Kinetic study of immobilized ω-transaminase for the asymmetric synthesis of chiral amine

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Abstract. Biocatalysis is a powerful tool for the synthesis of high value products such as, chiral molecules and intermediates. Chiral amines can be synthesized by kinetic resolution of racemic amines or by the asymmetric synthesis from pro-chiral ketones. Kinetic parameter estimation for biocatalytic reaction is useful to evaluate process and technology options. In this work, the Michaelis–Menten kinetic parameters of immobilized ω-transaminase on methacrylate beads for the asymmetric synthesis of (R)-1-phenylethylamine (PEA) from acetophenone (ACP) and alanine (ALA) were determined. The parameters such as, Michaelis-Menten constant (Km) and maximum rate of reaction (Vmax) were measured from initial rate experiments by varying ACP concentrations (2 mM to 10 mM) at a fixed 100 mM ALA under mild reaction conditions (pH 7.5 and temperature 30 °C). The concentration of ACP and PEA were measured by gas chromatography. Lineweaver-burk plot was used to estimate the Vmax and Km from the initial rate data. The results yielded a Vmax of 6.87 mM/min and a Km of 2.52 mM. The Michaelis-Menten model is useful for understanding the kinetic properties of immobilized enzymes.

Keywords: Immobilization, parameter estimation, Michealis-Menten model, ω-transaminase

1. Introduction
Biocatalysis is today established as a tool for the syntheses of many interesting chiral molecules and intermediate compounds. Many of the syntheses have been driven by the ability of enzyme to catalyse reactions with high selectivity under mild conditions. Chiral amines can be produced by ω-transaminase (ω-TA; EC 2.6.1.18) class of aminotransferase enzymes, via kinetic resolution of racemic amines or asymmetric synthesis from pro-chiral ketones. The ω-TA enzyme catalyses the transfer of an amine group from a donor molecule to a carbonyl acceptor for the chiral amine. In principle, the maximum yield for the kinetic resolution and the asymmetric synthesis is 50% and 100%, respectively [1]. However, ω-TA is often hindered by unfavourable equilibrium [2], substrate and product inhibition in organic solvents, which therefore limits the yield.

To achieve industrially favourable yield, several process strategies are required to utilize ω-TA efficiently. Immobilize ω-TA onto different supports are the strategies to improve ω-TA properties. For example, ω-TA stability can be increased by entrapment on calcium alginate [4, 5], polymeric resins [6], methacrylate [7], LentiKats® [8], magnetic PVA-Fe3O4 nanoparticles [9] and by encapsulation in sol–gel/celite matrix [10]. However, immobilization can affect the kinetic properties of ω-TA. In a linear regression of the Michaelis–Menten model using Lineweaver–Burk plot, the substrate affinity (Km) of immobilized ω-TA on magnetic PVA-Fe3O4 nanoparticles was increased 2.5-fold (11.03 mM) from that of free ω-TA [9]. Similarly, the Km of immobilized whole cells ω-TA...
on calcium alginate was 6.05 mM (3.5-fold increase from that of free ω-TA), estimated by nonlinear regression of the Michaelis–Menten model [4]. The kinetics of immobilized ω-TA in methacrylate polymeric resin for the asymmetric synthesis of chiral amines have not yet been determined. Therefore, the objective of this study was to determine the kinetic parameters of immobilized ω-transaminase for the asymmetric synthesis of (R)-1-phenylethylamine (Figure 1).

![Figure 1](image_url). Reaction for the synthesis of acetophenone (ACP) and alanine (ALA) catalysed by immobilized ω-transaminase (ω-TA) [2]

2. Materials and methods

2.1 Materials

Materials used were acetophenone (ACP), alanine (ALA), and (R)-1-phenylethylamine (PEA) were purchased from Nacalai Tesque (Japan). (R)-ω-transaminase (ω-TA), pyridoxal 5’-phosphate (PLP), Diaion® HP-2MG (methacrylate polymeric resin), 4-bromoacetophenone, sodium phosphate dibasic (Na2HPO4), sodium phosphate monobasic, sodium hydroxide, ethanol and magnesium sulphate (MgSO4) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Methods

2.2.1 Immobilization of ω-Transaminase and PLP in Diaion beads resin

1 g of Diaion HP-2MG beads was washed with 0.1 M concentration of phosphate buffer solution with pH of 7.5 in a 20 mL vial. After that, 100 mg of ω-TA, 8 mg of PLP (0.8 mM) and 0.1 M phosphate buffer solution were added to the washed beads of Diaion HP-2MG. Then, the solution was stirred using the magnetic stirrer at 22°C. After 24 hr, the supernatant was removed and the wet ω-TA-PLP-beads was dried [7].

2.2.2 Kinetic studies

100 mg of immobilized ω-TA-PLP beads were added to a 20 ml vial in 0.1 M phosphate buffer solution (pH 7.5). Then, 0.5 mM PLP and 100 mM ALA were added to the vial. 10 mM ACP was added last for the reaction to take place. After the 3 min of reaction, 1 ml NaOH was added to stop the reaction. The procedure was repeated for 5 and 10 min. The ACP concentrations were then varied for 8 mM, 4 mM and 2 mM.

