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Evaluation of thrombin inhibitory activity of catechins by online capillary electrophoresis-based immobilized enzyme microreactor and molecular docking

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ABSTRACT

An online capillary electrophoresis (CE)-based thrombin (THR) immobilized enzyme microreactor (IMER) method was established to screen THR inhibitors in this study. S-2366 was used as chromogenic substrate for determination of THR activity and other kinetic constants. After continuously run for 50 times, the prepared IMER could still remain 89% of the initial immobilized enzyme activity. The Michaelis-Menten constant (K_m) of immobilized THR was measured as 0.514 mmol/L and the half-maximal inhibitory concentration (IC_{50}) and inhibition constant (K_i) of argatroban on THR were determined as 78.07 and 26.53 nmol/L, respectively, which indicated that CE-based THR IMER was successfully established and could be applied to screen THR inhibitors. Then the prepared IMER was used to investigate the inhibitory potency on THR of four main catechins in green tea including epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), and epigallocatechin gallate (EGCG). The results showed that ECG and EGCG had good THR inhibition activity and their inhibition rates at concentration of 200 μmol/L were 53.2 ± 3.8% and 55.8 ± 2.6%, respectively, which was in consistent with the results of microplate reader assay. Additionally, molecular docking results showed that the benzopyran groups of ECG and EGCG were inserted into the THR active pocket and interacted with residues LYS60F, TRP60D, TRY60A, IEU99, GLY216, HIS57 and SER195, but EC and EGC did not. Therefore, the developed CE-based THR IMER is reliable method for measuring THR inhibitory activity of natural inhibitors.

1. Introduction

Cardiovascular diseases (CVDs) take the lives of 17.7 million people every year, 31% of all global deaths [1]. Medicines such as β-adrenergic blockers had been intensively used for the treatment of various CVDs [2–4]. In reality, thrombosis are among the leading causes of CVDs. Thrombin (THR), a blood coagulation enzyme, plays an important role in processes of thrombosis [5]. Currently, lowering the activity of THR or preventing its generation is one of the most effective treatment strategies for thromboembolic diseases. Argatroban and dabigatran etexilate, direct THR inhibitors, have been clinically used to prevent and treat thrombosis-related indications; however, serious side effects like hemorrhage may accompany along with their treatment [6,7]. Recently, some THR inhibitors from natural products such as 15, 16-dihydrotanshinone I, cryptotanshinone and tanshinone IIA in Radix Salviae Miltiorrhizae; protocatechuic aldehyde and salvianolic acid C in Danshen injection; isochlorogenic acid C and senkyunolide I in Rhizoma Chuanxiong had been screened out with direct THR inhibition activities [8–10].

On the other hand, previous results suggested that polyphenolic compounds might be potential structural bases and source to find and project nature-based, safe, orally bioavailable direct THR inhibitors [11,12]. Green tea is a beverage rich in catethins such as epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), and epigallocatechin gallate (EGCG) (chemical structures were presented in Fig. 1). Above all, EGCG contributes to more than 50% of the total catechins. The bulk of the available data support the hypothesis that platelet inhibitory effects might explain the observed relations between tea consumption and reduced cardiovascular risks [13]. Studies also showed that catechin, EC and EGCG may have anticoagulant activity [14,15]. However, it remains uncertain whether green tea catechins can inhibit THR activity or not.

Abbreviations: THR, Thrombin; EC, Epicatechin; EGC, Epigallocatechin; ECG, Epicatechin gallate; EGCG, Epigallocatechin gallate; CE, Capillary electrophoresis; EMMA, Electrophoretically mediated microanalysis; IMER, Immobilized enzyme microreaction; pNA, para-nitroaniline; S-2366, L-Pyroglutamyl-L-prolyl-L-arginine-p-Nitroaniline hydrochloride; K_m, Michaelis-Menten constant; IC_{50}, Half-maximal inhibitory concentration; K_i, Inhibition constant; Tris, Tris (hydroxymethyl) aminomethane; BGE, Background electrolyte; EOF, Electroosmotic flow; CVDs, Cardiovascular diseases

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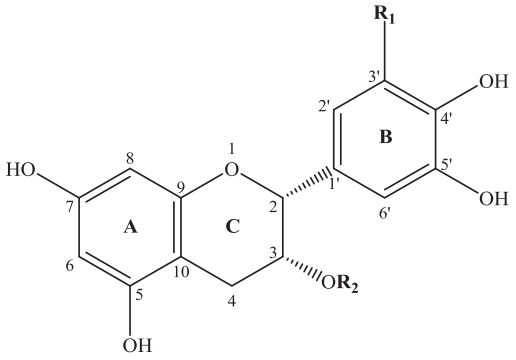

EC: R_1=H; R_2=H;
EGC: R_1=OH; R_2=H;
ECG: R_1=H; R_2= Galloyl group;
EGCG: R_1=OH; R_2= Galloyl group;

Fig. 1. Chemical structures of four catechins.

