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Research Article

Kinetic study of paracetamol on prolidase activity in erythrocytes by capillary electrophoresis with $Ru(bpy)_3^{2+}$ electrochemiluminescence detection

We explored the CE with $Ru(bpy)_3^{2+}$ electrochemiluminescence detection for the kinetic study of drug–enzyme interaction. Effects of four nonsteroidal anti-inflammatory drugs including aspirin, paracetamol, sodium salicylate and phenacetin on prolidase (PLD) activity in erythrocytes were investigated. Aspirin enhanced PLD activity whereas the other three had inhibiting effects. This may reveal their different effects on the collagen biosynthesis and catabolism that influence tumor invasiveness. Kinetic study of paracetamol on PLD showed that the value of Michaelis constant K_m for PLD was 1.23 mM. The mechanism of PLD inhibition by paracetamol is noncompetitive inhibition, and the inhibitor constant K_i value obtained in our research was 9.73×10^3 μg/L.

Keywords: Capillary electrophoresis / Electrochemiluminescence / Kinetic study / Paracetamol / Prolidase DOI 10.1002/elps.200600197

1 Introduction

$Ru(bpy)_3^{2+}$ electrochemiluminescence (ECL) characterized as having excellent stability of reagent, sensitivity and wide linear range, has been proven to be a suitable detector for CE [1]. CE-ECL offers a highly sensitive and selective technique for the detection of the analytes that contain amine groups, and a review concerning the analytical application of CE-ECL has been published [1]. Although CE-ECL has been proved to be a highly efficient and sensitive analytical method for biomaterial and pharmaceutical determinations using standard solutions [1], few papers concerning bioactive analytes existing in clinical samples have been reported.

In this paper, we explored CE-ECL for the kinetic study of drug–enzyme interaction in clinical samples, enlarging the application scope of the CE-ECL technique. The effects of four nonsteroidal anti-inflammatory drugs (NSAIDs) including aspirin, paracetamol, sodium salicylate and phenacetin, with their structures shown in Fig. 1, on prolidase (EC 3.4.13.9, PLD) activity in human erythrocytes were explored. The effects of NSAIDs on PLD activity are reflected by the amounts of proline produced by the enzymatic-catalyzed hydrolyzation of glycylproline, which can be expressed by the following equation:

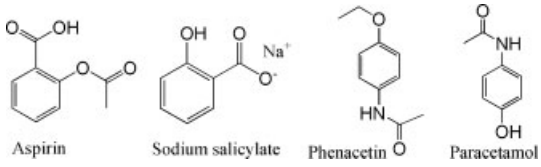

Figure 1. Structures of the four NSAIDs investigated in the present work.

$$\text{Glycylproline} + H_2O \underset{Mn^{2+}}{\overset{PLD}{\rightleftharpoons}} \text{glycine} + \text{proline} \quad (1)$$

Mn^{2+} is necessary as it is the cofactor of PLD, playing an important role in the activation and functional regulation of PLD [2]. As proline is rich in collagens, PLD mediates the breakdown of collagens on which the progression of tumour might critically depend [3, 4]. An increased PLD activity has been reported in lung and stomach cancer to increase the proline pool and balance the collagen degradation [3, 4]. Many studies show the potential cancer chemopreventive effects of aspirin and other NSAIDs [5–9], besides their common usage as anti-inflammatories, antipyretics and analgesics [10, 11]. But as for para-

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Abbreviations: ECL, electrochemiluminescence; **NSAIDs**, nonsteroidal anti-inflammatory drugs; **PLD**, prolidase; **PMT**, photomultiplier tube

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cetamol, the major metabolite of phenacetin, which is a carcinogen, conflicting results existed [6, 12, 13]. In order to evaluate the role of different NSAIDs on the collagen biosynthesis and cancer invasion, the effects of NSAIDs on PLD activity in human erythrocytes were investigated by CE-ECL, as proline can enhance $Ru(bpy)_3^{2+}$ ECL.

