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Kinetic Studies of the Toxicological Effect of 2-Chlorophenol and 4-Chlorophenol on Alkaline Phosphatase Activity *in vitro*

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The inhibition kinetic parameters of 2-chlorophenol and 4-chlorophenol on bovine intestinal mucosa alkaline phosphatase activity were studied by spectrophotometry. Both 2-chlorophenol and 4-chlorophenol inhibited alkaline phosphatase activity in a concentration dependent manner and the 50 % inhibitory concentration (IC_{50}) was estimated to be 23.29 mM for 2-chlorophenol and 30.02 mM for 4-chlorophenol, respectively. The apparent Michaelis-Menten constant (K_m) and apparent maximum reaction rate (V_{max}) for the hydrolysis of 4-nitrophenyl phosphate disodium salt hexahydrate by alkaline phosphatase was found to be 0.124 mM and 1.80 μM min^{-1}, respectively. Both K_m and V_{max} decreased distinctly in the presence of 2-chlorophenol and 4-chlorophenol, which indicated that the nature of the inhibition was uncompetitive type. The values of the inhibition constant (K_i) were estimated to be 18.89 mM for 2-chlorophenol and 32.68 mM for 4-chlorophenol. Therefore, 2-chlorophenol showed a stronger inhibition effect on alkaline phosphatase activity and displayed a higher affinity for the enzyme-substrate complex than 4-chlorophenol.

Key Words: Chlorophenols, Alkaline phosphatase, Inhibition effect, Kinetics.

INTRODUCTION

Chlorophenols are widely used as preservative agents, pesticides, antiseptics, disinfectants and in a variety of industrial applications[1,2]. Most of chlorophenols are 1isted as priority pollutant by the environmental protection agencies due to their high toxicity and low biodegradability. 4-Chlorophenol is used as an intermediate for the synthesis of insecticides, herbicides, preservatives, antiseptics and disinfectants. It is also used in making medicines, dyes, aroma compounds and other organic chemicals. 2-Chlorophenol is a result of the activities of the paper, pulp, pesticide and herbicide industries, it has been identified as potential carcinogen and it also has been designated as priority pollutant by the US EPA[3]. The toxicity of 2-chlorophenol is not fully understood and some studies have shown that it contributes to the loss of activity of some key biochemically reactive enzymes[4]. Therefore, it has important significance for investigating their toxicities and fates in the environment.

Alkaline phosphatase (ALP, EC 3.1.3.1) are non-specific phosphohydrolases that are found in many organisms from bacteria to mammals[5]. In mammals, alkaline phosphatase is a particularly ubiquitous enzyme that is very important in physiological functions and medical diagnosis. Both the catalytic mechanism and the structure of alkaline phosphatase have been studied and reviewed[6-9]. It is known that numerous compounds have inhibition effect on the activity of alkaline phosphatase. The inhibition mechanism of alkaline phosphatase from *Escherichia coli* by L-phenylalanine was demonstrated to be uncompetitive type[10,11] and that of human placental and germ-cell alkaline phosphatase by L-Leu and L-Phe was also found to be uncompetitive type[12]. Moreover, the non-competitive inhibition of alkaline phosphatase from *Escherichia coli* by okadaic acid was demonstrated by Meštrovic and Pavela-Vrancic[13]. However, to our knowledge, kinetics of alkaline phosphatase inhibition by 2-chlorophenol and 4-chlorophenol *in vitro* have not been elucidated. In this study, the inhibition kinetics of 2-chlorophenol and 4-chlorophenol on bovine intestinal mucosa alkaline phosphatase were investigated *in vitro*. This research aims to elucidate the toxicological effect of chlorophenols on alkaline phosphatase and to provide a more accurate estimate of its toxicity and availability.

EXPERIMENTAL

Alkaline phosphatase (ALP, ≥ 10 DEA units/mg solid, from bovine intestinal mucosa) and 4-nitrophenyl phosphate

disodium salt hexahydrate (*p*-NPP, ≥ 99.0 %, enzymatic, used as substrate) were purchased from Sigma-Aldrich Inc (St. Louis, USA). Tris base (> 99.9 %, ultra pure grade) was purchased from Amresco Inc (Solon, USA). 2-Chlorophenol and 4-chlorophenol (analytical grade) were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). All other reagents were of analytical grade. Ultra-pure water (18.2 MΩ cm) was used throughout experiments.

