Enzyme and Microbial Technology 67 (2014) 27–31

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

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

Activation of immobilized enzymes by acoustic wave resonance oscillation

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ARTICLE INFO

Article history:
Received 20 March 2014
Received in revised form 12 August 2014
Accepted 14 August 2014
Available online 4 September 2014

Keywords:
Immobilized enzymes
Resonance oscillation
Acoustic waves
Galactose oxidase
Amino acid oxidases
Enzyme activation

ABSTRACT

Acoustic wave resonance oscillation has been used successfully in the development of methods to activate immobilized enzyme catalysts. In this study, resonance oscillation effects were demonstrated for enzyme reactions on galactose oxidase (GAD), D-amino acid oxidase (DAAO), and L-amino acid oxidase (LAAO), all of which were immobilized covalently on a ferroelectric lead zirconate titanate (PZT) device that could generate thickness-extensional resonance oscillations (TERO) of acoustic waves. For galactose oxidation on immobilized GAD in a microreactor, TERO generation immediately increased enzyme activity 2- to 3-fold. Eliminating TERO caused a slight decrease in the activity, with ~90% of the enhanced activity retained while the reaction proceeded. Contact of the enhanced enzyme with a galactose-free solution caused almost complete reversion of the activity to the original low level before TERO generation, indicating that, not only TERO-induced GAD activation, but also preservation of the increased activity, required a galactose substrate. Similar activity changes with TERO were observed for enzyme reactions on DAAO and LAAO. Kinetic analysis demonstrated that TERO helped strengthen the interactions of the immobilized enzyme with the reactant substrate and promoted formation of an activation complex.

1. Introduction

Catalysis by immobilized enzymes has the important advantage of allowing separation of the enzyme from the reactant and product solution, but their poor catalytic performance has been a significant obstacle. Therefore, the development of a method for activating immobilized enzymes is needed.

Bulk acoustic resonance oscillations (ROs) generated on a single domain ferroelectric crystal by applying radiofrequency (rf) electric power are characteristic of periodic (dynamic) lattice distortion [1,2]. When applied to thin film metal catalysts deposited on ferroelectric crystals [3], acoustic waves lowered the activation energy of metal-catalyzed gas phase reactions, thus enhancing the catalytic reactions [4–11]. For example, thickness-extensional mode RO (TERO) with the lattice vibration mode perpendicular to the surface increased the catalytic activity of a Pd catalyst for ethanol oxidation by a factor of 1880 by lowering the activation energy of the reaction from 156 to 12 kJ mol^{-1} [3]. For the liquid-phase aldol condensation reaction of benzaldehyde and acetophenone to chalcone in a microreactor, TERO increased the activity by a factor of 8.2 and decreased the activation energy of the aldol condensation reaction by 70% [12].

To develop a method for artificially activating catalysis in an immobilized enzyme, resonance oscillation effects were applied to immobilized glucose oxidase (GOD) for glucose oxidation [13,14]. Results indicated that the TERO-induced dynamic lattice distortion vertical to the surface promoted catalysis of the immobilized GOD 3- to 5-fold. Interestingly, the catalytic properties of a bulky immobilized enzyme, *e.g.*, a super molecule, are influenced by the TERO-induced dynamic lattice distortion of substrates. The enzyme used was GOD only; but uncertainty remained about the applicability of that activation method to other enzyme systems.

In the present study, TERO was applied to immobilized galactose oxidase (GAD), D-amino acid oxidase (DAAO), and L-amino acid oxidase (LAAO). Acoustic wave TERO not only accelerated immobilized enzyme reactions, but also helped maintain the activated state.

2. Experimental

A ferroelectric lead zirconate titanate (PZT) polycrystalline ceramic device (25 mm in diameter, Murata Manufacturing Co., Ltd.) covered with 0.4-mm thick brass electrodes was used to produce radiofrequency electric power. The PZT had a resonance frequency of 2.2 ± 0.3 kHz and a lattice vibration of

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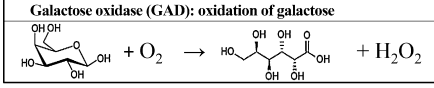
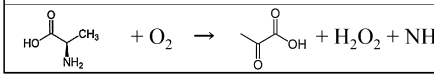
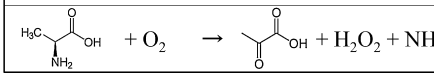
http://dx.doi.org/10.1016/j.enzmictec.2014.08.005
0141-0229/

Table 1
Enzymes and reactions.

Galactose oxidase (GAD): oxidation of galactose

D-amino acid oxidase (DAAO): oxidation of D-alanine

L-amino acid oxidase (LAAO): oxidation of L-alanine


thickness-extensional mode resonance oscillation (TERO) normal to the surface. Enzymes used were galactose oxidase (GAD) (*Dactylium dendroides*, 13.6 U mg^{-1}), D-amino acid oxidase (D-AAO) (Porcine kidney, 8.2 U mg^{-1}), and L-amino acid oxidase (L-AAO) [*Crotalus atrox* (western diamondback rattlesnake), 2.5 U mg^{-1}] from Sigma-Aldrich Co., Ltd. A covalent-bond method was used to immobilize the enzymes. The ferroelectric device was first dipped into 0.5 vol% 3-aminopropylethoxysilane in toluene for 2 h, dried, and then immersed in 2.5 vol% aq. glutaraldehyde for 2 h. After washing with distilled and ion-exchanged water, the device was immersed in a phosphate buffer solution (pH 7.0) of each enzyme. Concentrations of the solutions were 200 and 100 U mL^{-1} for GAD and the two AAO enzymes, respectively.

