Communication to the Editor

**A Rapid Method for Determining Kinetic Parameters of Enzymes Exhibiting Nonlinear Thermal Inactivation Behavior**

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A rapid method is developed to analyze the kinetics of thermal inactivation of enzymes that exhibit a nonlinear biphasic log(activity)-time relationship. Thermal destruction experiments on alcohol dehydrogenase from baker’s yeast demonstrate the applicability of the method. The method is based on physical considerations (as opposed to mathematical curve fitting/regression methods) and also serves as a quick check of results obtained using nonlinear regression. It is superior to fitting nonlinear enzyme inactivation data by first-order kinetics or taking the initial and final slopes of the inactivation data. In fact, the method is of general validity and can be applied to any decay process that can be represented by a sum of exponentials. © 1996 John Wiley & Sons, Inc.

**Key words:** inactivation • thermal inactivation • enzymes • alcohol dehydrogenase • kinetics

**INTRODUCTION**

Thermal processes (e.g., sterilization, pasteurization, and blanching) are of great importance in the food and pharmaceutical industry and hence thermal inactivation and thermal stabilization of enzymes and proteins has received much attention.\(^1\)\(^-\)\(^1\)\(^4\) In most studies on thermal inactivation of enzymes, the reaction rate constants and thermodynamic parameters are determined based on the assumption that the inactivation follows first-order kinetics.\(^3\)\(^,\)\(^4\)\(^,\)\(^1\(^1\)\) However, in general, large deviations from first-order kinetics are observed in the log(residual enzyme activity)-time curve (i.e., enzyme activity remaining vs. heating time at a constant temperature).\(^4\)\(^-\)\(^6\)\(^,\)\(^8\)\(^,\)\(^1\(^3\)\) These deviations could be due to the formation of enzyme groups with differing heat stabilities or the presence of stable/labile isoenzymes,\(^4\)\(^,\)\(^5\) or due to a series-type enzyme inactivation kinetics.\(^8\)\(^,\)\(^1\(^0\)\(^,\)\(^1\(^3\)\) Alternatively, the initial and final slopes of the inactivation data are taken.\(^5\) These procedures lead to inaccurate values of the kinetic parameters as well as the activation energies.

Nonlinear enzyme activity-time relationships have recently been observed for various enzymes: horseradish peroxidase,\(^4\) Bacillus licheniformis α-amylase,\(^5\) acid phosphatases,\(^8\) and in our own experiments on alcohol dehydrogenase from baker’s yeast. A simple and rapid method to determine the enzyme inactivation rate constants accurately from the nonlinear activity-time thermal destruction curves taking the physics of the inactivation process into account would, therefore, be very useful and would complement solely mathematical curve fitting/regression exercises. Such a method is proposed in the present work. The method was tested on thermal inactivation data of alcohol dehydrogenase from baker’s yeast. Because sugars are commonly used as stabilizers for biological systems,\(^1\)\(^,\)\(^2\) experiments were carried out both in the absence and presence of various concentrations of sucrose (30 and 80 wt %) and the kinetic parameters determined for all cases using the proposed method.

**THEORETICAL ASPECTS**

**Method for Analysis of Thermal Inactivation Data**

Nonlinear biphasic ln(activity)-time curves would result when the enzyme system consists of two groups, heat-stable and heat-labile, with differing heat stability (enzyme aggregates formed during inactivation, each with its own thermostability or alternatively, heat-stable and heat-labile isoenzymes). There exists sufficient experimental (e.g., electrophoretic) evidence that this is true and heat-stable and heat-labile isoenzymes have even been isolated in several cases.\(^4\)\(^,\)\(^9\) It is assumed that each group or isoenzyme independently follows first-order kinetics. With these in mind, the residual enzyme activity can be expressed as

\[
E/E_0 = A_1\exp(-k_1t) + A_2\exp(-k_2t)
\]

where \(E_0\) is the initial enzyme activity at zero time, \(E\) the enzyme activity after thermal processing at a constant temperature \(T\) for a time \(t\), \(k_1\) and \(k_2\) the inactivation rate constants of the heat-labile and heat-stable groups, and \(A_1\) and \(A_2\) the heat-labile and heat-stable fractions of the initial enzyme activity. Note that the kinetic parameters are expected to be functions of temperature, pH, additive concentrations, etc. We need to determine \(k_1\), \(k_2\), \(A_1\), and \(A_2\). At time zero,

\[
E/E_0 = 1 = A_1 + A_2.
\]

