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Real-time assay of immobilized tannase with a stopped-flow conductometric device

Fu-Shiang Chang [a], Po-Chung Chen [a], Richie L.C. Chen [a], Fu-Ming Lu [a], Tzong-Jih Cheng [a,b,*]

[a] Department of Bio-Industrial Mechatronics Engineering, College of Bioresources and Agriculture, National Taiwan University, Taipei, Taiwan
[b] Department of Biomedical Engineering, National Taiwan University Hospital, College of Medicine, National Taiwan University, Taipei, Taiwan

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Abstract

A stopped-flow manifold was developed to assay and characterize immobilized tannase (EC 3.1.1.20). The immobilized enzyme reactor was inserted within the tube-type electrode pair (cell constant = 103.2 cm^{-1}) for a real-time conductometric measurement. Tris buffer (2 mM, pH = 7.0) was used as the carrier for sensitivity improvement. The activities and kinetic parameters (K_m values) for propyl gallate, methyl gallate and tannic acid were investigated.

Keywords: Conductometric; Enzyme kinetics; Tannic acid; Stopped-flow

1. Introduction

Tannase (EC 3.1.1.20) is a crucial enzyme for degrading hydrolyzable tannins; the commercialized enzymes are usually expressed by microorganisms such as bacteria, fungi and yeast [1]. Tannase has various important applications in food industries including tea cream reduction, beer chill proofing, wine-making, the enzyme is also pivotal in synthesizing antioxidants of gallic acid derivatives [2]. The enzyme is frequently immobilized for repetitive use, stability improvement or to prevent product contamination [3]. Rapid analytical methods for the activity of immobilized enzyme are demanded especially in industrial applications.

Several analytical methods had been proposed to assay tannase. Spectrophotometric methods required specific substrates [4] and/or laborious post-reaction procedures [5], method using chromogenic reagent, rhodanine, was therefore developed to improve the sensitivity and selectivity [6,7]. The rhodanine method was adopted as a flow-injection analytical method for gallotannins [8]. However, the chromogenic reaction was prone to be affected by sample matrix, and a spike-calibration procedure must be used to improve the precision and dynamic range [8].

In the present conductometric approach, the enzyme activity was measured directly in a real-time manner. With the proposed stopped-flow manifold, the activity and kinetics of immobilized tannase can be easily determined and characterized, which were certainly valuable for industrial applications and academic researches.

2. Materials and methods

2.1. Chemicals

Tannase (EC 3.1.1.20, from *Aspergillus oryzae*, 56 U/mg), tannic acid, *n*-propyl gallate, methyl gallate, gallic acid monohydrate and 25% glutaraldehyde solution were purchased from Wako Co. Controlled pore-size aminopropoyl glass bead (aminopropoyl-CPG, 80–120 mesh, 70 nm of average pore size) was purchased from Sigma Co. as the supporting material for enzyme immobilization. Other chemicals were of analytical grade and used as received.

2.2. Immobilization of tannase

Tannase (20 mg) was covalently immobilized onto the surface of 0.1 g of aminopropoyl-CPG using glutaraldehyde as

* Corresponding author. Department of Bio-Industrial Mechatronics Engineering, College of Bioresources and Agriculture, National Taiwan University, Taipei, Taiwan. Fax: +866 2 33665345.
E-mail address: tzongjih@ntu.edu.tw (T.-J. Cheng).

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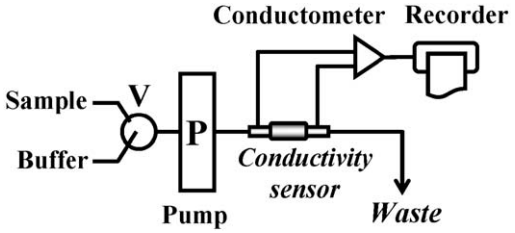

Fig. 1. Schematic diagram of the stopped-flow manifold. P: peristaltic pump (1 ml/min); V: flow-switching valve.

the cross-linking reagent [9]. Typically, 0.1 g of glass beads was washed with 0.2 M carbonate buffer (pH 10.0), and the beads were immersed in 4 ml of carbonate buffer. Excess amount of glutaraldehyde (1 ml of 25% glutaraldehyde solution) was added into the bead suspension to activate the amino groups. The Schiff-base formation reaction was proceeded at ambient temperature for 2 h under mild orbital shaking.

After washing with 0.1 M phosphate buffer (pH 6.8), the activated glass bead slurry was labeled with tannase (20 mg in 1 ml of phosphate buffer) at 4 °C for over 12 h. The resulting immobilized enzyme slurry was washed with phosphate buffer containing 3% (w/v) NaCl to remove non-covalent adsorption.

The unreacted amino groups on the surface of glass beads were blocked by adding 5 ml phosphate buffer containing 2% glycine. The blocking process was proceeded at ambient temperature for 2 h under the aforementioned orbital shaking condition. The resulting slurry was washed with phosphate buffer and then stored at 4 °C until use.

