Characterization and inhibition study of MurA enzyme by capillary electrophoresis

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Abstract

A capillary electrophoresis-based enzyme assay for UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA) is described. This method, based on UV detection, provides baseline separation of one of the reaction products, enolpyruvyl-uridine 5′-diphospho-*N*-acetylglucosamine (EP-UDP-GlcNAc), from substrates phosphoenolpyruvate (PEP) and uridine 5′-diphospho-*N*-acetylglucosamine (UDP-GlcNAc) within 4 min. The other product, phosphate, is not detectable by UV at 200 nm. Quantitation of individual components, substrates or product, can be accomplished based on the separated peaks. This methodology was used to determine the Michaelis constant, K_m, and product formation rate constant, K_{cat}, for MurA. Additionally, the CE method was used to evaluate the inhibition effects on MurA using one specific compound as an example. By following similar procedures, the apparent K_m values in the presence of different inhibitor concentrations were determined. The inhibition constant, K_i, can be determined from these apparent K_m values. In addition, this CE method can be used to study the inhibition mechanism. The principle of this approach is generally applicable to other enzyme studies. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: MurA enzyme; Enzymes

1. Introduction

Capillary electrophoresis (CE) is a powerful technique that has been applied to the separation and characterization of a variety of biomolecules, including amino acids [1], peptides [2], proteins [3,4], carbohydrates [5], and nucleic acids [6]. In addition, CE has found unique applications in the analysis of enzymes [7–12], co-enzymes [13], substrates [14], and other chemical species [15] that can be linked to enzymatic reactions. Specifically, this technique has become a valuable tool for the determination of enzyme activities [16], the study of enzyme kinetics [17], and the screening of potential enzyme inhibitors [18].

The MurA enzyme (UDP-*N*-acetylglucosamine enolpyruvyl transferase, E.C. 2.5.1.7) is involved in the first committed step in the synthesis of the peptidoglycan component of the bacterial cell wall [19]. The reaction catalyzed by MurA involves the transfer of enolpyruvate from phosphoenolpyruvate (PEP) to the 3-OH group of uridine 5′-diphospho-*N*-acetylglucosamine (UDP-GlcNAc) (Fig. 1). The

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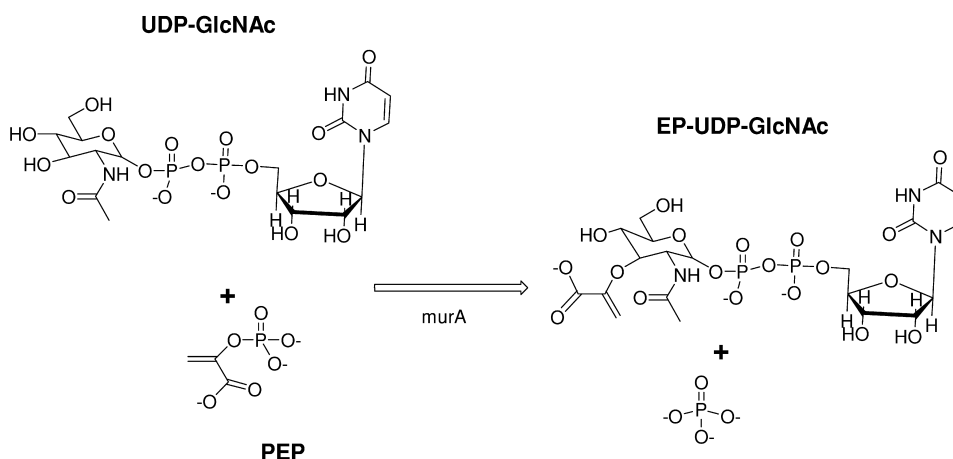
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Fig. 1. Schematic of the MurA-catalyzed transfer of enolpyruvate from phosphoenolpyruvate to uridine 5′-diphospho-*N*-acetylglucosamine.

antibiotic fosfomycin is known to specifically modify and inactivate MurA [20]. As a result, MurA has received significant attention and is a potential target for the development of new antibacterial agents [21,22].

