Study of substrate inhibition by electrophoretically mediated microanalysis in partially filled capillary

Kateřina Papežová a, Tomáš Němec a, Radka Chaloupková b, Zdeněk Glatz a,∗

a Department of Biochemistry, Faculty of Science, Masaryk University, Kamenice 5, 625 00 Brno, Czech Republic
b National Centre for Biomolecular Research, Faculty of Science, Masaryk University, Kamenice 5, 625 00 Brno, Czech Republic

Received 23 June 2006; received in revised form 6 September 2006; accepted 8 September 2006

Abstract

Substrate inhibition is a common phenomenon in enzyme kinetics. We report here for the first time its study by a combination of the electrophoretically mediated microanalysis (EMMA) methodology with a partial filling technique. In this setup, the part of capillary is filled with the buffer best for the enzymatic reaction whereas, the rest of the capillary is filled with the background electrolyte optimal for separation of substrates and products. In the case of haloalkane dehalogenase, a model enzyme selected for this study, the enzymatic reaction was performed in 20 mM glycine buffer (pH 8.6) whereas 20 mM β-alanine–hydrochloric acid buffer (pH 3.5) was used as a background electrolyte in combination with direct detection at 200 nm. The whole study was performed on poorly soluble brominated substrate – 1,2-dibromoethane. As a result it was first necessary to find the compromise between the concentrations of the enzyme and the substrate preserving both the adequate sensitivity of the assay and at the same time the attainable substrate solubility. By means of the developed EMMA methodology we were able to determine the Michaelis constant (K_M) as well as the substrate inhibition constant (K_SI). The value of K_M and K_SI obtained were 7.7 ± 2.5 mM and 1.1 ± 0.4 mM, respectively. Observation of the substrate inhibition of haloalkane dehalogenase by 1,2-dibromoethane is in accordance with previous literature data.

Keywords: Enzyme kinetics; EMMA; Substrate inhibition; Haloalkane dehalogenase

1. Introduction

Enzyme kinetics is an important area of modern enzymology. Its study brings essential knowledge for biochemistry as well as for biotechnology, pharmacology, medicine etc. [1–3]. Due to the large diversity in the chemical and physical characteristics of enzyme substrates and products, many different methods have been applied for this purpose so far [3–6]. Although photometric methods are undoubtedly the most frequently used, separation methods including capillary electrophoresis (CE) gain their position in this field [6].

Fourteen years ago a new procedure for the evaluation of enzymatic reactions in CE was proposed and developed by Bao and Regnier [7], the electrophoretically mediated microanalysis (EMMA). In contrast with the classic enzyme assay by CE where only the determination of products or substrates is performed by means of this efficient separation technique, the EMMA methodology used the capillary both as a separation medium and as a reaction chamber. Compared to spectrophotometric and other assays, the EMMA-based methods are rapid, can be automated, and require only small amount of reagents that is especially important in the case of enzymes. Recently three reviews dealing with EMMA methodology have been published. The first review covers the basic principle, procedure and different variants of EMMA [8]. The second and third reviews summarise, the applications of EMMA on the enzyme and non-enzyme systems [9,10].

Since its introduction the EMMA methodology has been utilized in a number of enzymologically related systems – for assays of enzyme activities [7,11–15], determinations of substrate concentrations [15–17], Michaelis constants [18–23], inhibitors and inhibition constants [24–27]. Recently we published the combination of EMMA with the partial filling technique for the kinetics studies of enzymes dealing with the high
mobile inorganic anions as substrates or products [22,27]. In this paper we proposed the additional extension of its applicability on the study of substrate inhibition of enzymatic reactions. This phenomenon has been observed by means of EMMA methodology by Nováková et al. for the first time during the kinetic study performed on phenol sulfotransferase [23]. However the evaluation of substrate inhibition constant has been not finally performed.

2. Experimental

2.1. Chemicals

1,2-dibromoethane was obtained from Aldrich (Milwaukee, WI, USA); β-alanine and glycine were obtained from Sigma (St. Louis, MO, USA). All other chemicals and solvents were of analytical reagent grade, supplied by Fluka (Buchs, Switzerland). The background electrolyte was prepared by adding hydrochloric acid (HCl) to 20 mM β-alanine solution down to pH 3.5. The glycine buffer was prepared by addition of 0.1 M sodium hydroxide (NaOH) to 20 mM glycine solution up to pH 8.6. All solutions were prepared with Milli Q (Buckee, WI, USA). Academic water (Millipore, Milford, MA, USA) and filtrated by a 0.45 μm membrane filter. Fresh enzyme and substrate solutions were prepared in 20 mM glycine buffer (pH 8.6) each day.