2.3. Conductometric flow cell

The tubular flow cell (total length = 7.5 cm; cell constant = 103.2 cm^{-1}) was assembled by connecting two stainless tube (2.5 cm × 2 mm i.d.) with a section (6 mm) of silicon tube (2 mm i.d.). Both ends of the enzyme reactor were stopped with cotton wool to prevent the leakage of the enzyme slurry.

2.4. Stopped-flow analytical system

The immobilized enzyme slurry was packed into the home-made conductometric flow cell. The low-pressure flow system (Fig. 1) comprised a controllable peristaltic pump (SMP-23S, Tokyo Rikakikai Co., Japan), a conductivity meter (SC-170, Suntex Instruments Co., Taiwan), the enzyme reactor and the tubing system (1 mm i.d.) for flow-injection analysis. Sample was introduced into the system manually or by a switching valve. The conductometric signals were monitored (Recorder model 101A-1878, Cole-Parmer) after stopping the carrier flow.

2.5. Impedance analysis

Impedances of the conductometric flow cell were measured with frequency response analyzer (Autolab PGSTAT30, Eco Chemie B.V., Netherlands) under the control of a built-in software (Frequency Response Analysis™ ver. 4.9). Sinusoidal input voltage was set at 0 V with 0.15 V of peak-to-peak amplitude, and directly imposed on the conductometric flow cell. The frequency range was from 1 Hz to 10 kHz.

3. Results and discussion

3.1. Effect of frequency

The frequency responses with pH buffers filled in the conductometric flow cell were investigated (Fig. 2). Along with the increase of frequency, the phase shift decreased to a

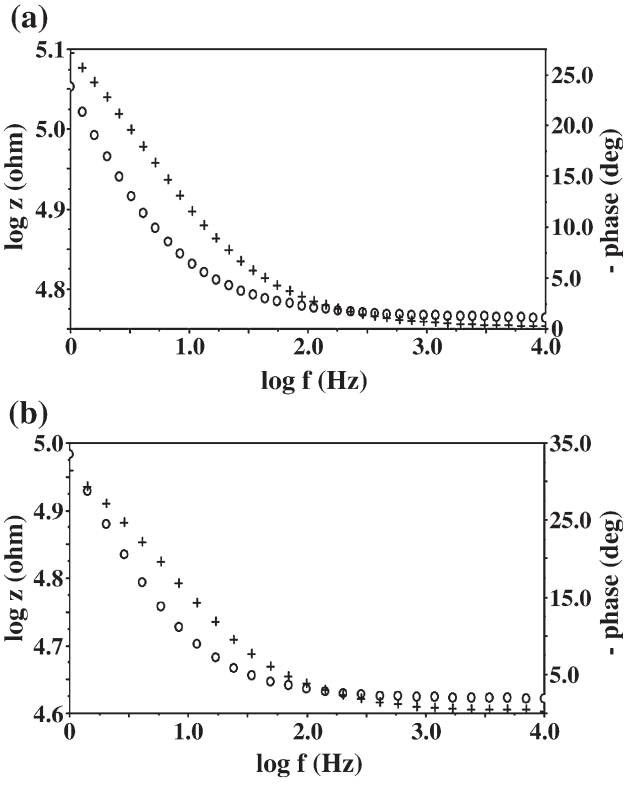

Fig. 2. Frequency response of the conductometric enzyme reactor. (a) Filled with 2 mM pH 7.0 Tris buffer. (b) Filled with 2 mM pH 7.0 phosphate buffer. ○: impedance; +: phase delay.

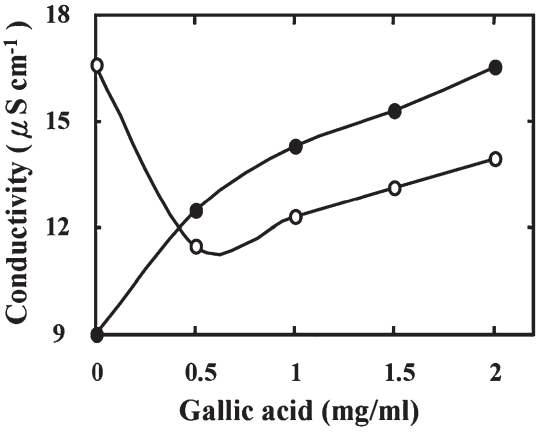

Fig. 3. Effect of gallic acid content on the conductivity of buffer. Gallic acid was dissolved in phosphate (○) and Tris (●) buffer (2 mM, pH 7.0).