Several methods, including high-performance liquid chromatography (HPLC) and spectroscopic methods, have been devised to monitor this enzymatic reaction and associated kinetics. In one study, the substrate, UDP-GlcNAc, was labeled with ^{14}C and the reaction mixture was subjected to anion-exchange chromatography employing an in-line radioactivity detector [20]. In another study, a size-exclusion HPLC method was used to evaluate the stoichiometry of the enzyme–substrate complex using a ^{14}C-labeled substrate [23]. An additional method involves quantitation of the UDP-GlcNAc-dependent release of radiolabeled inorganic phosphate from PEP [20]. An alternative to the radiolabel approach is to assay MurA using a coupled reaction by adding an excess amount of the enzyme MurB (UDP-*N*-acetylenolpyruvylglucosamine reductase E.C. 1.1.1.158) and its cofactors to the reaction mixture. The presence of MurB reduces the MurA reaction product to uridine diphosphate (UDP)-muramic acid and couples the transferase activity with NADPH oxidation, which can be monitored at 340 nm [23]. Alternatively, the release of inorganic phosphate can be monitored using a colorimetric

method that employs malachite green and monitors absorbance at 660 nm [24,25].

None of the methods described above are satisfactory for characterizing inhibitors of the MurA enzyme. Some of them require the use of radioactive materials. Radiolabeling the substrate is neither economic nor environmentally friendly. Others need to stop the reaction and to add reagents into the system, making it difficult to conduct kinetic analysis. For example, the colorimetric malachite green assay requires the addition of reagents to the reaction mixture for the purpose of detection. This means that a new reaction mixture has to be prepared for each data point on the reaction progression curve. This will add significant amount of work and the data will be less accurate as a result.

The HPLC methods for monitoring MurA activity suffer from the fact that all components of the injected reaction mixture have to be eluted and that the column condition re-equilibrated before subsequent injections can be made. This procedure generates a significant time lag between injections, making it difficult or impossible to monitor the progression of enzyme reactions from a single reaction mixture.

In the initial screening of potential MurA inhibitors by high throuput screening, a malachite green assay was used to monitor the activity of the enzyme [25]. In that assay, the reaction was carried

out in a 96-well plate and stopped prior to analysis, which increase throughput of the assay. As this was the initial screening, only a ''yes'' or ''no'' answer is sufficient, with subsequent characterization of inhibitors still being required. Other methods for monitoring the reaction such as coupling the reaction with other reagents, such as MurB and NADPH (nicotinamide adenine dinucleotide phosphate, reduced form), complicates the reaction system and is prone to interference by inhibitors. Consequently, there is a need for a better and more universal method for the analysis of this enzyme.

CE-based assays are ideal for enzyme kinetics studies. First, CE offers fast analysis time and requires extremely small amounts of sample. The combination of these two characteristics allows CE to continuously monitor the enzyme reaction progress from a single reaction system without disturbing the reaction. Unlike HPLC, where a significant time lag exists between runs, the capillary can be quickly flushed between injections in CE. Therefore, it is not necessary to wait for all sample components to migrate out of the capillary before subsequent injections can be made. This significantly reduces the total analysis time. The nanoliter volume of sample required for analysis makes CE a perfect tool for enzyme analysis. This is even more advantageous when the enzyme, substrate and inhibitors are very expensive.

Second, CE provides the capability of highly efficient separations of the reaction products from the substrates in short times. This is advantageous in situations like the MurA reaction where spectroscopic methods fail to detect differences between the substrate and products. Because of this separation, it is possible to monitor both the loss of substrate and the formation of products simultaneously. Either the loss of the substrate or the increase of the product can be used to study the enzyme kinetics. This separation offers the advantage that the presence of inhibitors, which may interfere with detection, will be separated from the reactant or products being measured.

Third, CE takes aqueous samples directly from the reaction mixture without the need for stopping the reaction prior to analysis. This is of particular importance for kinetic studies when the reaction proceeds quickly. This allows multiple samples to be taken from a single reaction mixture at different time points and avoids the need for multiple reactions to construct a single reaction time course. By using a single reaction mixture, the precision and accuracy of the results are significantly increased.

