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Analytical aspects of enzyme reversible inhibition

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ABSTRACT

A simple graphical method for the determination of reversible inhibition type, inhibition constant (K_i) and estimation of fifty percent of inhibition I_{50} of an enzyme reaction is described. The method consists of plotting experimental data as "degree of inhibition" versus the inhibitor concentration at two or more concentrations of substrate. Diagnosis of inhibition type is based on determination of I_{50} and the observation of the shift of the inhibition curves. Relationship between I_{50} and inhibition constant K_i was discussed. A simplified hyperbolae equation of degree of inhibition showing kinetic orders of 1 and zero at low and high concentrations of inhibitors respectively is proposed. The relative error of inhibitor concentration increased drastically when degree of inhibition reached values of 90%. Examples of published inhibition reports as well as an experimental example of amperometric biosensor based on tyrosinase inhibition by benzoic acid were in agreement with the proposed theoretical approach.

1. Introduction

Reversible enzyme inhibition requires graphical plots for diagnostic of inhibition type and for the determination of inhibition constant K_i. Hence, Lineweaver–Burk [1], Dixon [2] and Cornish-Bowden [3] were proposed, but none of these plots alone give satisfactory results. During several years, it was very common to use the Dixon's representation to determine the inhibition type and the inhibition constants for competitive and noncompetitive inhibitions [2]. However, this method is unable to distinguish between competitive and mixed inhibition types. The Cornish-Bowden method is an improved method for determining inhibition constant of uncompetitive inhibition [2], but it is not able to determine the inhibition constant for competitive inhibition. Indeed, secondary plot of Lineweaver–Burk representation or the use of both of Dixon and Cornish-Bowden plots are widely reported in biochemical research laboratories in order to solve the problem of determination of type of inhibition and K_i.

Also I_{50} (the concentration of inhibitor which causes 50% inhibition) is commonly used in pharmacological practice. This I_{50} is often regarded as a value simply equal to K_i but that is only true in the case of noncompetitive inhibition. In order to improve human health, many drugs based on enzyme inhibition have been evaluated, demonstrating the importance of enzyme inhibitors. I_{50} is essential for describing the extent of inhibition, in order to study the effect of drugs on enzymes. In other words, it is very useful to compare the values of I_{50} measured in different laboratories for the same substrate and enzyme, to assess the effectiveness of inhibitory compounds. The pharmacological treatment of some diseases is currently based on enzyme inhibitors like cancer, diabetes type II and neurologic disorders [4–6]. The relationship between the inhibition constants K_i and I_{50} for competitive, uncompetitive and noncompetitive inhibition was discussed [7,8]. Plots reporting the degree of inhibition versus concentration of inhibitor are often found in papers dealing with bioassays and biosensors based on enzyme inhibition. Although the huge number of papers are published in this field [9–15], the study of analytical aspects of enzyme inhibition is scarce.

In this work, we applied the "degree of inhibition" plot for the determination of I_{50} and the type of inhibition. We propose a simplified equation, valid for all types of inhibition, for the estimation of dynamic range and relative error of inhibitor concentration. A practical example of inhibition of tyrosinase biosensor by benzoic acid is discussed.

2. Materials and methods

2.1. Materials

Tyrosinase (EC 1.14.18.1) from mushroom (3933 units mg) was purchased from Sigma. All reagents were analytical grade and were available from Sigma.

2.2. Instrumentation

Amperometric measurements were performed with a PalmSens potentiostat interfaced to a computer. A10 mL electrochemical cell

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with a conventional three electrode system consisting of carbon paste electrode as the working electrode, a platinum wire as the auxiliary electrode, and an Ag/AgCl electrode as the reference electrode were used.

2.3. Application on biosensor based on tyrosinase

Tyrosinase (EC 1.14.18.1) is a copper-containing monooxygenase enzyme that catalyzes the conversion of phenolic substrates to catechol and the successive oxidation to quinone [16]. Thus, the quinone can be electrochemically reduced to allow amperometric detection of phenol

$$\text{Phenol} + \text{Tyrosinase (O2)} \rightarrow \text{Catechol}$$

$$\text{Catechol} + \text{Tyrosinase (O2)} \rightarrow \text{O-quinone} + H_2O$$

$$\text{O-quinone} + H^+ + 2e^- \rightarrow \text{Catechol}$$

2.4. Immobilization of tyrosinase

A biosensor based on the immobilized tyrosinase (Tyrs) enzyme is described for the detection of catechol. The immobilization was prepared by using the cross-linking immobilization. The enzyme solution was first prepared by mixing 15 μL of Tyrs (104 unit/μL), 7.5 μL of BSA (1%) and 7.5 μL of glutaraldehyde (0.25%). The mixture was spread on the surface of a carbon paste electrode, and then it was dried at room temperature. Before use, the enzyme electrode was placed under stirring for 10 min with buffer solution to remove enzyme not firmly immobilized. After use, the biosensor was stored in phosphate buffer solution overnight at 4 °C.

