Effects of Hg and Cu on the activities of soil acid phosphatase[*]

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Abstract: Comparative study on the activity and kinectic properties of acid phosphatase (ACPase) of three soils amended with Hg and Cu at constant temperature and humidity was carried out. The results indicated that the inhibition on ACPase of the three sample soils by Hg and Cu varied with the content of soil organic matter and pH, where, Soil 1 was the most seriously contaminated due to its lowest content of organic matter and the lowest pH among three samples, Soil 2 took the second place, and Soil 3 was the least contaminated. Except Soil 3, the activity of soil ACPase tended to increase along with the contact time under the same type and the same concentration of heavy metal. In particular the V_{max} values of ACPase in all three samples decreased with increasing Hg and Cu concentration, whereas the K_m values were affected weakly. According to the change of V_{max} and K_m values, Cu and Hg had the same inhibition effect on soil ACPase. Both of them may be a type of compound of non-competitive and anti-competitive inhibition. Statistic analyses indicated that activities of soil ACPase and V_{max} values could serve as bioindicator to partially denote the heavy metal Hg and Cu contamination degree.

Key words: Acid phosphatase (ACPase), Activity, Heavy metal, Kinetic property, Soil

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INTRODUCTION

Soil enzyme activities are sensitive to both natural and anthropogenic disturbances (Giller *et al.*, 1998). The negative impacts of heavy metal concentrations with European community standard on microbial biomass and activity indicated that the sensitivity of the soil to these impacts was greater than plants or animals (Brookes, 1995).

Heavy metal is one of the major pollutants in soil environment caused by industry, agriculture, traffic emission. Mercury (Hg) deposits slowly throughout the body and plays a major role in causing many distressing, even life threatening symptoms, Hg can be transferred rapidly inside the axon of the nerve (axonal transport) to the spinal cord and brain stem. As the nerves become weakened and mercury-toxic, a wide variety of illnesses can occur, often associating with neurological symptoms. Hg can inhibit the activities of soil urease and invertase (He *et al.*, 2001; 2002) and damage microorganism seriously (Cenci *et al.*, 1985).

As a microelement, copper (Cu) is indispensable to all organism in aerobic environment, but an excessive concentration of Cu can be toxic, forming free radicals which cause the peroxidation of lipids, and the oxidation of the proteins, polysaccharide and nucleic acids directly, Cu can also combine with the –SH in proteins, resulting in inhibiting enzyme activities. Chen *et al.*(2002) found that after Cu enters soil, the diversity of microbial communities could change with the increase or a decrease of the microbial biomass and the rate of respiration, and damage of soil enzyme activity could occur. The activities of cellulase and glucosidase are inhibited at copper concentrations above 200 μmol/L of suspensions of montmorillonite and Al-montmorillonite (Geiger *et al.*, 1998a; 1998b). It is known that excess of Cu is often introduced into soil through the use of chemical

compounds for disease treatments.

Numerous studies have investigated the correlation between the soil enzyme activities and the concentration of heavy metals. Among the trace elements that have been tested, Ag, Cr, and Cd have generally caused the greatest inhibition in enzyme assays (Frankenberger and Tabatabai, 1981; 1991a; 1991b; Eivazi and Tabatabai, 1990; Deng and Tabatabai, 1995). Some studies focused on the ED_{50} (i.e. the heavy metal concentration at which enzyme activity is half of the inhibiting level) to describe metal toxicity to enzymes (Doelman and Haanstra, 1989; Renella *et al.*, 2003). Doelman and Haanstra (1989) found that phosphatase activity was very similar to ED_{50} values in function of Cd, Cu and Zn. Based on the sensitivity of soil enzymes to heavy metal, it was proposed that enzyme activities could be used as bioindicator to evaluate the degree of soil contamination by heavy metal (Banerjee *et al.*, 1997). More information is needed to study the kinetics of soil enzyme activities in the presence of heavy metal.

The objectives of this study were to investigate the effects of Hg^{2+} and Cu^{2+} on the activities and kinetic property of soil acid phosphatase (ACPase) of three contrasting soil.

MATERIALS AND METHODS

2,6-dibrominequinone chlorimide was purchased from Huacheng Industry Ltd. of Tokyo, Japan. Phenyldisodium phosphate, sodium acetate, $HgCl_2$, and $CuSO_4 \cdot 5H_2O$, etc. were all A.R.

Soils 1, 2 and 3 were collected from Lin'an City, Deqing County and Hujiachi District of Zhejiang University, respectively. Soils are classified as mountainous red soil, mountainous soil, wetland soil according to Soil Taxonomy, sampled at the depth of 0~20 cm, dried in the shade after plant root and mixture were removed, then passed through 1 mm diameter sieve and kept at low temperature (4 °C).

Methods followed for soil characterization: pH by water extraction method, OM by $K_2Cr_2O_7$ volumetry method, TN by kjeidahi nitrogen determination, TP by colorimetric method, CEC by sulfuric acid-barium chloride method, particle size by pipette method. Soil was digested with $KMnO_4$-H_2SO_4, then Hg was determined by cold vapor absorption. Soil

was first digested with HF-$HClO_4$-HNO_3, then Cu was determined by atomic absorption spectrophotometry.

$HgCl_2$ and $CuSO_4 \cdot 5H_2O$ solution was added to 600 g soil samples respectively which were then air-dried and passed through 1 mm diameter sieve, the final concentration of Hg^{2+} was 0, 0.5, 1.0, 2.0, 5.0, 10.0, 20.0 mg/kg soil, and Cu^{2+} was 0, 10, 20, 50, 100, 200, 500, 800 mg/kg soil, which were mixed sufficiently, incubated at 80% of water-holding capacity and 25 °C with the beakers covered. The samples were assayed after 20, 40, 60 d of incubation. Each treatment was carried out in triplicate. Determination of dynamics parameter of ACPase (He and Zhu, 1997) was as follows: 1 ml methylbenzene was added to 5 g soil sample, after 15 min, 10 ml solution of phenyldisodium phosphate of different concentrations (0.001, 0.0025, 0.005, 0.01 mmol/L) and 10 ml acetic acid (pH 5.0) salt buffer solution were added, and the mixture was cultured at 37 °C. At scheduled time intervals, samples were collected and the ACPase activities were measured.