Finally, several sensitive detection methods, such as UV–Vis, laser-induced fluorescence (LIF) and mass spectroscopy, are available for CE. Therefore, detection can be accomplished without the use of radiolabeled materials.

In summary, CE is an excellent tool for studying enzyme reactions, kinetics, and the influence of potential enzyme inhibitors. We report here the results using CE to study the MurA enzyme and an inhibitor of this enzyme identified by high throughput screening.

2. Materials and methods

2.1. Materials

MurA, from *Escherichia coli* K12, was cloned and purified, and the MurA inhibitor PGE-553828, was synthesized at Procter & Gamble Pharmaceuticals (Mason, OH, USA). The borate buffers were made using Milli-Q water (Milford, MA, USA). Sodium hydroxide solutions (0.1 and 1.0 M) were obtained from J.T. Baker (Phillipsburg, NJ, USA). Uridine 5′-diphospho-*N*-acetylglucosamine, phosphoenolpyruvate, boric acid, Tris–HCl, KCl and dithiothreitol were obtained from Sigma (St. Louis, MO, USA).

2.2. CE instrumentation

CE experiments were performed using a Bio-Rad BioFocus 3000 capillary electrophoresis system (Bio-Rad Labs., Hercules, CA, USA). The instrument is fully automated and is controlled by the vendor's software. A 24 cm (19.6 cm to detector)× 50 μm I.D. uncoated fused-silica capillary (Polymicro, Phoenix, AZ, USA) was used for the analysis. Prior to use, the capillary was flushed sequentially with 1.0 M NaOH for 5 min, deionized water for 5 min, and CE run buffer for 5 min. The CE run buffer was 50 mM borate adjusted to pH 10 with 0.1 M NaOH. Prior to use, the CE run buffer was passed

through a 0.2-µm filter. Between injections the capillary was flushed with the CE run buffer for 20 s. All samples were injected hydrodynamically at 10 p.s.i. s (1 p.s.i.=6894.76 Pa). The applied voltage for each run was 20 kV. Detection of the reaction product was achieved at the cathodic end of the capillary at a wavelength of 200 nm.

2.3. Enzyme activity assay

Initial enzyme reactions were run to determine the capability of CE to separate the substrates from the reaction products. Stock solutions of 1 m*M* PEP and 1 m*M* UDP-GlcNAc were prepared by dissolving the solid compound in a reaction buffer consisting of 50 m*M* Tris–HCl (pH 8), 2 m*M* KCl, and 2 m*M* dithiothreitol. A stock solution of MurA enzyme was prepared in reaction buffer at a concentration of 112 µ*M*. Immediately before use, a 0.448 µ*M* solution of MurA was prepared by diluting the original solution 250-fold with reaction buffer. A 70 µl reaction mixture was prepared by combining 7 µl of 1 m*M* PEP, 7 µl of 1 m*M* UDP-GlcNAc, 51 µl of reaction buffer, and 5 µl of 0.448 µ*M* MurA. The enzymatic reaction was incubated at 37°C and monitored by CE with UV detection.

For kinetic measurements, the same general procedure was used at various substrate concentrations except that the volume of MurA used was halved, giving a final enzyme concentration of 0.016 µ*M*. Because the reaction of MurA involved two substrates, PEP and UDP-GlcNAc, the initial reaction rates were measured by maintaining the concentration of one substrate in excess, such as 100 µ*M*, while altering the concentration of the other. The total reaction volume was maintained at 70 µl by varying the amount of reaction buffer added to the mixture. The enzymatic reaction mixtures were kept at 37°C and injected into the CE instrument every 4 min for approximately 30 min.

2.4. Data analysis

The data collection and peak area integration were performed by Bio-Rad instrument control software associated with their Biofocus 3000 instrument. The results of the integrated peak areas were entered

either into Microsoft Excel for analysis or Sigma Plot software for curve fitting.