Although proline can be detected by UV or fluorescence detection [14, 15], the derivatization process of proline limited the application of these methods, especially in bioanalysis. CE-ECL has been successfully applied to the detection of underivatized proline by many groups [16–18], and proved to be a relatively simple and sensitive method. This enables CE-ECL as a simple and competent tool to the drug–PLD interaction analysis. The lower value of the linear range of proline detected by our method was ten times lower than that of glycylproline detected by CE-UV, which has been applied to PLD activity assay by Zanaboni's group [19] by detecting the disappearance of glycylproline by UV absorption. Also, results obtained by CE-ECL are more accurate than that by Chinard's method [2] which is widely used in PLD assay, because the Chinard's reagent reacts with other amino acids and sugar existing in plasma besides proline [20], which makes the results ambiguous. The pretreatment and determination procedures were simplified in CE-ECL compared to MALDI-TOF MS [20], and the analysis time was shortened to less than 7 min in this method, while ITP [21] needed more than 20 min in PLD activity assay.

2 Materials and methods

2.1 Chemicals

Tris(2,2'-bipyridyl) ruthenium(II) chloride hexahydrate was obtained from Aldrich Chemical (Milwaukee, WI, USA). Glycylproline was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Aspirin was obtained from Sigma Chemical (St. Louis, MO, USA). The physiological buffer saline (PBS) consisted of 8.500 g/L NaCl, 0.4370 g/L $NaH_2PO_4 \cdot 2H_2O$ and 2.579 g/L $Na_2HPO_4 \cdot 12H_2O$. All solutions were prepared with water, purified by a Milli-Q system (Millipore, Bedford, MA, USA) and stored at 4°C.

2.2 Instrumentation

ECL detection was carried out with an MPI-A CE ECL detector (Xi'an Remax Electronics, Xi'an China in association with the Changchun Institute of Applied Chemistry, Changchun, China), using a three-electrode system consisting of a 500 μm-diameter platinum disk as working electrode, an Ag/AgCl (KCl saturated) electrode as reference electrode and a 1 mm-diameter platinum wire as counterelectrode. The detection potential was set at 1300 mV. The detection cell was filled with 5.0×10^{-3} mol/L $Ru(bpy)_3^{2+}$ in 3.0×10^{-2} mol/L (pH 7.5) phosphate buffer. The potential of the photomutiplier tube (PMT) was set at −750 V. A capillary (Yongnian Optical Conductive Fiber Plant, Yongnian, China) of 25 μm id, 360 μm od and 44.2 cm length was used. It was rinsed with 0.1 mol/L NaOH, water and running buffer, each for 10 min before use. The sample was injected for electromigration at 10 kV for 10 s. The separation voltage was 18 kV.

2.3 Enzyme reaction procedure

Erythrocytes were extracted from anticoagulant human blood with PBS. Erythrocytes lysate was diluted four-fold with 3.0×10^{-2} mol/L (pH 7.6) phosphate buffer. A part of the diluted solution (40 μL) was mixed with $MnCl_2$ and glycylproline, and then the mixture was diluted to 400 μL with phosphate buffer. The mixture was incubated at 37°C for 30 min to ensure the reaction proceeded at initial velocity. After a water bath at 80°C for 10 min to terminate the reaction, it was centrifuged at 4000 rpm for 10 min. The supernatant was electromigration injected at 10 kV for 10 s for CE analysis.

2.4 Determination of Michaelis constant K_m and the maximum velocity of the enzymatic reaction V_{max}

Five substrate concentrations (1.00×10^{-3}, 1.43×10^{-3}, 2.50×10^{-3}, 5.00×10^{-3} and 2.50×10^{-2} mol/L) were used with the incubation time of 20 min for enzyme-catalysis analysis by Michaelis–Menten equation [22, 23].

3 Results and discussion

3.1 Determination of proline

The ECL intensity produced by the reaction of $Ru(bpy)_3^{2+}$ and proline highly depends on the concentration of $Ru(bpy)_3^{2+}$ and pH. ECL intensity increased very slowly when the concentration of $Ru(bpy)_3^{2+}$ was above 5.0×10^{-3} mol/L, and in order to reduce the consumption of reagent, 5.0×10^{-3} mol/L $Ru(bpy)_3^{2+}$ was chosen as the electrochemiluminescence reagent. The maximum pH was found at slightly basic conditions, and 7.5 was chosen in our experiment as ECL intensity decreased significantly below pH 6.5 and above pH 8.5, and also, the peak width of proline was rather broad at acidic conditions.