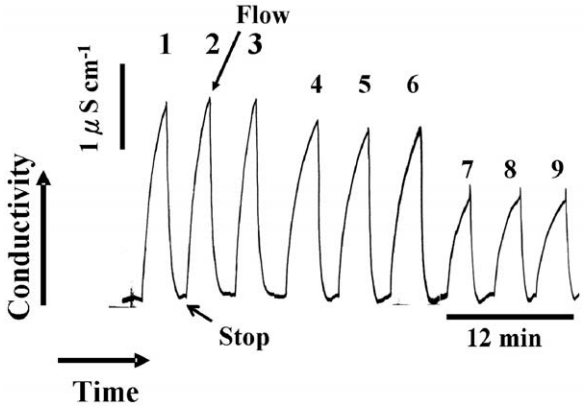

Fig. 4. Typical stopped-flow responses to different contents of tannic acid. 1, 2, 3: 2 mg/ml; 4, 5, 6: 1 mg/ml; 7, 8, 9: 0.5 mg/ml. The tannic acid solutions were dissolved in 2 mM Tris buffer, pH 7.0.

negligible value, which imply the disappearance of parasite capacitance. As the consequence, the impedance decreased also to its conductive component. Similar phenomena were observed with either Tris or phosphate buffer. With frequency exceeding 200 Hz, the problematic parasite capacitance can be neglected. Therefore, a commercial conductor meter with frequency higher than 200 Hz served well for the following studies.

3.2. Effect of pH buffer

The conductivities of pH buffers containing different concentrations of gallic acid were compared (Fig. 3). The proton exchange process between Tris and gallic acid molecules led to the increase in solution conductivity.

$$\text{Tris} + \text{Gallic acid} \rightarrow [\text{Tris} \cdot \text{H}]^{+} + \text{Gallate}^{-} \quad (1)$$

But the conductivity change of phosphate buffer (2 mM, pH 7.0) in the lower gallic acid concentration was in the opposite direction.

$$\text{HPO}_4^{2-} + \text{Gallic acid} \rightarrow \text{H}_2\text{PO}_4^{-} + \text{Gallate}^{-} \quad (2)$$

The negatively charged buffer system was ambiguous for quantification of the hydrolysis reaction and the background signal of phosphate buffer was higher than that of Tris buffer, therefore Tris buffer (2 mM) was used in the following experiments. The pH was optimized to be around 7.5.

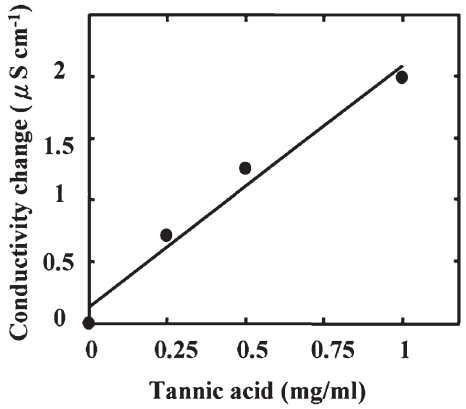

Fig. 5. Calibration curve of tannic acid.

Table 1
Kinetic parameters of immobilized tannase

Substrate[a]	K_m (mM)	V_{max} (μS cm^{-1} min^{-1})
Propyl gallate	7.28	0.59
Methyl gallate	14.97	1.43
Tannic acid[b]	1.37	1.93

[a] The substrates were dissolved in Tris buffer (2 mM, pH=7.0).
[b] Tannic acid is a mixture polyphenols containing mostly gallotannin, and its units of K_m is represented by mg ml^{-1}.

3.3. Stopped-flow signals

Fig. 4 shows typical stopped-flow signals by introducing tannic acid solutions into the enzyme reactor (1.0 ml/min), the conductivity increased immediately after stopping the sample flow. The linear detection range of conductivity was specified from 0 to 20 μS cm^{-1} in this system. Initial reaction rate of the immobilized tannase was estimated by the rate of conductivity change, which can be determined within 3.0 min. This parameter was useful in quantifying tannic acid content, as shown in Fig. 5, the linear range was up to 1.0 mg ml^{-1} (R^2=0.97). Lower detecting limit (S/N>3) for tannic acid measurement was 0.1 mg ml^{-1}, and the relative standard deviation for 1.0 mg ml^{-1} tannic acid was 2% (n=3).

3.4. Tannase kinetics

The initial rates for three different substrates were measured to determine the Michaelis constants (K_m) and the maximum reaction rate (Table 1). As compared with the K_m values of propyl gallate, K_m of immobilized tannase was slightly higher than that of free enzyme (K_m=6.24 mM, as measured with the present system), which indicated the reduction of substrate affinity after immobilization. As revealed by Table 1, the enzyme kinetics showed significant substrate dependency and the kinetics-based system was therefore inappropriate for sensing tannic acids with poor-defined phenolic compositions [10]. However, the system was very suitable in assaying the activity of immobilized enzyme and studying the kinetics.

4. Conclusion

Convenient and universal methods are demanded to quantify and characterize important hydrolytic enzymes including tannase. Compared with an oxidoreductase, hydrolase-catalyzed enzymatic reactions generally lack physical and/or chemical parameters of easy detection. Assays based on thermal measurement required sophisticated instrument and well-controlled (thermal-isolated) conditions [11]; pH-based methods were in itself out of pH control [10]. On the other hand, the instrumentation and condition control of a conductometric system are extremely simple and economic, and the resulting signal can be

easily handled in real-time. Based on conductometric principle, the proposed stopped-flow manifold is proven to be effective in assaying immobilized tannase, analytical applications to other enzymes are also expected.

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