3. Results and discussion

3.1. CE method development

A typical electropherogram of a reaction mixture injected after an incubation time of 60 min is shown in Fig. 2. Baseline separation of the product, EP-UDP-GlcNAc, from the other reaction mixture components was achieved in a run time of less than 4 min. Initially, electrophoretic separation of the reaction mixture and identification of the individual components were studied. The migration times of the enzyme (MurA), substrate (UDP-GlcNAc) and the reaction buffer (50 m*M* Tris–HCl, pH 8) were first determined by CE with UV detection. The other substrate, PEP, could not be detected at 200 nm. Also, one of the reaction products, inorganic phosphate, was not detectable by UV at 200 nm. The other reaction product, EP-UDP-GlcNAc, was identified by comparison of the electropherograms of the individual components to that of the reaction mixture. Positive identification of this product, as well as
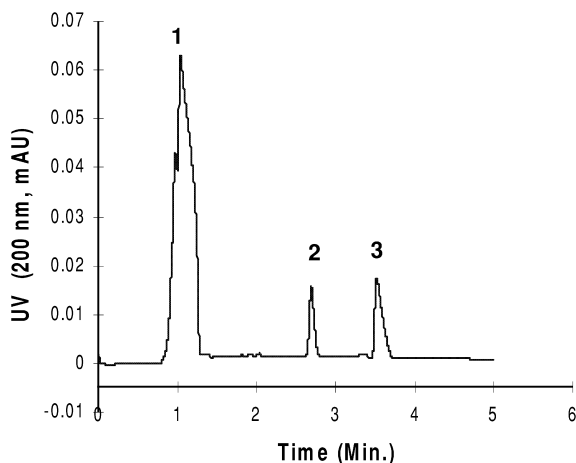

Fig. 2. Typical electropherogram illustrating the separation of the enzymatic reaction mixture containing (1) the reaction Tris–HCl buffer (peak 1); (2) the substrates (UDP-GlcNAc, peak 2); and the product (EP-UDP-GlcNAc, peak 3). Separation was performed using 50 m*M* borate buffer (pH 10), 24 cm capillary, and applied voltage of 20 kV. Detection was at 200 nm.
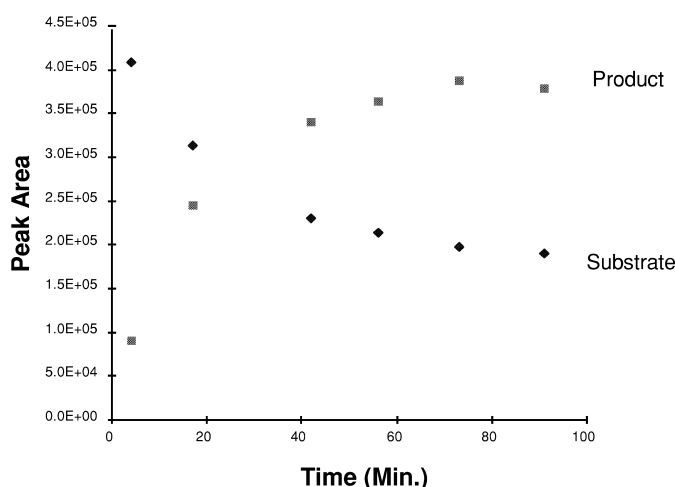
Fig. 3. Reaction progression curves for MurA. Curves are based on peak areas and illustrate the increase of product (EP-UDP-GlcNAc), and disappearance of substrate (UDP-GlcNAc).

the substrates, was achieved by observing the increase in the product peak and the decrease of the substrate peak as the reaction proceeded. Fig. 3 shows a reaction progression curve over 90 min with both substrate concentrations at 100 μM and the enzyme concentration at 0.032 μM. The reaction progression curve illustrates that both the formation of product and the disappearance of substrate can be monitored and quantitated according to their respective peak areas. During the course of the reaction, a continuous decrease in substrate concentration was observed which corresponded with an increase in product formation. A linear region of approximately twenty min at the beginning of the reaction corresponds to the first-order reaction region. The slope of this region is the initial reaction rate.