At the optimal detection conditions of proline, a linear range between 1.00×10^{-5} and 3.75×10^{-3} mol/L ($R^2 = 0.999$) was achieved. The equation for the regression

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line (Fig. 2) was $y = 8.87 \times 10^8 x - 4.86 \times 10^4$, where y is ECL intensity (counts) and x is the concentration of proline (mol/L). The LOD was determined to be 5.0×10^{-6} mol/L (S/N = 2). As the PLD activity in erythrocytes is about 17-fold of the average value of 900 U/L in plasma (producing 9.00×10^{-4} mol proline *per* minute *per* litre of plasma) [2], the detection limit and the linear range enable this method to be fit for the kinetic study of PLD.

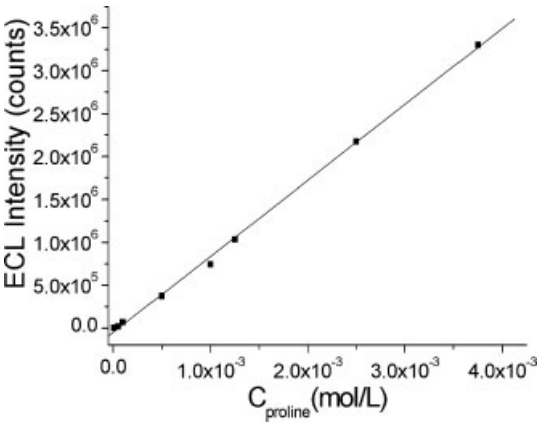

Figure 2. Calibration curve for the determination of proline. Phosphate buffer (30 mM, pH 7.5); 5.0 mM $Ru(bpy)_3^{2+}$; detection potential, 1300 mV (*vs.* Ag/AgCl); potential of PMT, −750 V; capillary, 25 μm id and 44.2 cm length; sample injection, 10 kV for 10 s; separation voltage, 18 kV.

3.2 Effects of four NSAIDs on PLD

Different effects of NSAIDs on PLD were found in our experiment. As shown in Fig. 3, aspirin enhanced the PLD activity in erythrocytes to a large extent, and the activity of PLD reached 1.4-fold of the control when the concentration of aspirin was 25 μg/mL. The activity remained at 90% when the concentration of aspirin was 150 μg/mL. Other NSAIDs all presented inhibition of PLD, though to different extents. The highest inhibition percentage of PLD on paracetamol, sodium salicylate and phenacetin were 41.6, 16.8 and 6.22%, respectively, but at lower concentrations (<100 μg/mL), phenacetin presented higher inhibition extent than paracetamol and sodium salicylate. On the basis of our results, it can be concluded that aspirin and three other NSAIDs had different effects on the collagen biosynthesis and catabolism. By accelerating the activity of PLD, aspirin enhanced the hydrolysis of glycylproline to release more proline that can be used in the resynthesis of collagen. The resynthesis and deposition of collagen on the base membrane prevent the invasion of cancer cells. The other three NSAIDs inhibit the collagen resynthesis by inhibiting the activity of PLD, thus increasing the risk of cancer invasion.

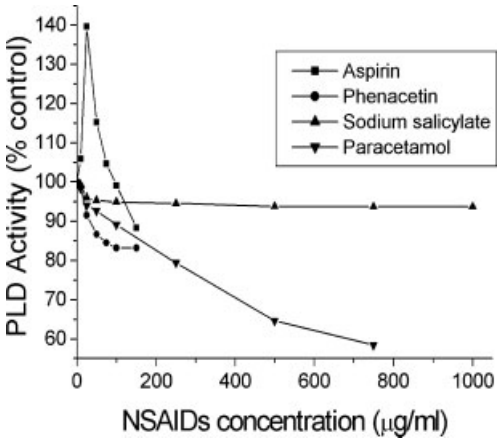

Figure 3. Effects of NSAIDs on PLD activity. Cofactor, 1.0 mM Mn^{2+}; substrate, 10 mM glycylproline; 30 mM phosphate buffer (pH 7.6); incubation, 37°C for 20 min.

