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Use of the contour approach for visualizing the dynamic behavior of intermediates during O-nitrophenyl-β-D-galactoside hydrolysis by β-galactosidase

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ABSTRACT

ONPG disappearance and ONP appearance were synchronously measured during ONPG hydrolysis by β-galactosidase using *in situ* on-line UV–vis spectroscopy. Intermediate formation was determined by the formula $d[ONPG]/dt - d[ONP]/dt$. The combined effects of temperature and time on ν_{inst} and ν_{inc} during the conversion of ONPG to ONP were expressed by the isogram method in which contour plots were used. Based on this approach, new insights were obtained into the irreversible-continuous conversion of ONPG to ONP during hydrolysis. The intermediate was a moving mass that flowed in three-dimensional space from the substrate to the product. The results of this study support the use of the isogram method for understanding the mechanisms of enzyme-catalyzed reactions via the dynamic resolution approach.

1. Introduction

The transition-state theory is widely used to understand the mechanism of enzyme-catalyzed reactions. This theory suggests that catalysis occurs because transition states are more tightly bound by the enzyme than the substrates and enzymatic reactions proceed through intermediates [1–4]. Thus, one of the key problems in determining the mechanism of an enzyme-catalyzed reaction is the identification and characterization of the enzyme–substrate intermediate (ES complex) [5–7]. Since it is generally very difficult to measure the formation of intermediates [3,8–12], many modifications of the transition-state theory have been proposed [1–7], one of which is the quasi-steady-state hypothesis [13,14], in which it is assumed that there is no change in the concentration of intermediates during the reaction process. This simplifies the calculation of kinetic parameters. This has been used for studying ONPG hydrolysis by β-galactosidase [15,16]. Actually, this theory is very similar to the classical steady-state hypothesis in which $d[ES]/dt$ is assumed to be zero [1,2]. However, over the past years, many questions have been raised regarding the quasi-steady state hypothesis [17–20].

In particular, enzyme-catalyzed reactions are always analyzed by constructing a simple differential equation based on the reaction velocity, which is derived from a single independent variable. In general, the reaction velocity is measured under fixed reaction conditions where [S], [E], temperature, and pH are constant and only changes in the velocity with time are measured. Thus, the kinetic equation is only reflective of the changes in the reaction rate with time under fixed conditions. It is well-known that temperature and reaction time are two important factors that influence enzyme catalysis [1,2,17,21–23]. Moreover, from the physical point of view, temperature is a function of the state, and its effects on the catalytic process must be determined during a certain time course [24,25]. Thus, the effects of temperature and time on the reaction velocity should be considered in combination with other factors. The principle of time–temperature superposition has been widely applied to biological engineering [24–26]. Unfortunately, this idea has long been neglected in the study of enzyme-catalyzed reactions. Because the different combination between temperature and time will result in different kinetic curves for reaction velocity, there is no common differential equation that can fit well all of data. These difficulties can be overcome by using the contour plot technique. The reaction path (directional tendency) is better visualized geometrically in 2D by means of the contour plots. Recently, we applied this approach to determine the combined effects of temperature and time on the catalysis of PNPC (p-nitrophenol-D-cellobioside) by CBHI (1,4-β-D-glucan-cellobiohydrolase) [27].

Based on the transition-state theory, the formation and breakdown of an intermediate should be on a time scale with the rate changes between substrate disappearance and product formation, and represent the distinct conformational states [7]. Thus, if the rates of substrate disappearance and product appearance can be measured *in situ* on-line, it may simplify the

Abbreviations: ONPG, O-nitrophenyl-β-D-galactoside; ONP, O-nitrophenol; ν_{inst}, instantaneous rate; ν_{inc}, instantaneous increment; AUC, area under the curve.

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estimation of transient intermediate kinetics [28,29]. In the present study, ONPG hydrolysis by β-galactosidase was selected as a model system to evaluate this hypothesis.