Traditionally, enzyme inhibitory activity tests were carried out by microplate reader assay with multiwell plate [8–10], but requirement of large amount of enzyme is the main disadvantage of this method. To compensate, capillary electrophoresis (CE) has been employed in assays for studying enzyme kinetics, evaluating enzyme activity, and for inhibitor screening due to its high separation efficiency, extremely small sample requirement, short analysis duration, and ease of automation [16–18]. Generally, CE-based enzyme assays can be divided into pre-capillary and in-capillary that incubation, separation and analysis are consecutive processes performed in the same capillary [19–21]. In-capillary assays mainly include electrophoretically mediated microanalysis (EMMA) and immobilized enzyme microreactor (IMER). In IMER method, Enzyme could be immobilized in inlet of capillary by various methods such as adsorption [22], cross-linking [23], encapsulation [24] and magnetic beads [25,26]. Among them, adsorption is cheap, easy to carry out, as well as mildly and moderately harmful to enzyme. Furthermore, comparing with EMMA, enzyme could be reused by IMER method. In reality, the inhibitory potency of argatroban on THR using the chromogenic substrate L-Pyroglutamyl-L-prolyl-L- arginine-p-Nitroaniline hydrochloride (formula: pyroGlu-Pro-Arg-pNA·HCl, MW: 539.0) (S-2366), which can be readily split by THR, had been determined by EMMA principle [27]. However, there are no reports about THR inhibitor assay using IMER method.

The aim of this study was to develop an online CE-based IMER for THR inhibitor screening. THR was immobilized in inlet of capillary by adsorption using a simple, short time, and automation operation. The enzymatic reaction was triggered by mixing with chromogenic substrate S-2366, whose product is para-nitroaniline (pNA) that can be detected at wavelength of 405 nm. Through measuring the peak area of product pNA, the Michaelis-Menten constant (K_m) of immobilized THR was measured. Argatroban, a direct THR inhibitor, was used to validate the CE-based THR IMER for inhibitor screening, and the values of half-maximal inhibitory concentration (IC_{50}) and inhibition constant (K_i) were calculated. Afterwards, the method was applied to investigate the inhibitory potency of EC, EGC, ECG and EGCG on THR. In addition, molecular docking as a support method was used to demonstrate the bind mode between these catechins and active sites of THR.

2. Materials and methods

2.1. Reagents

Bovine THR was obtained from Sigma-Aldrich (Shanghai) Trading Co., Ltd. (Shanghai, China). EC, EGC, ECG, and EGCG were purchased from Chengdu Biopurify Phytochemicals Ltd. (Chengdu, China). Argatroban was purchased from Meilune Biological Technology Co., Ltd. (Dalian, China). S-2366 was obtained from Chromogenix. (Milano, Italy). Tris (hydroxymethyl) aminomethane (Tris) was obtained from Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China). HCl were analytical grade reagents and purchased from Chengdu Kelon Chemical Reagent Factory (Chengdu, China). The water used for all the experiments was purified by water purification system (ATS-H20, Antesheng Environmental Protection Equipment Co., LTD., Chongqing, China). Unless otherwise specified, all other chemicals and solvents were guaranteed reagent grade.

2.2. Apparatus

The capillary electrophoresis experiments were performed on an Agilent 7100 3D CE system (Agilent Technologies, Palo Alto, CA, USA) equipped with a diode array detector and Agilent ChemStation software. The bare fused-silica capillary (Yongnian Ruifeng Chromatographic Device Co., Ltd., Hebei, China) used in all the experiments was 75 μm id and 50 cm total length with 41.8 cm effective length. Background electrolytes (BGE) buffer and catechins solutions were ultrasonicated in a KQ-100B ultrasonic cleaner (Kunshan ultrasonic instruments Co., Ltd, Kunshan, China). The pH of running buffer was measured by a FE28 pH meter (Mettler-Toledo Instruments, Shanghai, China). Enzyme inhibitory assay was carried out by iMark™ microplate absorbance reader (Bio-Rad Laboratories, Inc., California, USA.)

2.3. Preparation of buffers and solutions

The BGE solution containing 20 mmol/L Tris was carefully adjusted to pH 7.4, 8.0 and 8.5 with 1 mol/L HCl, respectively. A stock solution of THR (500 U/ mL) was prepared in water. This stock solution was stored at −20 °C in aliquot of 100 μL. A stock solution of S-2366 was solved in 20 mmol/L Tris-HCl solution (pH 7.4) at a concentration of 4 mmol/L. Catechins in green tea have multiple phenolic hydroxyl groups, so they can be easily dissolved in water. Stock solutions of EC, EGC, ECG and EGCG were solved in water at a concentration of about 1 mmol/L. Stock solution of argatroban was solved in methanol at a concentration of 10 mmol/L.

2.4. Preparation of THR IMER

In this experiment, the characteristic of THR easily absorbing on surface of silica was exploited to immobilize THR in inner wall of capillary. The immobilized THR zone is short as conventional sample injection length. So the influence of immobilization of THR on inner surface of capillary to electroosmotic flow (EOF) could be negligible. New fused silica capillaries were pretreated with methanol, HCl (1 mol/L), and NaOH (1 mol/L) for 30 min each, water for 10 min and BGE for 5 min. Subsequently, an automated program was set to prepare the IMER: THR solution (125 U/L) was introduced with a voltage +10 kV for 8 s, remained for 30 s, flushed using BGE with a pressure of −100 mbar for 60 s, and then flushed with a pressure of 935 mbar for 180 s. The immobilized THR capillaries could be immediately used after being prepared. When the activity of the immobilized enzyme decreases severely, the microreactor can be renewed by rinsing the capillary with NaOH (1 mol/L) for 20 min, water for 10 min, BGE for 5 min and then repeating the preparation program.

2.5. CE conditions of enzymatic reaction in IMER

The temperature of the capillary cartridge was set at 25 °C. The enzyme kinetics was carried out by a reaction that the substrate was injected into the enzyme microreactor of the capillary inlet by applying