3.2. Enzyme K_m determination

A typical single substrate based enzymatic reaction can be expressed as:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \overset{K_{cat}}{\rightarrow} E + P \tag{1}$$

where where E is the enzyme, S is the substrate, ES is the complex between the enzyme and the substrate, P is the product. The k_1 and k_{-1} are constants associated with the formation and redissociation of the enzyme–substrate complex. K_{cat} is the reaction rate constant associated with the formation of the final product, i.e.:

$$V = \frac{d[P]}{dt} = K_{cat}[ES] \tag{2}$$

where V is the rate of product formation and t is time, [P] and [ES] are the molar concentrations of the product and the enzyme–substrate complex, respectively.

When certain conditions, such as the substrate concentration is significantly higher than that of the enzyme, are met, there is a well-known relationship between the initial reaction rates and the substrate concentration, i.e.:

$$V = \frac{V_{max}[S]}{K_m + [S]} \tag{3}$$

where [S] is the substrate concentration, V_{max} is the maximum reaction rate and K_m is a constant, which is defined as:

$$K_m = \frac{k_{-1} + k_{cat}}{k_1} \tag{4}$$

Eq. (3) is called the Michaelis–Menten equation and K_m is also called Michaelis constant, which is a unique parameter of a specific enzyme. It reflects the binding affinity of the enzyme for a specific substrate. This parameter can be obtained experimentally

by determining the initial reaction rates at different substrate concentrations and then plot the rates against the substrate concentration.

For enzyme reaction system requiring more than one substrate, the enzyme kinetics is much more complicated. However, under certain experimental conditions, it is possible to simplify the reaction system to a single substrate case. For example, MurA catalyzes a reaction with two substrates, i.e.:

UDP-GlcNAc + PEP → EP-UDP-GlcNAc +

phosphate
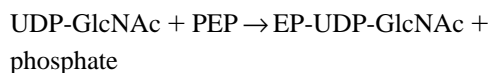
The kinetics of this system is quite complicated. However, we may saturate the system with one substrate constant at relatively high concentration, the reaction kinetics will be similar to a single substrate system as described by the Michaelis–Menten equation. Therefore, by taking more data points at the early stage of the reaction, i.e., the first 20 min for MurA reaction, we obtained a linear relationship between the area of the product peak and the reaction time. This slope represents the initial reaction rate of the reaction under this specific condition. By measuring the rates at different substrate concentrations, we obtained a relationship between the initial rate and the substrate concentration. Since the reactions were run under conditions such that one of the substrates, UDP-GlcNAc, was saturated, the above reaction is equivalent to a one substrate reaction. Thus, the K_m for this substrate can be determined.

Following similar steps, the K_m for each substrate can be determined under conditions in which the other substrate is kept constant at saturating concentrations. Specifically, to determine the K_m for PEP, reaction mixtures containing 100 μM UDP-GlcNAc were used and the concentration of PEP was varied from 1 to 10 μM. The reaction progression curves at different PEP concentrations, monitored by measuring the appearance of the product EP-UDP-GlcNAc, are illustrated in Fig. 4, where the product peak area is plotted as a function of the reaction time. The initial reaction rates of these reactions were determined based on the initial linear portion of these curves (Fig. 4). Then, these initial reaction rates were plotted against PEP concentrations as shown in Fig. 5. The K_m value was calculated by
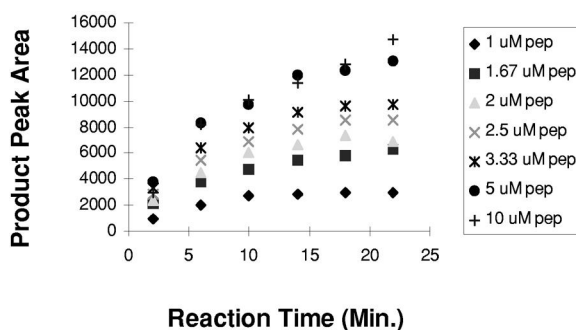

Fig. 4. Reaction progression curves based on peak area of product (EP-UDP-GlcNAc) for various concentrations of PEP used for the determination of K_m. PEP concentrations were varied from 1 to 10 μM with UDP-GlcNAc held constant at 100 μM. Experimental conditions are all the same as described in Section 2.3.

