50 μm i.d., 360 μm o.d.) with total/effective length of 45/37 cm were used for production of the IMER as well as separation/detection column. The CE running buffer was 20 mM phosphate buffer at pH 7.8. Prior to analysis, the IMER capillary was filled with the running buffer and equilibrated at 200 V/cm until a stable current and baseline was achieved. The substrate solution, with or without inhibitor, was hydrodynamically injected into the enzyme reactor at 10-cm height for 3 s. After incubation by suspension in the column of buffer for a desired period, a high voltage of −10 kV was applied to separate the substrate and products. The enzyme activity or inhibition was determined by measuring the peak height of the product (NADH), which was detected by UV absorption at a wavelength of 340 nm.

2.4. Off-line enzyme and inhibition assay

For off-line assay, a reaction mixture of 120 μL was prepared containing G6P and NAD^+ of different concentrations in 50 mM Tris/HCl buffer (pH 7.8). For inhibition study, the reaction mixture also contained different concentrations of an inhibitor. The reaction was started by quickly adding 50 μL of enzyme (1 U/mL) into the mixture. Aliquots of 10 μL were periodically removed from the reaction mixture, and the enzyme reaction was terminated by addition of 2 μL 0.1 M HCl to each aliquot. The sample was injected into a capillary (50 μm i.d., 365 μm o.d.) at a height of 10 cm for 3 s. The product NADH was separated by CE and detected by UV absorption at 340 nm. The CE running buffer was 20 mM phosphate buffer (pH 7.8).