2.5. Enzyme activity assays

Tyrosinase activity was measured by injecting different concentrations of catechol as substrate and by using amperometric measurement. The assays of the enzyme activity were performed in an electrochemical cell containing 10 mL of 0.1 mol l^{-1} phosphate buffer, pH 7.0 at 25 °C. The applied potential was fixed to 0 V.

2.6. Competitive inhibition by benzoic acid

To perform each measurement, the carbon paste electrode modified with Tyrs was dipped into the electrochemical cell containing 10 mL of 0.1 mol l^{-1} phosphate buffer solution (pH 7.0) maintained under constant magnetic stirring. The applied potential was fixed to 0.0 V. Once the baseline was established (15 min approximately), a defined concentration of catechol was added to the measuring cell. A large reduction current was observed due to the addition of catechol. The addition of benzoic acid solution caused a decrease in current.

3. Results and discussion

3.1. Relationship between I_{50}, K_i and concentration of substrate

Inhibitors can bind to enzymes following the expressions shown in Scheme 1 [17].

In the case of competitive inhibition (Scheme 1a), the inhibitor binds to free enzyme with a greater affinity than to the EI complex.

Eq. (1) below represents the Michaelis–Menten equation in absence of inhibitor

$$V_0 = \frac{V_{max}[S]}{K_m + [S]} \quad (1)$$

V_{max} = Maximum velocity.
V_0 = Velocity in the absence of the inhibitor.
K_m = Michealis constant of the substrate(S).
$[S]$ = Substrate concentration.

Eq. (2) below represents the Michaelis–Menten equation in presence of competitive inhibitor

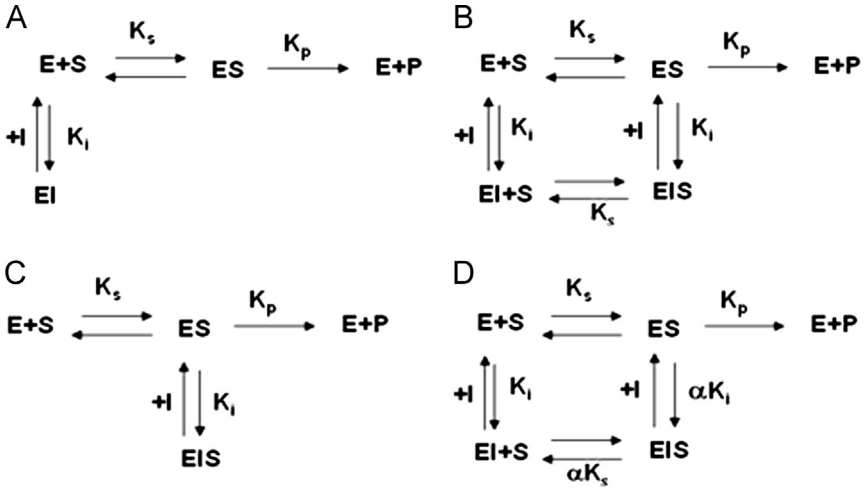
$$V_i = \frac{V_{max}[S]}{K_m(1 + [I]/K_i) + [S]} \quad (2)$$

V_i = Velocity in the presence of inhibitor.
$[I]$ = Inhibitor concentration.
K_i = Dissociation constant of EI.
When $I = I_{50}$, and $V_0 = 2V_i$, then

$$\frac{V_{max}[S]}{K_m + [S]} = \frac{2V_{max}[S]}{K_m(1 + [I_{50}]/K_i) + [S]}$$

By rearrangement

$$\frac{I_{50}}{K_i} = \left(1 + \frac{[S]}{K_m}\right) \quad (3)$$



Scheme 1. . Scheme of enzyme inhibition in case of reversible inhibition: competitive (a), non-competitive (b), un-competitive (c) and mixed type inhibitions (d).