A 5.0×10^{-5} M alkaline phosphatase stock solution was prepared in 0.1 M Tris-HCl buffer solution (pH 9.8) and kept at -20 ºC; the desired alkaline phosphatase concentration for the experiment was obtained by diluting the stock solution with tris-HCl buffer solution. A 0.1 M 4-nitrophenyl phosphate stock solution was also prepared in 0.1 M tris-HCl buffer solution (pH 9.8) and kept at 4 ºC; the desired 4-nitrophenyl phosphate concentration was also obtained by diluting the stock solution with tris-HCl buffer. A 0.01 M 2-chlorophenol and 0.01 M 4-chlorophenol stock solutions was prepared in the anhydrous ethyl alcohol, respectively, the desired concentration for the experiment was also obtained by diluting the stock solution. A 0.1 M $MgCl_2$ solution was prepared in ultra-pure water.

Assay of alkaline phosphatase activity: Alkaline phosphatase activity was measured spectrophotometrically at 405 nm based on formation of yellow colour *p*-nitrophenol produced by hydrolysis of 4-nitrophenyl phosphate in alkaline solution at 37 ºC[14]. In our experiment, the assay for alkaline phosphatase activity was carried out at 37 ºC in 0.1 M tris buffer (pH 9.8) containing 2 mM $MgCl_2$ (used as activator). The absorbance of *p*-nitrophenol in the reaction mixture was continuously measured spectrophotometrically at 405 nm using a SP-723C spectrophotometer (Shanghai, China) equipped with a 1 cm cell and a SDC-6 thermostat bath (Ningbo, Zhejiang). To achieve temperature equilibration, the mixture was added into the colorimetric tube and was incubated for 5 min prior to addition of alkaline phosphatase solution. The exact procedure is given in Table-1.

TABLE-1
PROCEDURE FOR THE DETERMINATION OF ALKALINE PHOSPHATASE ACTIVITIES

Sequence of solution addition	Final concentration	
	Control system (without inhibitor)	System treated with chlorophenol (with inhibitor[a])
Tris-HCl buffer	0.1 M, pH 9.8	0.1 M, pH 9.8
p-NPP	0.20 to 0.64 mM	0.20 to 0.64 mM
$MgCl_2$	2 mM	2 mM
Inhibitor[a]	–	2.0 to 20 mM
Equilibrate at 37 ºC for 5 min, then add:		
Alkaline phosphatase	0.60 nM	0.60 nM
Determination of the absorbance of *p*-nitrophenol in the reaction mixture for 15 min at 37 ºC.		

[a]2-CP = 2-chlorophenol or 4-CP = 4-chlorphenol; *p*-NPP = 4-Nitrophenyl phosphate.

The 4-nitrophenyl phosphate concentration for measuring IC_{50} was 0.40 mM and in experiments determining Michaelis-Menten constants and inhibition constants was varied from 0.20-0.64 mM. Each assay was made in triplicate. The relatively activity of alkaline phosphatase in the presence of inhibitor was calculated as follows:

$$\text{Relatively activity (\%)} = \frac{A}{A_{control}} \times 100\ \% \quad (1)$$

where, A is the absorbance in the presence of inhibitor; $A_{control}$ is the absorbance in the absence of inhibitor.

Computation of kinetic parameters: Michaelis-Menten constants (K_m) were determined by Lineweaver-Burk plots, using initial velocities obtained over a substrate concentration range of 0.20-0.64 mM. The initial reaction velocity of 4-nitrophenyl phosphate hydrolysis was defined as the change in product concentration with reaction time, it was calculated as follows:

$$\nu = \frac{A}{\varepsilon bt} \times 10^3 \quad (2)$$

where, ν is the hydrolysis reaction rate, μM min^{-1}; A is the absorbance of *p*-nitrophenol produced by hydrolysis of 4-nitrophenyl phosphate at 405 nm; ε is the absorption coefficient of *p*-nitrophenol at 405 nm, in our experiment the value of ε is 19.7 mM^{-1} cm^{-1}; b is the length of absorption cell, cm; t is the reaction time, min.

