Talanta 79 (2009) 1130–1134

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Flow injection analysis of angiotensin I-converting enzyme inhibitory activity with enzymatic reactors

Le Hoang Lam [a], Tomoko Shimamura [a,*], Munetaka Ishiyama [b], Hiroyuki Ukeda [a]

[a] Faculty of Agriculture, Kochi University, Monobe B-200, Nankoku 783–8502, Japan
[b] Dojindo Laboratories, Tabaru 2025-5, Kamimashiki, Kumamoto 861-2202, Japan

ARTICLE INFO

Article history:
Available online 20 February 2009

Keywords:
ACE
Antihypertension
3HB-GGG
FIA
Enzymatic reactor

ABSTRACT

Assay of angiotensin I-converting enzyme (ACE) inhibitory activity always draws much attention because of diverse applications in the field of antihypertension and related pathogenesis. Recently, the use of a new synthetic substrate, 3-hydroxybutyrylglycyl-glycyl-glycine (3HB-GGG), for the assay of ACE inhibitory activity has been confirmed. To construct a rapid, economical, and automatic determination system of ACE inhibitory activity using 3HB-GGG, a flow injection analysis (FIA) system with enzymatic reactors was developed in this study. Enzyme reactors were composed of aminoacylase and 3-hydroxybutyrate dehydrogenase immobilized separately on CNBr-activated Sepharose 4B. The assay condition was optimized in terms of the conversion of 3HB-G into NADH by the enzymatic reactors when the reaction solution containing 3HB-G generated from 3HB-GGG (after the incubation with ACE) was repetitively injected into the FIA system. Under the optimized conditions, 3HB-G was converted to 3HB, and then 3HB was oxidized by NAD^+ to form NADH. The developed system successfully detected practical ACE inhibitors with a great sensitivity, high sampling frequency (10 samples h^{-1}) and a durable stability of the enzymatic reactors.

1. Introduction

Angiotensin I-converting enzyme (ACE) is a dipeptidyl carboxypeptidase associated with the renin–angiotensin system [1]. One of its key actions is the regulation of blood pressure together with water and salt metabolism, since it cleaves angiotensin I into a potent vasopressor angiotensin II. Besides, ACE inactivates bradykinin, a hypotensive peptide, by sequential removal of two C-terminal dipeptides [2]. The increase in serum ACE activity results in blood-pressure elevation and related diseases, such as sarcordosis, silicosis, and hyperthyrodis [3]. Therefore, the assay of ACE as well as the assay of ACE inhibitory activity was necessary to identify antihypertensive compounds in order to prevent high-blood pressure and related pathogenesis.

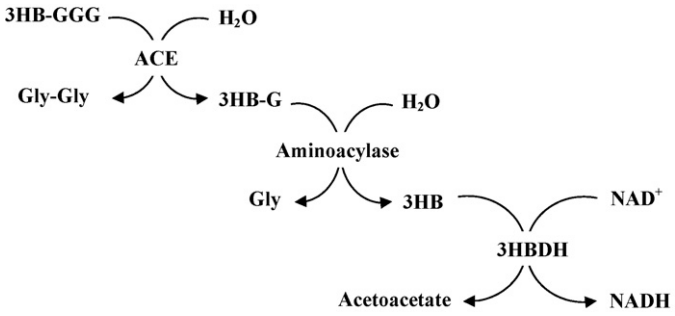
In the field of functional food, studies on active components (principally peptides hydrolyzed from various food sources) that can inhibit the ACE activity with the aim to control hypertension through diet have been carried out [2]. In order to facilitate the identification and isolation of ACE inhibitors, numerous methods for the measurement of ACE activity have been reported. In most of those works, the principle of the ACE assays was based on a hydrolysis of the synthetic peptide hippuryl-His-Leu (HHL) [4], which has been used widely. However, this conventional method often encounters interferences because of the use of invisible wavelength 228 nm and the extraction of unexpected components with organic solvent.