3.3 PLD inhibition by paracetamol

Typical electropherograms of PLD inhibition by paracetamol are shown in Fig. 4. The concentrations of the substrate glycylproline and the cofactor Mn^{2+} were 1.0×10^{-2} and 1.0×10^{-3} mol/L, respectively. Enzymatic reaction proceeded in the medium of 30 mmol/L phosphate buffer at pH 7.6. As shown in Fig. 4, the peak area of proline, produced from the enzymatic reaction, was reduced gradually with increase in the concentration of paracetamol. In addition, no sample pretreatment and no interference existed, ensuring the exactness of the kinetic parameters determined in our experiment.

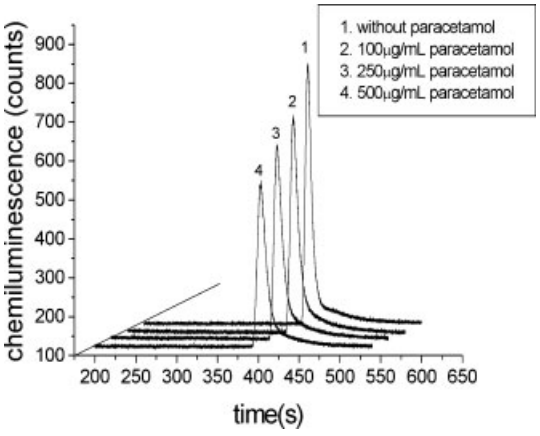

Figure 4. Electropherograms obtained after enzymatic reaction with different concentrations of paracetamol. The concentration of paracetamol in the enzymatic reaction system was 0, 100, 250 and 500 μg/mL, respectively. Cofactor, 1.0 mM Mn^{2+}; substrate, 10 mM glycylproline; 30 mM phosphate buffer (pH 7.6); incubation, 37°C for 20 min.

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3.4 Michaelis–Menten analysis

The inhibition mechanism of PLD by paracetamol was assessed using Michaelis–Menten analysis. The Michaelis–Menten equation [22, 23] reflects the relationship between the initial velocity (v) and substrate concentration [S]

$$v = \frac{[S]V_{max}}{[S] + K_m} \quad (2)$$

Here, v is the initial reaction velocity with the substrate concentration [S]. As the enzymatic reaction proceeds at initial velocity at first, and the velocity then declines gradually according to the enzymatic dynamics, the enzymatic reaction time was controlled within 30 min to make sure that the reaction was proceeding at initial velocity. $1/v$ was calculated by the electrochemiluminescence peak areas of proline, with the concentration of proline divided by the reaction time. V_{max} is the maximum velocity of the enzymatic reaction, and K_m is the Michaelis constant representing the concentration of substrate at half of the maximum velocity. The liner regression curve describing the relationship between $1/v$ and 1/[S], namely Lineweaver–Burk plot, can be obtained by inversing the equation to the form

$$\frac{1}{v} = \frac{K_m}{V_{max}}\frac{1}{S} + \frac{1}{V_{max}} \quad (3)$$

Five glycylproline concentrations ranging from 1.00×10^{-3} to 2.50×10^{-2} mol/L were used in Michaelis–Menten analysis. Three Lineweaver–Burk plot lines were achieved and shown in Fig. 5, for paracetamol concentrations of 0, 100 and 500 μg/mL. These lines intersect on the X-axis, with the correlation coefficients (R^2) of 0.996, 0.994 and 0.990, respectively. The K_m value was 1.23 mM and did not change by the addition of paracetamol, while V_{max} declined when the concentration of paracetamol increased from 0 to 500 μg/mL. The V_{max} values of the three reaction systems were 1.41×10^{-5}, 1.33×10^{-5}, and 1.08×10^{-5} mol·L^{-1}min^{-1}. Paracetamol combines with both PLD and PLD–glycylproline complex to prevent the releasing of proline. The mechanism of PLD inhibition by paracetamol is noncompetitive inhibition. K_m values of PLD, detected by different groups vary according to different sources of PLD and different incubating conditions, as listed in Table 1.