2.2. Enzyme preparation

The His-tagged hydrolytic haloalkane dehalogenase from Sphingomonas paucimobilis UT26 (LinB) purified to homogeneity [28,29] was used as a model enzyme. To overproduce LinB, the enzyme was expressed in Escherichia coli BL21 under isopropyl-β-thiogalactopyranoside (IPTG) induction. Transformed E. coli was cultured in 21 of Luria broth at 37 °C. IPTG was added to the cells up to a final concentration of 1 mM when the culture reached an optical density of 0.6 at 600 nm. The cells were harvested after 4 h incubation (at 30 °C), resuspended in 50 mM phosphate buffer pH 7.5 containing 1 mM β-mercaptoethanol and 10% glycerol and disrupted by sonication. After centrifugation at 100,000 × g for 1 h, the supernatant was applied on a Ni-NTA Sepharose column (QIAGEN, Hilden, Germany) equilibrated with 20 mM phosphate buffer (pH 7.5) containing 0.5 M sodium chloride and 10 mM imidazole. The unbound proteins were washed out by equilibrating buffer and the His-tagged LinB was eluted by the same buffer containing 0.5 M imidazole. The purified enzyme was stored in 50 mM phosphate buffer (pH 7.5) containing 1 mM β-mercaptoethanol and 10% glycerol at 0–4 °C.

2.3. Apparatus

The Agilent 3D Capillary Electrophoresis System (Waldbronn, Germany) with a diode-array UV–VIS detector was used to carry out all CE separations. The CE system autosampler was heated using a recirculating water bath for 25 °C to have all solution preincubated on the temperature of the capillary. Data were collected on a HP Compaq personal computer using the Agilent 3DCE ChemStation Software. A Polymicro Technology (Phoenix, AZ, USA) 75 μm fused silica capillary was used for all separations.

2.4. Study of haloalkane dehalogenase reaction by EMMA

A 75 μm fused silica capillary (64.5 cm total length, 56.0 cm effective length) was washed with 20 mM β-alanine–HCl (pH 3.5) as a background electrolyte for 3 min. The in-capillary enzymatic reaction was performed by injection of 20 mM glycine buffer (pH 8.6) – 50 mbar for 4.0 s; the enzyme solution in 20 mM glycine buffer (pH 8.6) – 50 mbar for 4.0 s; the solution of 1,2-dibromoethane in 20 mM glycine buffer (pH 8.6) – 50 mbar for 4.0 s; 20 mM glycine buffer (pH 8.6) – 50 mbar for 4.0 s and the background electrolyte – 50 mbar for 4.0 s consecutively into the capillary. The reaction was initiated by the application of separation voltage −29.9 kV (negative polarity). The temperature of the capillary was 25 °C. Samples were detected at 200 nm with a bandwidth 10 nm. The peak areas were measured by means of ChemStation software and were corrected on the migration times. The concentrations of bromide were calculated from the calibration graph. The capillary was washed 1 min with deionised water after each run.

3. Results and discussion

3.1. Substrate inhibition

A number of substances may cause a reduction in the rate of an enzyme-catalysed reaction. Some of these are non-specific protein denaturants. Others, which generally act in a fairly specific manner, are known as inhibitors. Loss of activity may be either irreversible, when the loss of activity is time independent and cannot be recovered during the timescale of interest or reversible, when activity may be restored by the removal of the inhibitor [1–3].

A special case of reversible inhibition is substrate inhibition which occurs in about 20% of all known enzymes. Normally an increase in substrate concentration increases the velocity of the enzyme reaction. Some enzymes, however, display the phenomenon of excess substrate inhibition. This means that large amounts of substrate can have the opposite effect and actually slow down the reaction. It is primarily caused by binding more than one substrate molecule to an active site meant for just one. The different parts of the substrate molecules bind usually to different subsites within the substrate binding site. If the resultant complex is inactive, this type of inhibition causes a reduction in the rate of reaction at high substrate concentrations. It may be modelled by the following scheme:

\[
\begin{align*}
\text{E} + \text{S} & \rightarrow \text{ES} \\
\text{ESS} & \rightarrow \text{P}
\end{align*}
\]

Please cite this article as: Kateřina Papežová et al., Study of substrate inhibition by electrophoretically mediated microanalysis in partially filled capillary, Journal of Chromatography A (2006), doi:10.1016/j.chroma.2006.09.022
Michaelis–Menten equation for substrate inhibition can be described as

\[ v_0 = \frac{V_{\text{max}}[S]}{K_M + (1 + ([S]/K_S_I))[S]} \]  

where \( v_0 \) and \( V_{\text{max}} \) are the initial and maximum velocities, respectively \( K_M \) is the Michaelis constant, \([S]\) is the concentration of substrate and \( K_{S_I} \) is the substrate inhibition constant.