A newly synthesized substrate, 3-hydroxybutyrylglycyl-glycyl-glycine (3HB-GGG), has been proposed for ACE inhibition assay [5,6]. It was the measurement of ACE inhibitory activity based on the detection of 3-hydroxybutyric acid (3HB) derived from 3HB-GGG by the action of ACE and aminoacylase. The generated 3HB was detected spectrophotometrically by a commercial F-kit [5]. Subsequently, the costly F-kit was substituted by a more effective measurement of 3HB with a water-soluble tetrazolium salt (WST-1) [6]. Those established assays using 3HB-GGG were applicable for the evaluation of ACE inhibitory activity of practical samples. In addition, they were more selective and convenient than the conventional method using HHL.

In this study, a double-line FIA system with enzymatic reactors for ACE inhibition assay using 3HB-GGG was developed with the aim to construct a rapid, economical, and automatic analysis system. The principle of the assay is shown in Scheme 1. The FIA system with aminoacylase reactor and 3-hydroxybutyrate dehydrogenase (3HBDH) reactor was used to measure 3HB-G derived from 3HB-GGG by the hydrolysis of ACE. Aminoacylase and 3HBDH were immobilized on CNBr-activated Sepharose 4B. In the FIA system, 3HB-G was a sample subjected to a hydrolysis by aminoacylase to form 3HB and Gly. The product, 3HB, was subsequently oxidized by NAD^+ to form NADH and acetoacetate with the catalysis of 3HBDH. The formed NADH was spectrophotometrically detected at 340 nm.

* Corresponding author. Tel.: +81 88 864 5193; fax: +81 88 864 5189.
E-mail address: tomokos@kochi-u.ac.jp (T. Shimamura).

0039-9140/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2009.02.016



Scheme 1. Principle of assay of ACE inhibitory activity using FIA system with enzymatic reactor.

2. Experimental

2.1. Reagents and chemicals

CNBr-activated Sepharose 4B was obtained from GE Healthcare (Uppsala, Sweden). 3-Hydroxybutyrate dehydrogenase (EC 1.1.1.30, 140 units/mg, from *Pseudomonas sp.*) and aminoacylase (acylase I, EC 3.5.1.14, 4300 units/mg, from pig kidney) were purchased from Wako (Osaka, Japan). The F-kit was purchased from R-Biopharm (Darmstadt, Germany). ACE (EC 3.4.15.1, 3.4 units/mg, from rabbit lung), DL-*ß*-hydroxybutyric acid sodium salt (DL-3HB), and Val-Tyr were purchased from Sigma (St Louis, MO, USA). 3HB-GGG, 3HB-G, and WST-1 were obtained from Dojindo Laboratories (Kumamoto, Japan). All chemicals and solvents were of analytical grade and were prepared with water purified by a Milli-Q system (Millipore, Tokyo, Japan).

Five kinds of antihypertensive drinks (Tokuho, in Japanese) containing either Val-Tyr or a mixture of Ile-Pro-Pro and Val-Pro-Pro were purchased from local supermarkets and kindly gifted from Senmi Ekisu Co. (Ehime, Japan).

2.2. Apparatus

The diagram of the double-line FIA system (PD-2000, Ogawa Co., Kobe, Japan) with the enzymatic reactors was shown in Fig. 1. The phosphate buffer (0.1 M, pH 8.0) and NAD^+ solution were pumped with two plunger pumps at a total flow rate up to 3.0 mL min^{-1}. The samples were injected into the stream through a manually operated six-way valve. The absorbance was measured at 340 nm with a Chratec colorimeter (KCM-0306, Ogawa Co., Kobe, Japan) and recorded with a potentiometric recorder (Servocorder SR 6211, Graphtec, Tokyo, Japan). The peak height reflected the concentration of 3HB-G in the sample.

The sample solutions were ultrafiltrated with Centricut (cut-off size 10 kDa, Kurabo Industries, Osaka, Japan) before being injected into the FIA system. The ultrafiltration process was performed with a Hitachi compact centrifuge (Himac CF 16 RX, Tokyo, Japan).