2. Materials and methods

2.1. Enzyme and materials

β-Galactosidase was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Its tetramer molecular weight (MW) is estimated to be 465 kDa, since it has four subunits, and each has a MW of 116.3 kDa [30]. ONPG and ONP were also purchased from Sigma. The UV spectra were measured with a UV-3100 UV–vis-NIR recording spectrophotometer equipped with a thermostat temperature controller (Shimadzu, Japan). The measurements were performed in the range 200–400 nm using a 1.0-cm path length quartz cell and a scan speed of 240 nm/min. Reagents for the enzyme assays were purchased from Sigma. The recorded data represent the means from at least three measurements, and the overall coefficient of variation was less than 5%.

2.2. Effect of different combinations of temperature and time on ONPG hydrolysis

To express an enzyme's catalytic capacity and the effects of temperature on the reaction and to obtain more information on the entire catalytic process [31,32], experiments on ONPG hydrolysis by β-galactosidase were performed using different combinations of temperature and time (from 2.5 to 17.5 min at 2.5-min intervals and from 25 to 60 °C at 5 °C intervals). The reaction velocities were calculated as v_{inst} and v_{inc} and used to accurately determine the effects of temperature and time [27,33,34]. The rationale of this approach is to allow the influence of two critical variables (reaction temperature and reaction duration) to be individually analyzed by fixing one variable and altering the other and studying the resultant effect.

2.3. Use of the AUC of the UV spectra in the range 400–500 nm as an index to express ONP formation during hydrolysis

As shown in Fig. 1A, ONP had high absorbance in the 400–500 nm range and the absorbances of ONPG, β-galactosidase, and D-galactose in this region were remarkably close to zero. Thus, the $AUC_{400-500\ nm}$ can be used as an index to accurately calculate the amount of ONP formed during hydrolysis by β-galactosidase.

The absorbance of a single point on the spectrogram curve may reflect the response of the effects of the variables. However, since the experimental error will depend on averaging over a certain wavelength interval, in general, the larger the observed interval is, the smaller the error is [35]. Thus, when the total AUC is calculated from a larger curve, a smaller error is obtained. The AUC can be directly derived by a definite integral [36,37]. The numerical integration equation may be written as the summation

$$\mathrm{AUC} = \int_0^i A_i(\lambda) \cdot d\lambda_i$$

where λ_i is the wavelength ($i = 1, \ldots, n$) and A_i denotes the absorbance of the hydrolyzate at wavelength λ_i. Therefore, the AUC is the total intensity of the absorbance in the wavelength range $n-1$ to n. In comparison to the classical absorbance method, the AUC method appears to be more sensitive and accurate in determining ONP formation during hydrolysis.

2.4. Use of the isosbestic points at 344.5 nm of the UV spectra as an index to calculate ONPG disappearance during hydrolysis

As shown in Fig. 1B, three isosbestic points were observed (at ≈269, ≈302, and ≈344.5 nm) in the overlay plot of the UV spectra during hydrolysis. In general, the absorption coefficient of an isosbestic point can be used to estimate the amount of precursor that is quantitatively converted to the product [38]. However, as shown in Fig. 1B, the wavelengths of these isosbestic points may change in different assays in which the effective molar absorptivity (ε_M) of ONPG and ONP differs [38]. The experimental results suggested that the shift of the isosbestic point at 344.5 ± 0.05 nm is a little smaller than other two. Based on this, the molar absorbance of ONPG and/or ONP at 344.5 nm was selected as the index for estimating the concentrations of a mixture of ONPG and ONP during hydrolysis. This is the key requirement for determining ONPG disappearance as hydrolysis progresses. Details of the process are described below.