3.2. Electrophoretically mediated analysis of haloalkane dehalogenase

Like in the previous papers [22,27], haloalkane dehalogenase from \( S. \) paucimobilis was chosen as a model enzyme. It is an important enzyme involved in the biodegradation of the halogenated aliphatic hydrocarbons producing alcohol, halide and proton:

\[ \text{CH}_3(\text{CH}_2)_n \text{X} + \text{H}_2\text{O} \rightarrow \text{CH}_3(\text{CH}_2)_n \text{OH} + \text{H}^+ + \text{X}^- \]

but it can be also used in enantioselective organic syntheses [30–32].

The whole study was performed on brominated substrate – 1,2-dibromoethane, which inhibition property was recently confirmed by gas chromatography [33]. Therefore the identical EMMA setup could be applied in this work as in the previously mentioned publications. In this manner the capillary is first filled with a background electrolyte. Subsequently, a plug of the glycine buffer, a plug of the enzyme solution, a plug of the substrate solution, a plug of the glycine buffer and finally a plug of the background electrolyte are injected hydrodynamically into the capillary. Upon to the application of an electric field, the zones of enzyme and substrate interpenetrate and the enzymatic reaction takes place. Finally the resultant reaction product – bromide anion – is electrophoretically transported towards detector and quantified by means of direct detection at 200 nm.

Preliminary experiments were performed to find the suitable concentration of haloalkane dehalogenase for this study. Although \( K_M \) as well as \( K_{S_I} \) are not dependent on the amount of the enzyme in reaction mixture, its concentration influences the concentration of the substrate required for the saturation of enzyme to estimate \( V_{\text{max}} \). In view of the fact that the poorly soluble 1,2-dibromoethane (maximum water solubility 4.3 g per litre \( \approx 23 \text{ mM} \) [34]) was selected as a model substrate, the lowest concentration of enzyme as possible should be used to attain its complete saturation within the achievable substrate concentration range. For that reason the set of pilot experiments was performed in which the concentration of enzyme was changed from 0.025 to 1.0 mg of protein per ml, whereas five concentrations of substrate – 2.5, 5, 10, 15 and 20 mM were tested. Based on these experiments (data not shown) the study of substrate inhibition was finally performed using haloalkane dehalogenase solution of 0.08 mg of protein per ml; the concentrations of 1,2-dibromoethane were changed from 1 to 20 mM. Each concentration was analysed in triplicate using separate vial for each measurement to avoid possible substrate volatilization during the corresponding parallel experiments.

The influence of substrate concentration on the production of bromide can be seen from Fig. 1. showing the overlaid electropherograms obtained at increasing concentrations of the substrate. The inhibition effect was clearly detectable at concentrations higher then 11 mM. This phenomenon is well documented by Michaelis–Menten plot (Fig. 2) where the corrected bromide peak areas were taken as the initial reaction velocities and the concentrations of 1,2-dibromoethane in the substrate plugs were taken to be equal to the concentrations in the vial before sampling. Since the substrate inhibition only occurs at high substrate concentrations the low substrate part of this graph is identical to that of a normal enzyme. At high substrate concen-

Please cite this article as: Kateřina Papežová et al., Study of substrate inhibition by electrophoretically mediated microanalysis in partially filled capillary, Journal of Chromatography A (2006), doi:10.1016/j.chroma.2006.09.022
trations the plot falls as the velocity is reduced by the inhibitory effect.

In view of the fact that the enzyme no longer follows the Michaelis equation, giving a non-hyperbolic plot, the Lineweaver–Burk plot is no longer linear as well (Fig. 3). Instead towards the 1/velocity axis, this is the high substrate end, the line curves upwards to show the reduction in velocity. The straight line section of the plot, the low substrate end, can still be extrapolated to determine $K_M$ in the usual way. The other linear kinetic plots will, of course, also be removed from the straight and narrow in a similar fashion. Similarly as in the case of $K_M$ determination $K_{SI}$ can be obtained for example from the Dixon plot using the points laying in the linear range at high substrate concentrations where the inhibitory effect predominates. The $K_M$ value of 7.7 ± 2.5 mM and the $K_{SI}$ value of 1.1 ± 0.4 mM for 1,2-dibromoethane with $R^2 > 0.96$ in both cases were finally computed from electrophoretic data using SigmaPlot software.

4. Concluding remark

The study of the substrate inhibition of haloalkane dehalogenase by the combination EMMA methodology with a partial filling technique is described. This progressive EMMA modification is used for given purpose for the first time. Its application potential is thus spread on the other important field of enzyme kinetics which is the other significant contribution of CE to the studies of enzymatic reactions.

Acknowledgments

This work was supported by grant No. 203/06/0047 from Grant Agency of Czech Republic and by research project No. MSM0021622413 and by research centre LC06023 both from Czech Ministry of Education.

Authors express thanks to Jiří Damborský (Masaryk University, Brno) for helpful discussion.

References


Please cite this article as: Kateřina Papežová et al., Study of substrate inhibition by electrophoretically mediated microanalysis in partially filled capillary, Journal of Chromatography A (2006), doi:10.1016/j.chroma.2006.09.022