2.3. Enzyme immobilization

CNBr-activated Separose 4B was selected as the support for enzyme immobilization, because it provides a convenient way to immobilize ligands by the cyanogens bromide method [7]. Sepharose (0.25 g) was added to 25 mL of 1 mM HCl with occasionally gentle shaking for 15 min in order to swell the gel. After the HCl solution was filtered out, the retained gel was washed with 200 mL of phosphate buffer (0.1 M, pH 8.0). The obtained gel was put into the enzyme solution containing 1.3 mg of 3HBDH or 100 mg of aminoacylase in 1.0 mL of phosphate buffer (0.1 M, pH 8.0). The mixture was shaken adequately and kept at 4 °C overnight for enzyme immobilization. The mixture of enzyme and gel was stuffed into a glass tube (2.0 mm i.d., 150 mm length) by a peristaltic pump (ATTA Co., Tokyo, Japan). The two extremities were closed with glass wool and filter paper. The reactor was washed with phosphate buffer (0.1 M, pH 8.0) to remove the excessive enzyme. The protein concentration of the enzyme solutions was determined by measuring the absorbance at 280 nm before and after the immobilization. The coupling yields were calculated as the percentage disappearance of the amount of protein initially added to the reaction mixture [8]. They were 81% and 21% for 3HBDH and aminoacylase, respectively.

2.4. Assay of ACE inhibitory activity

To a 1.5-mL snap-lock microtube, 125 μL of 12.35 mM 3HB-GGG, 15 μL of sample (ACE inhibitors), and 50 μL of 0.2 U mL^{-1} ACE were added. The substrate solution was freshly prepared by dissolving 3HB-GGG in borate buffer containing 380 mM NaCl (pH 8.3), meanwhile the ACE solution was prepared with borate buffer (pH 8.3) [5]. The reaction mixture was stirred thoroughly for 20 s and then incubated at 37 °C for 30 min. Right after the incubation, the reaction was immediately terminated by ultrafiltration with Centricut (cut-off size 10 kDa, Kurabo Industries, Japan) for 10 min at 5000 rpm (10 °C) to remove ACE. The solution containing 3HB-G was then injected into the FIA system as a sample.

2.5. Optimization of the FIA system

Conditions of the FIA system were optimized in terms of the conversion of 3HB-G and 3HB by the action of aminoacylase and 3HBDH. The conversion of 3HB-G and 3HB was calculated based on the calibration curve of 3HB-G, 3HB, and NADH. The standard solutions of 3HB-G, 3HB, and NADH at various concentrations were alternately injected into the FIA system to make the calibration curves between the absorbance and the concentration of substances. The 3HB solution was prepared by dissolving the DL-3HB in buffer (the concentration of D-isomer was half of the DL-3HB

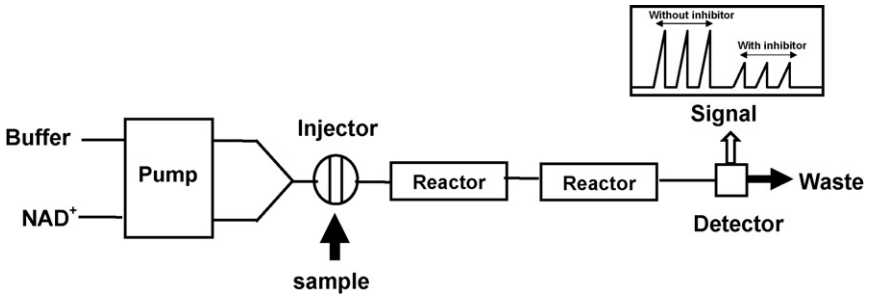


Fig. 1. Flow diagram of FIA system with enzymatic reactors for assay of ACE inhibitory activity. 0.1 M phosphate buffer, pH 8.0; 1 mM NAD^+ in 0.1 mM phosphate buffer, pH 8.0; injection volume, 20 μL.