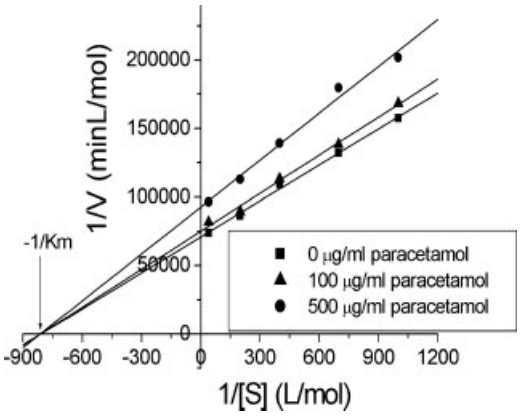

Figure 5. Lineweaver–Burk plots for the enzymatic reaction inhibited by 0, 100 and 500 μg/mL paracetamol. The concentrations of substrate were chosen as 1.00, 1.43, 2.50, 5.00 and 25.0 mM; cofactor, 1.0 mM Mn^{2+}; 30 mM phosphate buffer (pH 7.6); incubation, 37°C for 20 min.

3.5 Determination of inhibitor constant K_i

A noncompetitive inhibition mechanism of paracetamol was deduced from the Lineweaver–Burk plots. In the noncompetitive inhibition, Michaelis–Menten equation can be expressed as [24]

$$\frac{1}{V} = \frac{1}{V_{max}}\left(1 + \frac{K_m}{[S]}\right)\left(1 + \frac{[I]}{K_i}\right) \quad (4)$$

Table 1. K_m of glycylproline by PLD from different sources

Sources of PLD	Incubating conditions	Detection methods	K_m (mM)	References
Erythrocytes	pH 7.6, 37°C	CE-ECL	1.23	Our method
Escherichia coli	pH 8.0, 25°C	UV-Vis	1.4 ± 0.2	[25]
PLD I in erythrocytes	pH 7.8, 37°C	Chinard's	2.59 ± 0.13	[26]
Erythrocytes	pH 7.8, 37°C	Chinard's	4.99 ± 0.17	[27]
Porcine kidney	pH 8.0, 37°C	CE-UV	0.71	[19]
Human fibroblast cells	pH 8.0, 37°C	CE-UV	0.81 ± 0.046	[19]
Aureobacterium esteraromaticum PLD expressed in *E. coli*	–	–	0.67	[28]
Cultured fibroblasts	pH 7.8	Chinard's	4.1	[29]

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[I] is the concentration of the inhibitor, K_i is the inhibitor constant representing the combining ability of the inhibitor with the enzyme or enzyme–substrate complex. Dixon plot can be generated from this equation with [I] along the *X*-axis and 1/*v* along the *Y*-axis. Dixon plots of three different [*S*] values 1.43, 2.50 and 5.00 mM are shown in Fig. 6. Three K_i values calculated are 9.75×10^3, 9.72×10^3 and 9.73×10^3 μg/mL, respectively, with the correlation coefficients (R^2) 0.995, 0.992 and 0.999. The mean value of K_i is 9.73×10^3 μg/mL.

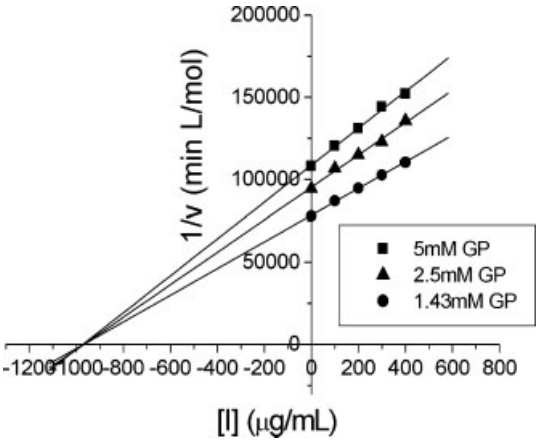

Figure 6. Dixon plots for the enzymatic reaction with [S] of 1.43 mM, 2.50 mM, and 5.00 mM. The concentrations of paracetamol were 0, 100, 200, 300, and 400 μg/mL; cofactor, 1.0 mM Mn^{2+}; 30 mM phosphate buffer (pH 7.6); incubation, 37°C for 20 min.

4 Concluding remarks

CE-ECL was successfully applied to study the effects of four different NSAIDs on PLD activity of erythrocytes. Different effects of four NSAIDs on the collagen biosynthesis and catabolism that influence tumour invasiveness were revealed, and the mechanism of PLD inhibition by paracetamol was also studied. CE-ECL has proved to be a simple and efficient method for the kinetic study of paracetamol on PLD activity in clinical samples.

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