Continuous Spectrophotometric Assay for β -Glucuronidase

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ABSTRACT

*A continuous spectrophotometric assay has been developed for detecting β -glucuronidase activity. In the assay, Para-nitrophenyl β -D-glucuronide is cleaved to yield a chromophoric product. With the commercial *E. coli* enzyme, it is demonstrated that the reactions can be continuously monitored by the increase of absorbance at 405 nm. The method is highly sensitive and able to detect less than 1.4×10^{-4} U/mL of the enzyme activity in solution. Such a new assay offers significant advantages over the existing discontinuous methods and should be useful for both routine enzyme assay and accurate kinetic studies.*

INTRODUCTION

β -glucuronidase (GUS, EC 3.2.1.31) catalyzes the hydrolysis of β -linked D-glucopyranosiduronic acids (β -glucuronides) to glucopyranosiduronic acid (glucuronic acid) and aglycone: β -D-glucuronide + $H_2O \rightarrow$ D-Glucopyranosiduronic Acid + R-OH. This enzyme is present in animals and some bacteria but is absent from higher plants (1,2,10). In animals, GUS degrades natural glucuronide substrates, such as heparan sulphate, dermatan sulphate, and chondroitin sulphate, to glucuronic acid and aglycone residues (5). GUS enzyme deficiency in humans leads to a disease known as mucopolysaccharidosis type VII that results from lysosomal storage of undegraded glycosaminoglycans (6,21,22). In enteric bacteria, which normally inhabit the vertebrate gut, the GUS activity is involved in the hydrolysis of glucuronide compounds derived from conjugation of endogenous and xenobiotic organic compounds with glucuronic acid in the liver. The released glucuronic acid residue is used as a carbon source for bacterial cells (25).

GUS assays are extensively performed in various fields. Polymorphonuclear leukocytes are quantitated by the release of lysosomal enzyme GUS (7). The biochemical analysis of the GUS gene activity in gingival crevicular fluid offers a noninvasive

means of assaying periodontal disease (13). GUS assays are routinely used as diagnostic purposes for the specific detection of *E. coli* and *Shigella* species in clinical and environmental samples (11,15,18,25). The GUS gene is the most widely used reporter gene in plant molecular biology because of the absence of background activity in higher plants. Cell-, tissue-, and organ-specific gene expression is now routinely studied using GUS fusions in transgenic plants (9,10,26).

Numerous applications demonstrate the importance of developing a simple, sensitive, and high-throughput assay for GUS activity. During the last three decades, a number of convenient substrates have been synthesized for the assay, taking advantage of the lack of structural specificity for the glucuronides (10,12,16,17,20,23,27). All of these synthetic substrates contain the sugar D-glucopyranosiduronic acid attached by glycosidic linkage to a hydroxyl group of a chromophoric, fluorogenic, or other detectable molecule. A variety of discontinuous assay protocols now exist for histochemical, spectrophotometric, or fluorometric analysis. However, these procedures are tedious and generate kinetic data that may not be accurate (24). Here, we describe an improved GUS assay procedure by continuous spectrophotometric analysis and demonstrate the utility of this assay with the enzyme from *E. coli*.

Table 1. Kinetic Parameters of GUS Measured by Different Assays

Assay	K_m (mM)	V_{max} (nmol/min)
Continuous spectrophotometric assay	0.078	1.55
Discontinuous spectrophotometric assay	0.197	1.2
Discontinuous fluorometric assay	0.24	0.026
	0.22 ^a	
	0.57 ^b	0.014 ^b

^aSee Reference 19.

^bSee Reference 23.

MATERIALS AND METHODS

E. coli GUS was obtained from Roche Molecular Biochemicals (Laval, QC, Canada) and used as received. Para-nitrophenyl β -D-glucuronide (PNPG) and para-nitrophenol were purchased from Sigma (St. Louis, MO, USA). 4-methylumbelliferyl β -D-glucuronide (MUG) and 7-hydroxy-4-methylcoumarin (MU) were purchased from Molecular Probes (Eugene, OR, USA).

Commercial GUS was diluted in 50 mM Na_2HPO_4 buffer, pH 7.0, containing 10 mM β -mercaptoethanol. The enzyme is stable in the buffer at 4°C for at least 20 h. All the assays were carried out at 21°C. The activity of the commercial GUS was estimated to be 70 U/mL using the continual spectrophotometric method at this temperature. The substrate used for this assay was PNPG, which generated para-nitrophenol (PNP) when cleaved by the enzyme. One unit of activity is defined as 1 μmol PNP formed per minute. Protein concentration was determined by the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin as the standard. The enzyme concentration is 3 $\mu\text{g}/\mu\text{L}$; thus, the specific activity is calculated to be 23.3 U/mg.