At sufficiently long times, the contribution of the heat-labile
The left-hand side of Eq. (5) is known at every value of \( t \). Thus, at long times, \( E/E_0 = A_0 \exp(-k_f t) \) \( (3) \) or \( \ln(E/E_0) = \ln(A_0) - k_f t \). \( (4) \) Thus, a semilog plot of \( E/E_0 \) vs. \( t \) for long times yields \( k_f \) as its slope and \( A_0 \) as intercept. Now the short-time decay can be readily interpreted:

\[ \frac{E}{E_0} - A_0 \exp(-k_f t) = A_s \exp(-k_r t) \] \( (5) \)

The left-hand side of Eq. (5) is known at every value of short-time, because \( A_s \) and \( k_r \) have been determined from Eq. (4) and \( E \) and \( E_0 \) are known from the experimental thermal inactivation curve at each time value. Therefore, a plot of \( \ln[E/E_0 - A_s \exp(-k_r t)] \) vs. \( t \) for short times should have \( k_r \) as its slope and \( A_s \) as its intercept. Knowing \( A_0 \) and \( A_s \), we can now check if their sum adds to 1. This deconvolution procedure enables a rapid and accurate determination of \( k_r \), \( k_s \), \( A_s \), and \( A_0 \) from thermal inactivation data.

**EXPERIMENTAL METHODS AND MATERIALS**

**Materials**

Pure alcohol dehydrogenase from baker’s yeast was purchased from Sigma Chemical Co. (St. Louis, MO); samples of baker’s yeast were a gift from Kothari Fermentation and Biochemicals Ltd. (New Delhi, India). NAD was purchased from Spectrochem Pvt. Ltd. (Bombay, India). Sodium dihydrogen phosphate and disodium hydrogen phosphate were bought from Qualigens, Glaxo India Ltd. (Bombay, India) and tetrasodium pyrophosphate from Loba Chemicals (Bombay, India). Ethanol was purchased from Merck (Darmstadt, Germany) and sucrose from BDH Ltd. (Poole, England). All reagents were of analytical grade.

**Methods**

**Cell Disruption and Centrifugation**

Yeast cells, 30 g wet weight, were thawed and suspended in 10 mM phosphate buffer, pH 7.5, to yield a final concentration of 60% (w/v). The cells were disrupted in a French press at a pressure of 1250 psi (85 atm). This procedure was repeated four times. After disruption, the pH was brought back up to 7.5 with 0.05M Na₂HPO₄ solution. A technical centrifugation was carried out at 15,000g for 20 min.

**Pure Enzyme**

Pure alcohol dehydrogenase was dissolved in 10 mM phosphate buffer, pH 7.5, to yield a concentration of 1.7 mg/mL. The sample size was 1–2 mL in all cases.

**Thermal Treatment**

Sucrose (30 and 80 wt %) was dissolved in the enzyme solution. The thermal treatment was performed in a constant temperature bath. The samples were placed in the bath for fixed periods of time, taken out, and immediately cooled in ice water. The residual enzyme activity was then measured. All sucrose percentages referred to in this work are weight percentages.

**Activity Measurement**

The activity of alcohol dehydrogenase was measured spectrophotometrically at 340 nm in pH 8.8 tetrasodium pyrophosphate buffer. The procedure is based on progressive oxidation of ethanol in the presence of NAD. The temperature was kept constant at 30°C. Enzyme activities were calculated by linear regression of absorbance at 340 nm vs. time data. In all cases, both the initial and the residual activity were measured. The experiments are reproducible and the errors do not exceed the size of the data symbols in Figure 1a,b. The measured enzyme activities, \( E \), were well within the sensitivity range of the measurement. The smallest value of the enzyme activity was on the order of 1 U/mL; the least count of the measurement technique was at least 10 times lower (0.1 U/mL).

**RESULTS AND DISCUSSION**

**Thermal Inactivation Curves for Alcohol Dehydrogenase**

The experimentally obtained thermal inactivation behavior of pure alcohol dehydrogenase and alcohol dehydrogenase from disrupted baker’s yeast at constant temperature (50, 65, 70°C) both in the absence and presence of sucrose is shown in Figure 1a,b. The natural logarithm of the fraction of remaining enzyme activity is plotted as a function of heating time. Nonlinear, biphasic behavior is clearly seen from the figure at all sucrose concentrations (0–80%) i.e., the inactivation curve exhibits a different slope at short times from that at long times. A definite tendency from distinct to less pronounced biphasic behavior is observed with increasing sucrose concentration (Fig. 1a,b). Similar behavior was found recently by De Cordt et al. in B. licheniformis α-amylase systems. It is also observed that pure alcohol dehydrogenase inactivates to a significantly greater extent than alcohol dehydrogenase from disrupted baker’s yeast containing several other proteins that have a stabilizing effect on the enzyme (Fig. 1; Tables I, II).