fitting the data in Fig. 5 to the Michaelis–Menten equation. An average value of 4.1 μM from two measurements (3.9 and 4.3 μM) was determined to be the K_m of PEP for MurA. As an alternative, K_m may be calculated using a Lineweaver–Burk plot of $1/\nu$ vs. $1/[S]$, which should give a linear relationship as:

$$\frac{1}{V} = \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}} \tag{5}$$

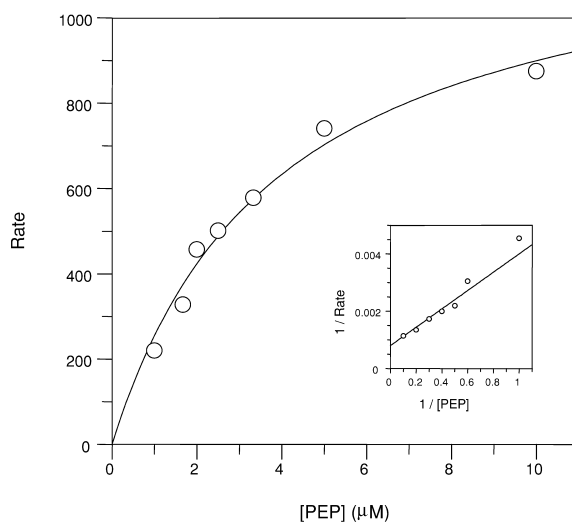An average of 4.2 μM from two measurements

Fig. 5. The initial reaction rate for the formation of product (EP-UDP-GlcNAc) as a function of PEP concentration. Inset shows the double-reciprocal plot related to PEP as the substrate.

(1.6 and 6.8 μM) was obtained. In this case, the two methods of analysis provided similar results (4.1 vs. 4.2 μM). However, the result obtained from the direct curve fitting of the reaction rates to the Michaelis–Menten equation is more accurate than that obtained from the Lineweaver–Burk plot due to two reasons. First, in the Lineweaver–Burk plot, most of the data ended up at the lower half of the linear curve leaving the upper half of the curve with few points. This uneven distribution of the data points makes linear curve susceptible to experimental errors associated with the few data points at the upper half. Second, statistically, errors associated with the two ends of a linear regression curve are larger than that associated with the middle points. The insert in Fig. 5 shows that the two points at the upper half of the curve are deviated from the linear curve. The values of these two points are larger than ideal making the intercept ($1/V_m$) smaller or the slope (K_m/V_{max}) larger than ideal values. If some of the data points are lower than the curve, the opposite will be true. Fortunately, our results indicated that the difference between fitting the data directly to the Michaelis–Menten equation or using the Lineweaver–Burk plot is small and is within acceptable range. Therefore, the result of either method is acceptable.

A similar study was performed to determine the K_m of the other substrate, UDP-GlcNAc, for MurA. In this case, the concentration of PEP was maintained at a saturating concentration of 100 μM while the UDP-GlcNAc concentration varied from 5 to 25 μM. An average of 6.3±4.3 μM ($n=3$) was determined to be the K_m of UDP-GlcNAc for MurA.

As mentioned previously, one of the advantages of CE-based assays is the ability to monitor both the disappearance of the substrate and the appearance of the product simultaneously during the course of the reaction. This means that either the substrate or the product can be used to determine the K_m values. Normally, product concentration alone is used. Occasionally, substrate concentration is also used as an alternative method. By using substrate concentration as well as the product concentration, we can verify the results within a single data set. When substrate concentration was used, the K_m value of UDP-GlcNAc for MurA was determined to be 5.7 μM (6.3 and 5.1 μM) by fitting the reaction data to the

Michaelis–Menten equation. This result is in good agreement with the result obtained monitoring the product concentration. Since PEP was not detectable by UV detection, the K_m value of PEP for MurA was not calculated using PEP concentration.