Table 1 shows the enzyme reactions that were investigated. Galactose oxidase catalyzes the oxidation of galactose to galactohexodialdose and hydrogen peroxide using molecular oxygen. The DAAO and LAAO oxidize D- and L-alanine to the corresponding acids, ammonia, and hydrogen peroxide.

The enzyme reactions were conducted using a flow-type microreactor, which was fabricated as described previously [12–14]. An enzyme-immobilized PZT device was fixed tightly with Viton rings in the reaction area of the microreactor. Substrate reactants were introduced into the reactor using a syringe pump and were allowed to flow around the immobilized enzyme catalyst at a liquid layer depth of 130 μm. One common product in all of the enzyme reactions was H_2O_2, therefore the amount of H_2O_2 generated over time was used to represent enzyme activity. The H_2O_2 was monitored chronoamperometrically using an electrochemical analyzer (BAS, ALS600D) with Pt counter and working electrodes and an Ag/AgCl/Cl reference electrode. To generate the acoustic wave resonance oscillation, the microreactor was furnished with electrode pins for the introduction of radiofrequency electric power. The radiofrequency power was generated from a function analyzer (NF Corporation, Multifunction Generator WF1974), amplified by an amplifier (NF Corporation, High-Speed Power Amplifier, 4005), and then introduced to an enzyme-immobilized RO device [13,14].

Galactose oxidation was conducted with a GAD concentration of 0.5 to 10 mM in potassium phosphate buffer, at a total flow rate of 20 μL min^{-1}, pH 6.0 to 8.0, and a reaction temperature of 278 to 300 K. For D-alanine and L-alanine oxidations, reaction conditions were: a concentration range of 2 to 10 mM in potassium phosphate buffer, pH 7.0, and a reaction temperature of 298 to 308 K. The frequency of electric power for TERO generation was 1.5–4.0 kHz with a scan speed of 1 s^{-1}. The applied RF electric power was 4.0 W.

3. Results and discussion

TERO-induced sonic wave effects, such as cavitation in the liquid phase and/or at the surrounding wall of the microreactor, may produce a local hot zone of high pressure and temperature. Thus, TERO was applied first to aqueous solutions without immobilized GAD, D-AAO, or L-AAO, and no H_2O_2 was produced. Next, TERO was applied to reactant solutions containing dissolved enzymes. TERO generation did not cause significant enhancement of the catalytic enzymatic reactions. Thus, no direct TERO-induced sonic wave effects on liquid phase reactions were observed.

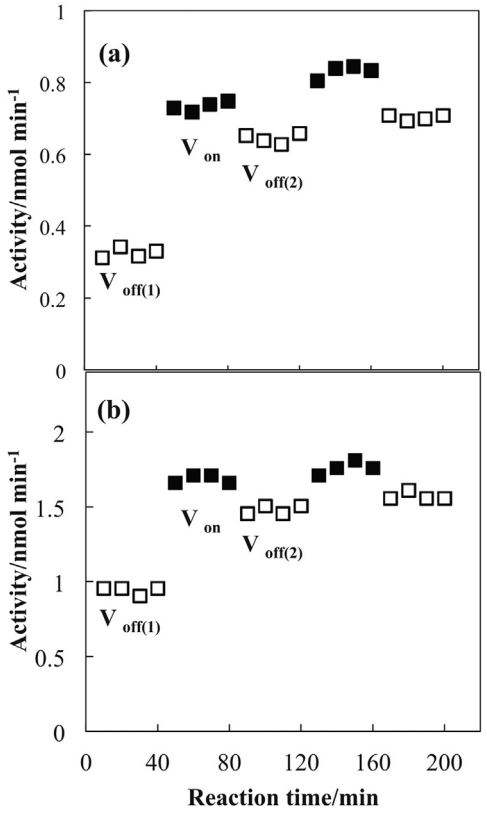


Fig. 1. Changes in the catalytic activity of immobilized GAD for galactose oxidation at (a) 283 K and (b) 300 K. Activity with TERO-off before TERO-on (□), activity with TERO-on (■), and activity with TERO-off after TERO-on (□) are designated $V_{off(1)}$, V_{on}, and $V_{off(2)}$, respectively.

3.1. GAD

Fig. 1 shows enzyme activity of immobilized GAD with and without application of TERO at reaction temperatures of 283 and 300 K. The original activity without TERO, $V_{off(1)}$, averaged 0.33 nmol min^{-1}. Upon generation of TERO, activity immediately increased to 0.73 nmol min^{-1}, indicated by V_{on}. The activation factor, defined as the ratio of V_{on} to $V_{off(1)}$, was approximately 2.3 at a reaction temperature of 283 K. When TERO was terminated, no significant activity decrease occurred, and activity ($V_{off(2)}$) remained approximately 88% of V_{on}. The second cycle of applying TERO and then ending it was nearly the same as in the first run. At a temperature of 300 K, the change in enzymatic activity with and then without TERO was similar to that observed at 283 K, except for a small activity enhancement of 1.8 upon application of TERO.

Thus, the characteristic TERO effects include significant activation of immobilized enzymes and maintenance of TERO-induced high activity without TERO.

The TERO-activated enzymes gradually lost the activated state upon contact with a substrate-free solution. To evaluate activity recovery, a recovery coefficient, *R*, was defined as the ratio of activity of [$V_{on} - V_{off(2)}$] to [$V_{on} - V_{off(1)}$]. Fig. 2 shows the comparison of the difference in activity recovery of immobilized galactose oxidase upon contact with galactose-containing and galactose-free