Spectrophotometric assays were performed on a Hewlett-Packard 8453 diode array spectrophotometer. The molar extinction coefficients of PNP at pH 7.0 (50 mM Na_2HPO_4 buffer, pH 7.0, containing 10 mM β -mercaptoethanol) and at pH 11.0 (0.4 M Na_2CO_3) were estimated as 9000 and 18 500/M/cm, respectively. To check the linearity of the enzyme activity by the continuous spectrophotometric

method, 20 μL diluted GUS (0.007 U/mL) and 100 μL 10 mM PNPG were added to the assay buffer (50 mM Na_2HPO_4 at pH 7.0 and 10 mM β -mercaptoethanol) to make the total volume 1 mL. The software supplied by Hewlett-Packard provides the rate of reaction directly by monitoring the absorbance at 405 nm. For the measurement of kinetic constants (K_m and V_{max}), substrate (PNPG) concentrations were varied from 0.03 to 1 mM with 20 μL diluted GUS (0.07 U/mL) in a total volume of 1 mL. For the discontinuous spectrophotometric assays, 20 μL diluted GUS (0.7 U/mL) were mixed with 100 μL 10 mM PNPG and 880 μL assay buffer. The mixtures (100 μL) were removed and added to 900 μL 0.4 M Na_2CO_3 , pH 11.0, at various time-points, and the absorbance at 405 nm was measured to check the linearity of the enzyme activity with time. Kinetic parameters were determined by varying the substrate concentrations from 0.05 to 2 mM with the same concentration of enzyme, stopping the reaction after 20 min in 0.4 M Na_2CO_3 , pH 11.0.

Fluorometric enzyme assays were performed on a Hitachi fluorescence spectrophotometer (F-2000). A standard calibration curve was drawn by varying the MU concentration (0.1–1.0 μM). The samples were excited at 365 nm, and emissions were monitored at 443 nm. A mixture containing 20 μL diluted GUS (0.14 U/mL), 230 μL buffer, and 250 μL assay solution containing 2 mM MUG was used to check the linearity of enzyme activity with time. Fifty microliters of this mixture were added to 1.95 mL 0.2 M Na_2CO_3 , pH 11.0, stopping solution at various time points and fluorescence emission

values at 443 nm were measured ($\lambda_{\text{ex}} = 365$ nm) (4). Kinetic parameters were determined by varying the substrate concentrations from 0.05 to 2 mM with the same concentration of enzyme and stopping the reaction after 10 min in 0.2 M Na_2CO_3 , pH 11.0.

The Michaelis-Menten equation (14)

$$V = \frac{V_{max} [S]}{K_m + [S]} \quad [\text{Eq. 1}]$$

is the rate equation for a one-substrate enzyme-catalyzed reaction. V is the initial rate of an enzymatic reaction that can be measured spectrophotometrically or fluorometrically. Weighted non-linear least-squares regressions were used to estimate the kinetic parameters V_{max} and K_m of the Michaelis-Menten equation. We also evaluated K_m and V_{max} from the double-reciprocal plot of the Lineweaver-Burk equation (4,14).

$$\frac{1}{V} = \frac{K_m}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}} \quad [\text{Eq. 2}]$$

RESULTS AND DISCUSSION

In the discontinuous spectrophotometric assay, the substrate PNPG is cleaved by GUS, yielding a chromogenic product, PNP, that has a maximal absorbance at 405 nm. This prompted us to investigate whether a continuous assay can be developed. With *E. coli* enzyme as a test, there was no change of absorbance at this wavelength when substrate or enzyme was omitted (Figure 1). In contrast, incubation of the enzyme with substrate led to a linear increase of absorbance with time (Figure 1), and the rates were directly proportional to the amount of enzyme used (Figure 2). This demonstrates the validity of this continuous assay procedure. Figure 3 shows that the plot of the rate versus concentration follows a typical hyperbolic trend. From the Lineweaver-Burk plot of Figure 3, we obtained values of K_m and V_{max} as 0.078 mM and 1.55 nmol/min, respectively, by the continuous method (Table 1) discussed earlier. The standard errors are less than 10%, indicating that the current method can be applied to generate accurate kinetic data.

The sensitivity of the continuous assay is compared with that of the discontinuous spectrophotometric and fluoro-

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metric methods. With the continuous spectrophotometric assay, the activity remains linear for at least 40 min when the commercial GUS was diluted up to 5×10^5 -fold (to 1.4×10^{-4} U/mL). In the discontinuous spectrophotometric assay, the enzyme activity remained linear with time when the enzyme was diluted by a factor 5×10^4 (to 1.4×10^{-3} U/mL). Further dilution resulted in nonlinear curves. Thus, the continuous spectrophotometric method is 10-fold more sensitive than the discontinuous spectrophotometric assay method. For fluorometric analysis, a calibration curve was drawn using MU as a standard. GUS activity remained linear at room temperature when the enzyme was diluted 5×10^5 -fold (to 1.4×10^{-4} U/mL). Further dilution generates nonlinear curves. The sensitivity of the fluorometric assay method depends on the nature of the substrate and quality of the fluorescence spectrophotometer. At room temperature with our fluorometer, using MUG as the substrate, we found that the sensitivity is comparable with the present continuous spectrophotometric method. It is noteworthy that, due to the low cost, PNPg is the most widely used substrate for the spectrophotometric assay and MUG is the most accepted fluorometric substrate. Though the sensitivity of the discontinuous methods is either 10-fold lower or at best comparable with the present continuous method, the amount of enzyme needed to perform each assay gives a more intriguing result. For the continuous spectrophotometric method, 0.14 U of this enzyme are required to check the activity and linearity of the assay simultaneously. On the other hand, for the discontinuous spectrophotometric and fluorometric methods, 14 and 2.8 U enzyme, respectively, are needed to perform the same analysis. It can therefore be concluded that the continuous spectrophotometric method is 100-fold and 20-fold more efficient than the discontinuous spectrophotometric and fluorometric methods, respectively.