The kinetic constants were estimated from Lineweaver-Burk plots of 1/ν against 1/[S], lines being fitted to the experimental points by the method of least squares. The inhibition constants (K_i) were derived from plots of reciprocal apparent Michaelis-Menten constant *versus* inhibitor concentrations.

RESULTS AND DISCUSSION

Estimation of IC_{50}: Plots of alkaline phosphatase relatively activity *versus* concentration of 2-chlorophenol and 4-chlorophenol were showed in Fig. 1. The concentration of inhibitor that inhibits 50 % of enzymatic activity, IC_{50}, was estimated by fitting the data of relatively activity *versus* inhibitor concentration with linear regression equation[15]. In our investigation, IC_{50} was determined at fixed substrate concentration (4-nitrophenyl phosphate 0.40 mM) and fixed enzyme concentration (alkaline phosphatase 0.6 nM).

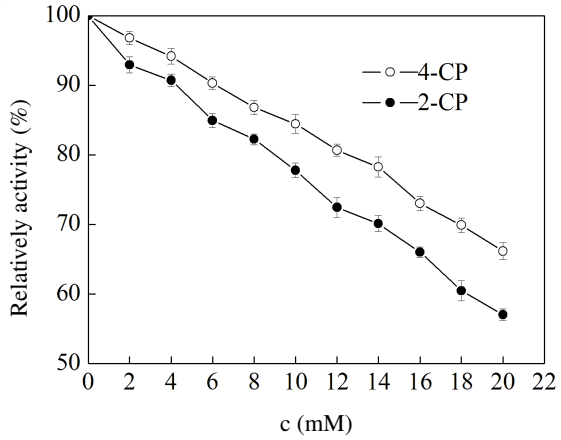

Fig. 1. Inhibition of alkaline phosphatase activity as a function of 2-chlorophenol (2-CP) and 4-chlorophenol (4-CP) concentration

From Fig. 1, it can be seen that both 2-chlorophenol and 4-chlorophenol inhibited alkaline phosphatase activity in a concentration dependent manner and a negative linear relationship was observed between alkaline phosphatase relatively

activity and the concentration of 2-chlorophenol and 4-chlorophenol, respectively. The linear regression equations were fitted as follows:

$$\text{Relatively activity (\%)} = -2.0834\,[\text{2-CP}] + 98.53\ (r = 0.9956) \quad (3)$$

$$\text{Relatively activity (\%)} = -1.6838\,[\text{4-CP}] + 100.54\ (r = 0.9969) \quad (4)$$

where, [2-chlorophenol] and [4-chlorophenol] is the concentration of 2-chlorophenol and 4-chlorophenol, respectively; r is the linear correlation coefficient. Consequently, when alkaline phosphatase relatively activity was 50 %, IC_{50} was calculated to be 23.29 mM for 2-chlorophenol according to eqn. 3 and IC_{50} was calculated to be 30.02 mM for 4-chlorophenol according to eqn. 4. The lower value of IC_{50} means stronger inhibition, hence, the inhibitory effect of studied chlorophenols on alkaline phosphatase activity follows the pattern: 2-chlorophenol > 4-chlorophenol.

Determination of kinetic parameters: Plots of reaction rate, ν *versus* alkaline phosphatase concentration in absence and presence of 2-chlorophenol and 4-chlorophenol are shown in Fig. 2, in this experiment, 4-nitrophenyl phosphate concentration was fixed at 0.40 mM. As shown in Fig. 2, the hydrolysis reaction rates increased linearly with the increasing of alkaline phosphatase concentration. In the absence and presence of 10 mM 2-chlorophenol and 10 mM 4-chlorophenol, three lines through the origin point have obtained, respectively. Obviously, the inhibition type of alkaline phosphatase induced by 2-chlorophenol and 4-chlorophenol was reversible inhibition[16].