The published K_m values for PEP and UDP-GlcNAc using other analytical methods are 0.4 and 15 μM, respectively [20]. The K_m values for PEP and UDP-GlcNAc obtained by CE were the same as those we obtained using this enzyme system and reaction conditions, detecting inorganic phosphate released during the enzyme reaction with a malachite green colorimetric assay, as described by Cogan et al. [25]. The K_m value, for UDP-GlcNAc, obtained in this study is close to that previously reported. However, the K_m value for PEP of 4.1 μM is approximately 10-fold higher than that previously described [20] but is significantly lower than the reported dissociation constant for PEP of 240 μM [26]. This discrepancy could be due to differences in experimental parameters such as temperature and purity of enzyme. However, it is difficult to identify all the possible causes of this discrepancy as the experimental details for the literature values of K_m are referenced as unpublished results [20].

3.3. Enzyme V_{max} determination

Theoretically, the V_{max} values can also be determined in conjunction with the determination of the K_m values. However, calculating the V_{max} values based on the product requires a calibration curve to convert the product peak areas of the reaction to product concentration. Due to the unavailability of pure reaction product in this study, we could not obtain a product calibration curve directly. Thus, we could not determine the V_{max} values simultaneously with the K_m value determination using the product concentration.

However, we can use the substrate concentrations to determine the V_{max} (or k_{cat}) values. Based on the concentration of one substrate, UDP-GlcNAc, the V_{max} was calculated as 8.6 μM/min (average of 7.4 and 9.7 μM/min). From this value, k_{cat}, which equals V_{max} divided by the total enzyme concentration, was calculated as 8.9 s^{-1} compared to a literature value of 3.8 s^{-1} [20].

3.4. Inhibition study

The ultimate purpose of developing this CE method for monitoring MurA activity was to use this assay system to characterize compounds that inhibit this enzyme. It is known that inhibition of this enzyme prevents bacterial cell wall synthesis and leads to the bacterial cell death [20,27]. For an inhibition reaction:

$$E + I \overset{K_i}{\rightleftharpoons} ES \tag{6}$$

where I stands for the inhibitory substance, EI is the complex of enzyme and the inhibitor, and K_i is a dissociation constant for inhibitor binding defined as:

$$K_i = \frac{[E][I]}{[EI]} \tag{7}$$

where [E], [I], and [EI] are the concentrations of E, I and EI, respectively.

This CE method has been used to evaluate potential inhibitors as well as to assess the inhibition mechanism. For example, using a colorimetric method for enzyme assay, PGE-553828 was found to have inhibition effects on MurA at concentrations above 80 μM, in the presence of 100 μM PEP and 20 μM UDP-GlcNAc. In this study, the inhibitory effects of PGE-553828 were further determined experimentally by repeating the K_m determination for UDP-GlcNAc at several inhibitor concentrations ranging from 0 to 240 μM. For each inhibitor concentration, PEP was present at 100 μM and UDP-GlcNAc was varied from 4 to 25 μM. The inhibition constant (K_i) was determined by plotting ν_0/ν_i vs. inhibitor concentration ([I]) according to [28]:

$$\frac{\nu_0}{\nu_i} = 1 + \frac{[I]}{K_i\left(1 + \dfrac{[S]}{K_m}\right)} \tag{8}$$

where ν_0 is the uninhibited initial velocity, ν_i is the initial velocity at a given inhibitor concentration ([I]), and [S] is the substrate concentration. Following linear regression, the slope, $1/\{K_i(1+[S]/K_m)\}$, was used to calculate the K_i value [28].

The inhibition effect of this compound in the concentration range of 0 to 240 μM is illustrated in Fig. 6, where the initial reaction rate is plotted as a
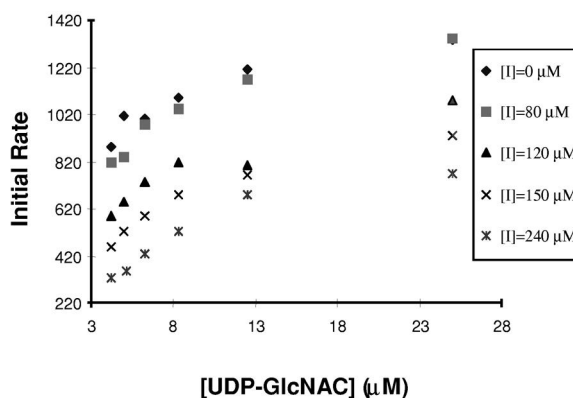