2.2.3 Gas chromatography analysis

The concentrations of ACP and PEA were measured by gas chromatography. Briefly, the sample after reaction was quenched by adding aqueous NaOH (5 M, 100 μL). After 30 min, 150 mM 4-bromoacetophenone was added as an eternal standard along with 150 μl of ethyl acetate for extraction. Magnesium sulphate was added to the solution and centrifuged at 5000 rpm for 20 min. The concentration of reactants and products was analysed by GC on a Clarus 500 (Perkin–Elmer) with a 25 m × 0.25 mm Agilent J&W CP-Chirasil-Dex CB column. A 2 μL sample was injected with a 30:1 split ratio and a thermal gradient from 120 to 200°C for 14 min. A carrier gas (Helium) was used at 1.4 mL/min and a flame ionization detector (FID) was carried out at 250°C [11].

2.2.4 Estimation of kinetic parameters

The enzyme was assumed to follow simple and known Michaelis-Menten kinetics. The experimental data was fitted with a Michaelis-Menten kinetic model as shown in Eq. 1. The kinetic parameters (Vmax and Km) can be estimated by a Lineweaver-Burke plot (double-reciprocal plot) from a linear
regression line through the values for 1/S against 1/V (Eq. 2) using Microsoft® Excel 2016. In the equations, V is the reaction rate and S is the substrate concentration. The effect of mass transfer resistance on the kinetic parameters of immobilized ω-transaminase was not considered in this study.

\[ V = \frac{V_{\text{max}} S}{K_m + S} \]  

(1)

\[ \frac{1}{V} = \frac{K_m}{V_{\text{max}}} \frac{1}{S} + \frac{1}{V_{\text{max}}} \]  

(2)

3. Results and Discussion

In this study, the kinetics of immobilized ω-TA on methacrylate beads in the asymmetric synthesis of PEA were measured using initial rate experiments. The concentration of substrate (ACP) was varied while the other (ALA) was kept constant. The initial rate of reaction is shown in Table 1. In kinetic studies, the Vmax occurs when all of the enzyme active sites are saturated with substrate. An enzyme with a low value of Km indicates a high affinity for its substrate, and vice versa [3]. The kinetic parameters (Km and Vmax) can be calculated from Lineweaver–Burk plot of initial reaction rate (1/V) against substrate concentration (1/[ACP]) as shown in Figure 2. In this study, the Vmax and Km for this reaction were 6.87 mM/min and 2.52 mM, respectively.

Table 1. Initial rate of reactions for the immobilized ω-TA in the amination of ACP by varying the ACP concentration at fixed 100 mM ALA

| ACP concentration, mM | Initial reaction rate, V, mM/min |
|-----------------------|----------------------------------|
| 2                     | 3.13                             |
| 4                     | 3.85                             |
| 8                     | 5.01                             |
| 10                    | 6.25                             |

Figure 2. Kinetic parameter estimation by Lineweaver Burk plot of 1/V (min/mM) versus 1/[ACP] (mM⁻¹)

Lineweaver-Burk plot of Michealis-Menten model is commonly used to calculate and compare the kinetic parameters of free and immobilized enzymes. The Km of immobilized ω-TA on magnetic
PVA-Fe3O4 nanoparticles increased to 11.03 mM from the free ω-TA (4.40 mM) [9]. In another study, Km values increased from 0.0951 mM (free laccase) to 0.272 mM (immobilized laccase) [13]. Similarly, the Km of the immobilized glucose isomerase was increased 1.3-fold from that of free isomerase [14]. The increase of Km value shows less affinity of the immobilized enzyme towards its substrate compared to the free enzyme. In most cases, it was due to diffusional limitation of the substrate and the conformational changes of enzyme caused by the supports to immobilize enzymes [6, 9].

One the other hand, different methodologies used to estimate the kinetic parameters will result in different values of the parameters. In this study, the Km of immobilized ω-TA calculated from the plot was higher than the free ω-TA reported in the literature. The Km of free ω-TA was 1.71 mM estimated by nonlinear regression of the Michaelis–Menten model [4]. The estimation analysis relies on the initial guesses of the parameters. In another study, Shin and Kim reported the Km of free ω-TA value was 0.54 mM [12]. In their study, the initial rate data was fitted nonlinearly to a more complex model (a ping-pong bi-bi mechanism). However, the model contains a significant number of parameters which requires large experimental data for accurate prediction of the parameters.

4. Conclusion
In this study, the kinetic parameters (Vmax and Km) of immobilized ω-TA in the asymmetric synthesis of chiral amine (PEA) was determined. The Vmax and Km estimated from Lineweaver-Burk plot were 6.87mM/min and 2.52 mM, respectively. The Michaelis-Menten model is useful for understanding the kinetic properties of immobilized enzymes.

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