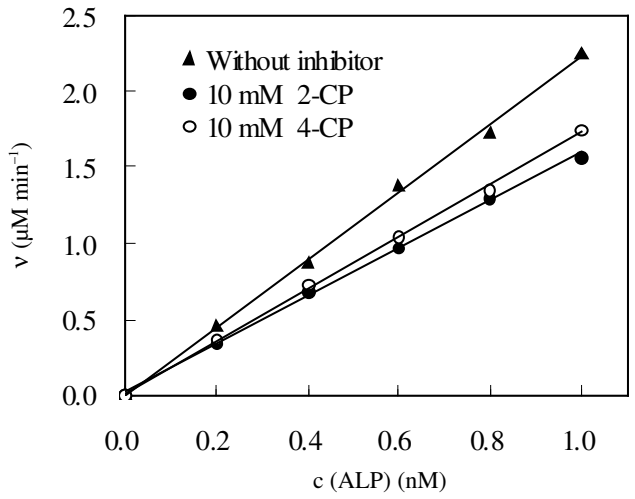

Fig. 2. Plots of hydrolysis reaction rates *versus* alkaline phosphatase (ALP) concentration

Reversible enzyme inhibitors have been classified as competitive, uncompetitive, non-competitive or mixed, according to their effects on K_m and V_{max}. The reversible inhibition type can be determined by the Lineweaver-Burk plot (double reciprocal plot) as follows[17]:

$$\frac{1}{\nu} = \frac{K_m}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}} \quad (5)$$

where, ν is reaction rate; K_m is Michaelis-Menten constant; V_{max} is maximum reaction rate; [S] is the substrate concentration.

Plots of 1/ν *versus* 1/[S] in the absence and presence of chlorophenols are shown in Fig. 3 and the kinetic constants, K_m and V_{max} obtained by fitting the data to the Lineweaver-Burk plot (eqn. 5) are displayed in Table-2.

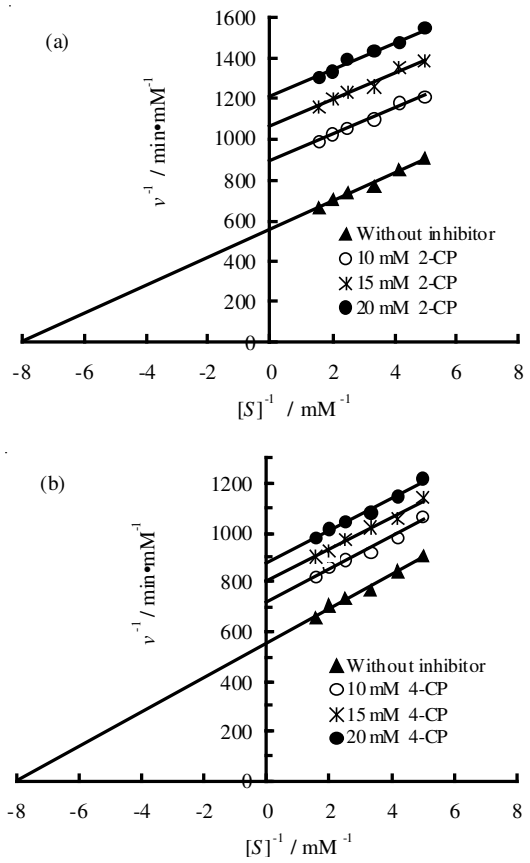

Fig. 3. Lineweaver-Burk plots of alkaline phosphatase activity in the absence and presence of 2-chlorophenol (2-CP) (a) and 4-chlorophenol (4-CP) (b)

TABLE-2
INHIBITION KINETIC PARAMETERS OF ALKALINE PHOSPHATASE INHIBITED BY 2-CHLOROPHENOL AND 4-CHLOROPHENOL

Inhibitor[a]	Concentration (mM)	K'_m (mM)	V'_{max} (μM min^{-1})	K_i[b] (mM)
2-Chlorophenol	10	0.074	1.12	18.89
	15	0.063	0.94	
	20	0.055	0.83	
4-Chlorophenol	10	0.091	1.39	32.68
	15	0.082	1.25	
	20	0.074	1.14	

[a]K_m and V_{max} was 0.124 mM and 1.80 μM min^{-1} for the control system, respectively. [b]K_i was calculated from plots of $1/K'_m$ *versus* [I].