Fig. 6. Plot of initial reaction rate for the formation of product (EP-UDP-GlcNAc) as a function of UDP-GlcNAc concentration at several concentrations (0–240 μM) of the test compound, PGE-553828.

function of substrate concentration (UDP-GlcNAc) at several concentrations of PGE-553828. The K_i value was determined according to Eq. (8) using the plot of ν_0/ν_i vs. [I] (Fig. 7). According to Eq. (8), the slope of the plots of ν_0/ν_i vs. [I] should decrease as the concentration of the substrate increases. This has been confirmed in our experiments as shown in Fig. 7. The average K_i for this compound was 38 ± 6 μM ($n=4$), this data was then reanalyzed, fitting the data to the Michaelis–Menten equation (assuming competitive inhibition) and the K_i was determined to be 41 μM. The ability to fit the data to this model combined with the convergence of the double reciprocal plots near the y-axis (data not shown), supports the conclusion that PGE-553828 acts as a competitive inhibitor with respect to UDP-GlcNAc. However, the intercept of the ν_0/ν_i vs. [I] plot should be 1. The fact that the intercepts are deviated from 1 indicates that this competitive model is not perfect. In addition, there could have been some experimental errors that contributed to this discrepancy.

4. Conclusions

It may be concluded that CE is useful for the analysis of the MurA enzyme activity. By analyzing the activity of this enzyme, it is possible to determine the enzyme kinetic parameters, such as K_m and V_{max} values. Further, this CE method can be used
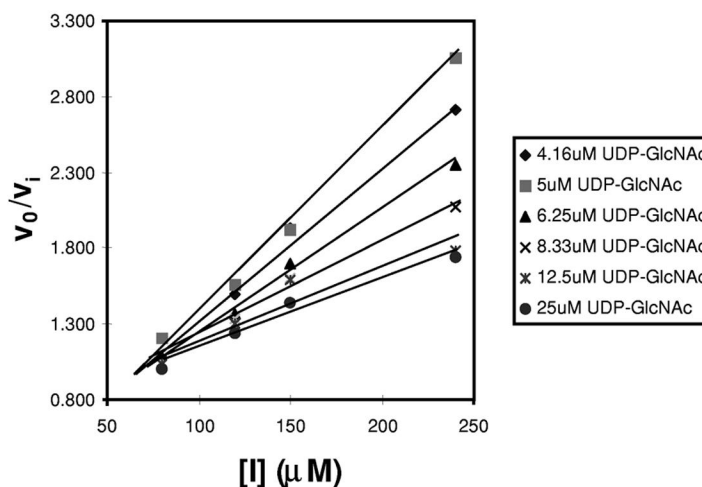
Fig. 7. Plot of the experimental values of the initial reaction rate ratio (ν_0/ν_i) vs. inhibitor concentration at different substrate concentrations.

to characterize the inhibitory effects of various compounds, thus providing useful information for selecting proper drug development candidates. This CE-based method has several advantages, such as no radiolabeled substrate is required for the assay and no secondary reaction is needed. The most important advantage is that CE requires so small amount of sample for each analysis that the amount of sample injected for each analysis does not change the reaction volume significantly. Therefore, continues sampling from a single reaction vial, even it contains only a few microliter volume of reaction mixture, does not have any adverse effect to the reaction mixture. Thus, the reaction can continue while multiple samples can be taken out. At the same time, the various components in a reaction mixture are separated immediately after they are injected into the capillary column, removing the need to stop the reaction before analysis. This avoids the potential errors resulted from failure to fully stop the reaction prior to sample injection in some other assay methods. The major limitation of the current method is that it takes several minutes, including the instrument cycling time, to finish one analysis. This is not suitable for some fast reaction systems because the reaction rate may be under estimated. In order to control the reaction rate, it is necessary to adjust the concentrations of the substrates and the enzyme. This limitation can be overcome if multiplexed capillary electrophoresis (MCE) system is employed for this

analysis [29]. In that case, multiple reactions with fixed time delay can be run simultaneously. Multiple time points can be obtained from a single MCE analysis.

Acknowledgements

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