**Determination of \( A_s \) and \( k_s \)**

The long-time slopes and intercepts of the experimental thermal destruction curves of both pure and impure enzyme at 50, 65, and 70°C are shown in Figures 1a,b and 2 and tabulated in Tables I and II. With increasing temperature,
the value of the heat-stable fraction \( A_i \) decreases while the inactivation rate constant of the heat-stable fraction \( k_i \) increases, for a constant sucrose concentration (Tables I, II). At constant temperature (e.g., 70°C in Table I and 50°C in Table II), the addition of sucrose leads to a significantly lower \( k_i \) and higher \( A_i \) compared to that obtained in the absence of sucrose.

**Determination of \( A_i \) and \( k_i \)**

Using Eq. (5), the deconvoluted short-time decay is plotted in Figure 2a,b for various sucrose concentrations (0, 30, 80%) and temperatures (50, 65, 70°C). Straight lines are obtained in accordance with the theoretical predictions. Values of the heat-labile fraction \( A_l \) and the heat-labile inactivation rate constant \( k_l \) obtained from Figure 2 are tabulated in Tables I and II. Both \( A_l \) and \( k_l \) decrease strongly with increasing sucrose concentration at constant temperature (Tables I, II). In the biological literature, very few data points are taken at short times\textsuperscript{16}; this makes the determination of \( A_l \) and \( k_l \) inaccurate, and sometimes even impossible. Hence, an important recommendation of this work is that more data points be recorded at short times in enzyme inactivation experiments to permit accurate resolution of the faster decay. Note that both \( A \) and \( k \) parameters need to be considered simultaneously (and not singly) for a correct interpretation. Thus, the presence of stabilizers at constant temperature or a decrease in temperature at constant stabilizer concentration has the effect of increasing \( A \) and altering \( k_i \) and \( k_j \). The proposed method clearly differentiates between these two effects.

**Justification of Method of Kinetic Parameter Determination**

The straight lines obtained in Figure 2 together with the fact that the sum of \( A_i \) and \( A_l \) is unity in all cases (Tables I, II) justifies the method employed to determine the kinetic parameters. At long heating times (e.g., 10 min with as high as 80% sucrose at a temperature of 65°C), only 1.5% of the heat-labile pure alcohol dehydrogenase enzyme is active. This value declines to 0.05% at 20 min. For the pure enzyme in the absence of sucrose even at a low temperature of 50°C, the percentage of active heat-labile enzyme after 10 min measures less than 0.1%. Hence, the assumption that

<table>
<thead>
<tr>
<th>( T ) (°C)</th>
<th>Sucrose wt %</th>
<th>( A_i )</th>
<th>( k_i \times 10^2 ) (min(^{-1}))</th>
<th>( A_l )</th>
<th>( k_l ) (min(^{-1}))</th>
<th>( k_l/k_i )</th>
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<tr>
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<td>0.13</td>
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<tr>
<td>70</td>
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<td>50</td>
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<td>0.68</td>
<td>1.10</td>
<td>0.30</td>
<td>0.26</td>
<td>23.6</td>
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</table>

**Table I.** Heat-stable and heat-labile fractions \((A_i, A_l)\), heat-stable and heat-labile inactivation rate constants \((k_i, k_l)\), and ratio of labile to stable rate constant for alcohol dehydrogenase from disrupted baker's yeast in the presence and absence of sucrose.

<table>
<thead>
<tr>
<th>( T ) (°C)</th>
<th>Sucrose wt %</th>
<th>( A_i )</th>
<th>( k_i \times 10^2 ) (min(^{-1}))</th>
<th>( A_l )</th>
<th>( k_l ) (min(^{-1}))</th>
<th>( k_l/k_i )</th>
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<td>0</td>
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<td>0.20</td>
<td>0.00</td>
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</table>

**Table II.** Heat-stable and heat-labile fractions, heat-stable and heat-labile inactivation rate constants, and ratio of labile to stable rate constant for pure alcohol dehydrogenase from baker's yeast in the presence and absence of sucrose.
CONCLUSIONS

A rapid method is developed to estimate kinetic parameters of enzymes exhibiting nonlinear biphasic thermal inactivation behavior. The applicability of the method is shown for thermal inactivation data on alcohol dehydrogenase from baker’s yeast. The method provides a quick check of results obtained using nonlinear regression techniques while retaining its physical features. It is superior to fitting nonlinear enzyme inactivation data by first-order kinetics or taking the initial and final slopes of the inactivation data. It is recommended that more data points be recorded at short times in enzyme inactivation experiments to permit accurate resolution of the faster decay. The method may find use not only in enzyme kinetics, but also in a wide variety of physical, chemical, and biological processes where nonlinear decays occur—radioactive decays, the relaxation of bidisperse colloids/polymer solutions upon removal of the external field, in fluorescence quenching studies, in virus-cell fusion processes. Thus, the method may be used in general for analyzing the dynamics of nonequilibrium systems relaxing toward equilibrium.

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References