When used for determining the type of enzyme inhibition, the Lineweaver-Burk plot can distinguish competitive, non-competitive and uncompetitive inhibitors. From Fig. 3, it can be seen that Lineweaver-Burk plots in the presence of 2-chlorophenol and 4-chlorophenol are straight lines parallel to those obtained in the absence of inhibitor, but with a higher y-intercept. The value of K_m and V_{max} for the hydrolysis of 4-nitrophenyl phosphate by bovine intestinal mucosa alkaline phosphatase, estimated from the Lineweaver-Burk plot was 0.124 mM and 1.80 μM min^{-1} for the control system, respectively, while both

K_m and V_{max} was decreased obviously in the system treated with chlorophenols (Table-2). The inhibition kinetic results indicated that the inhibition of bovine intestinal mucosa alkaline phosphatase by 2-chlorophenol and 4-chlorophenol is of an uncompetitive type. Therefore, both 2-chlorophenol and 4-chlorophenol acted as uncompetitive inhibitor of alkaline phosphatase, which means that the 2-chlorophenol and 4-chlorophenol could bind to the enzyme-substrate (ES) complex and caused a reduction of alkaline phosphatase activity.

In the present study, uncompetitive inhibition of bovine intestinal mucosa alkaline phosphatase by 2-chlorophenol and 4-chlorophenol was similar to uncompetitive inhibition of *Escherichia coli* alkaline phosphatase by L-phenylalanine[10,11] and human placental and germ-cell alkaline phosphatase by L-Leu and L-Phe[12], while it is different to non-competitive inhibition of alkaline phosphatase from *Escherichia coli* by okadaic acid[13]. The K_m value, which depends on the hydrolysis reaction conditions and the type of substrate, for bovine intestinal mucosa alkaline phosphatase with 4-nitrophenyl phosphate as substrate was found to be near to the value for bone alkaline phosphatase with β-naphthyl phosphate (0.110-0.118 mM)[18] and the value for intestinal alkaline phosphatase with *p*-nitrophenyl phosphate (0.1 mM) as substrate[19].

Since the inhibition type is uncompetitive, the apparent Michaelis-Menten constant, inhibition constant and inhibitor concentration is given by the following equation[20]:

$$K'_m = \frac{K_m}{1+\frac{[I]}{K_i}} \quad (6)$$

where, K_m and K'_m is the apparent Michaelis-Menten constant in the absence and in the presence of inhibitor, respectively; K_i is the inhibition constant for binding to the enzyme-substrate complex; [I] is the concentration of inhibitor. Taking the reciprocal for eqn. 6, gives:

$$\frac{1}{K'_m} = \frac{1}{K_m} + \frac{1}{K_m K_i}[I] \quad (7)$$

Based on the data of K_m, K'_m and [I], the value of K_i was calculated from the plots of $1/K'_m$ *versus* [I] (eqn. 7) as displayed in Table-2. As shown in Table-2, the value of K_i, the corresponding dissociation constants of the ESI (enzyme-substrate-inhibitor) complex, was found to be 18.89 mM for 2-chlorophenol and 32.68 mM for 4-chlorophenol. The lower value of K_i indicated a higher affinity for the enzyme-substrate complex. Therefore, the affinity of studied chlorophenols for the enzyme-substrate complex follows the pattern: 2-chlorophenol > 4-chlorophenol.

The active sites of alkaline phosphatase contain two Zn^{2+} ion and one Mg^{2+} ion, as well as residues Asp-91, Ser-92 and Arg-166, which are involved in substrate binding[12]. The activity of alkaline phosphatase depends on the presence of Zn^{2+} and Mg^{2+} ions in the catalytic site. Several studies have identified Zn^{2+} ion 1 as of primary importance for catalysis, its removal causing a complete loss of enzyme activity[21]. Uncompetitive inhibition of alkaline phosphatase by chlorophenols (2-chlorophenol and 4-chlorophenol) may be induced by reversible binding of the phenolic hydroxyl group binding to the active-site Zn^{2+} ion 1 and interferance with the hydrolysis of the phosphoenzyme intermediate, thereby, causing the decrease in alkaline phosphatase activity.

Conclusion

The present data indicate that both 4-chlorophenol and 2-chlorophenol act as uncompetitive inhibitors of alkaline phosphatase activity. Inhibition effects can be caused by binding to the enzyme-substrate complex. Compared with 4-chlorophenol, 2-chlorophenol shows a stronger inhibition effect on alkaline phosphatase activity and displays a higher affinity for the enzyme-substrate complex. The results presented here may provide a useful database for future investigations of the toxicological mechanisms of representative chlorophenols. However, more detailed experiments will be required to provide further insight as to how chlorophenols cause damage in organisms.

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