First, we constructed a standard curve of ONPG based on its absorbance at 344.5 nm using various known concentrations of ONPG. The data were fitted by linear regression within the ONPG concentration range of 100 μM, as shown in

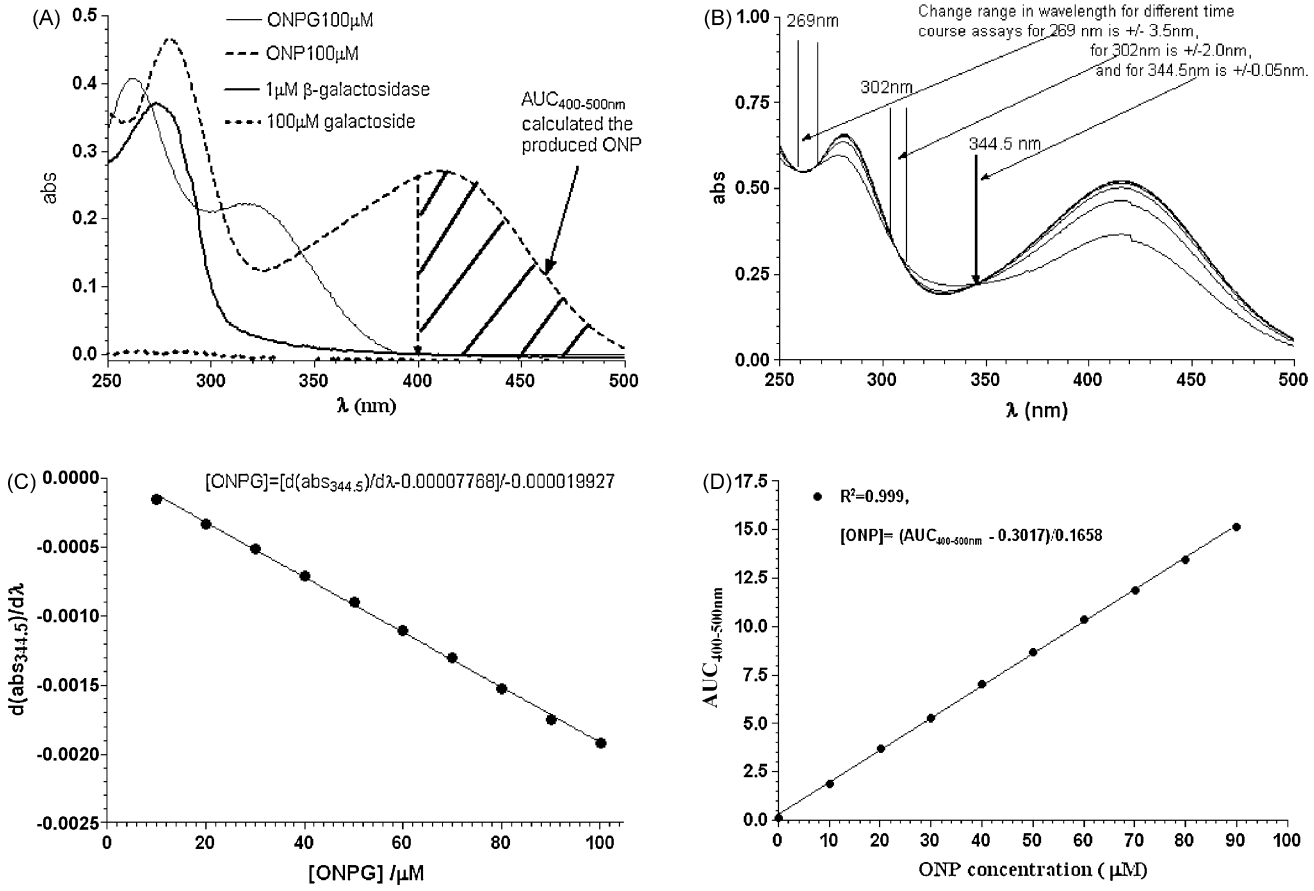

Fig. 1. (A) Comparison of the UV spectra of ONPG, ONP, β-galactosidase, and galactoside. (B) UV spectra of ONPG (200 μM) hydrolyzed by β-galactosidase (1.0 μM) at 40 °C at different times (from 2.5 to 17.5 min at 2.5-min intervals, from bottom to top). (C) Standard curve of ONPG. (D) Standard curve of ONP.