The kinetic parameters (i.e., K_m and V_{max}) were also obtained with the use of the discontinuous methods (Table 1). Kinetic studies are rarely done on *E. coli* GUS. There are a few reports, with some of them are only giving the spe-

cific activity of GUS (4,12,19). Moreover, there are various sources of GUS used, and all of the reports carried out their assay at 37°C where the enzyme is more active. Thus, it is not justified to compare directly the specific activities of the GUS enzymes obtained from different sources. The literature values of K_m and V_{max} for *E. coli* GUS are 0.57 mM and 14.7 pmol/min, respectively, at

37°C (4). The present report gives $K_m = 0.224$ mM and $V_{max} = 26$ pmol/min at room temperature. In both of these cases, the substrate is MUG and is analyzed fluorometrically. By the discontinuous spectrophotometric method using PNPg as a substrate, the $K_m = 0.197$ mM (literature value is 0.220 mM) (12) and $V_{max} = 1.2$ nmol/min (no literature value available). The present

Figure 1. Dependence of reaction velocity on substrate and enzyme concentration. Assay was carried out in the standard assay buffer in the presence of 1 mM PNPg (●); 1.4×10^{-3} U GUS (▼); 1 mM PNPg and 1.4×10^{-3} U GUS (■); 1 mM PNPg and 3.5×10^{-3} U GUS (→); and 1 mM PNPg and 7×10^{-3} U GUS (▲).

Figure 2. Linearity of the GUS assay. Initial velocity is plotted against varying concentration of *E. coli* GUS added to the standard assay mixture.

data from the continuous spectrophotometric method show $K_m = 0.078$ mM and $V_{max} = 1.55$ nmol/min. The difference in K_m values analyzed spectrophotometrically could be accounted for by the difference in ϵ at different pHs. Experimental pH for the present continuous spectrophotometric method is 7.0 in which ϵ_{405} of PNP is 9000/M/cm, in contrast to 18 500/M/cm at pH 11.0 that was used for the discontinuous spectrophotometric method. This might affect the K_m value by 2- to 3-fold because only 50% less product is formed in the continuous spectrophotometric assay than that in the discontinuous spectrophotometric assay.

All the existing spectrophotometric or fluorometric assays are discontinuous (16,25). As the reaction proceeds, aliquots are removed, and the absorbance or fluorescence is then measured. In these assays, each reaction proceeds for 10–20 min and requires sampling and analysis of multiple single points per assay. Thus, these existing procedures are tedious and time consuming. In addition, for the fluorescence assay, one has to draw a standard calibration curve with MU. When data are obtained by discontinuous methods, a number of errors that are often overlooked and that may invalidate the results thus derived may be in-

troduced. The most common of such errors is probably the failure of workers to ensure that product formation proceeds linearly for the duration of the incubation period (3,8,24). In contrast, the current continuous spectrophotometric assay is simple and rapid. One can measure the GUS activity of the six samples simultaneously within 2 min. This is due to the advantage of the continuous assay in which the kinetic parameters are derived while the linearity of the assay is checked simultaneously. Thus, the method provides significant advantages over existing discontinuous methods for routine enzyme assays used for either diagnostic purposes or for studies of plant gene expression and regulation.

As discussed earlier, the concentration of enzymes in the assay systems before dilution to the stopping buffer are 100- and 20-fold higher in discontinuous spectrophotometric and fluorometric methods, respectively. The continuous assay could be carried out with low enzyme activity for as short as 30 s. Such kinetic data are obtained under steady-state conditions in which only the forward reaction proceeds and data should be accurate. In contrast, discontinuous assays are performed with much higher concentration of enzyme for at least 10 to 20 min, during which

Figure 3. Plots of initial velocity versus PNP concentration. Lineweaver-Burk plot of corresponding data (inset).

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significant product can be accumulated. This may result in inaccuracy of the kinetic data; thus, it is generally accepted that the continuous assay methods are to be preferred over discontinuous assay methods if accurate kinetic data are required (3,8,24).

In conclusion, the current continuous spectrophotometric assay is significantly simpler, faster, more economical, and more sensitive than the existing discontinuous assays. The experimentalist can easily enjoy the beauty of this assay method. Such a method should be highly desired for both routine GUS assays and for accurate kinetic analysis of the enzyme.

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