The Michaelis-Menten constant K_m characterized the rapidity of enzyme combining with substrate in enzyme dynamics, while the maximum velocity V_{max} was a measurement of the total enzyme, which can characterize the velocity of enzyme-substrate compound decomposing into enzyme and products (He *et al.*, 2001):

$$\frac{1}{t}\ln\frac{[S]_0}{[S]} = -\frac{1}{K_m}\frac{([S]_0-[S])}{t} + \frac{V_{max}}{K_m}. \quad (1)$$

It is a linear equation. Herein t is the reaction time; $[S_0]$ and $[S]$ are substrate concentration at the beginning and end respectively.

Linearly fitting $\frac{1}{t}\ln\frac{[S]_0}{[S]}$ vs $\frac{[S]_0-[S]}{t}$ at different time, the slope was $-1/K_m$, intercept was V_{max}/K_m, abscissa axis intercept was V_{max}.

RESULTS AND DISCUSSION

Effect of Hg and Cu on activities of soil ACPase

Figs.1 and 2 show the effect of Hg and Cu on activities of soil ACPase. As the figures show, the decrease of three different kinds of soil ACPase
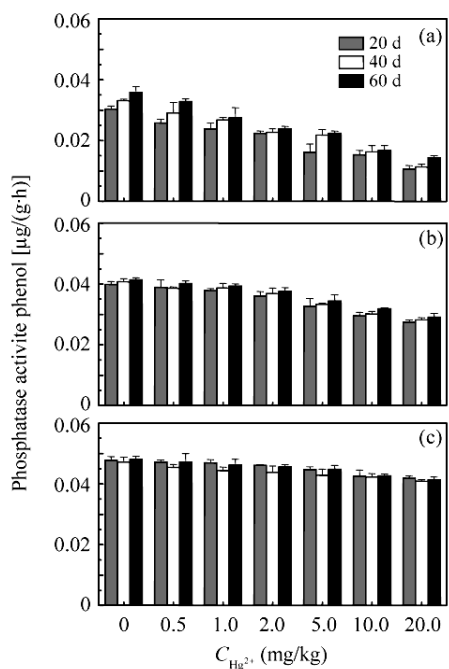
Fig.1 Effect of Hg on activities of acid phosphatase
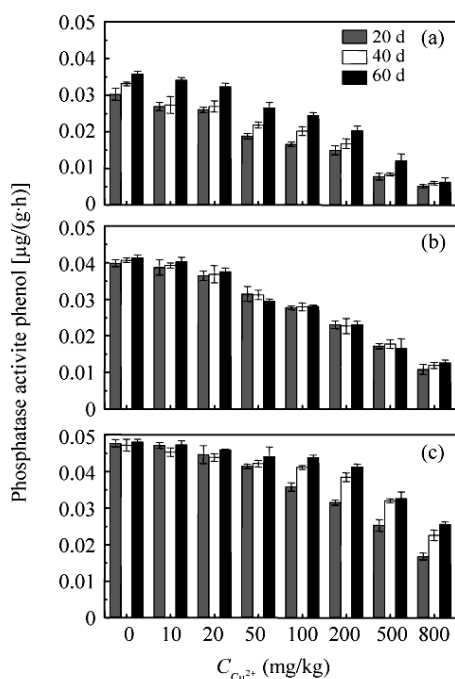(a) Soil 1; (b) Soil 2; (c) Soil 3

Fig.2 Effect of Cu on activities of acid phosphatase
(a) Soil 1; (b) Soil 2; (c) Soil 3

activities caused by Hg and Cu was different, Soil 1 decreased most, Soil 2 next and Soil 3 lowest, which was related to the soil character (Kandeler et al., 1997; Marzadori et al., 1996; Chen et al., 2000). Soil can

buffer exotic heavy metal action, which was the essence reflection between soil and heavy metal ion; so that, it was certainly related to the variety and character of the soil solid phase component. Due to the distinct component and character of different soils, the ability and intensity of the relationship between heavy metal and soil were also diverse. Entry of uniform quantity of heavy metal into different soils, causes different ecological and environment effect (Tu et al., 2000). Soil organic matter content and pH have considerable effects on their buffer action. It was reported that high content of soil organic matter could protect the heavy metal (just protect the enzyme) considerably (He et al., 2001; Lin, 1995). Based on the characteristic of soil organic matter particle with minus charges, when heavy metal enters soil, it is absorbed by organic matter, so the biotoxicity of the heavy metal decreased. There is negative correlation between the content of soil organic matter and the biotoxicity of heavy metal. High pH is also good for the buffering action of heavy metal, whereas low pH (lower than 6) caused the buffering action to be reduced greatly. Because in acidic condition, soil heavy metal exists in ion form, so low pH could cause the content and activity of free heavy metal to increase, which resulted in increasing the biotoxicity of heavy metal. Through adding Cu to three soils (acidic, neutral, alkaline) and analyzing the form change of Cu. Of the three soils, acid soil had the greatest amount of exchangeable Cu, which was 9% of external Cu, alkaline soil had the lowest amount, which was only 1.1% of external Cu; but for residue Cu, the result was completely opposite, the amount was 18.2% and 46.9% of external Cu respectively. The amount of both exchangeable and residue Cu in neutral soil was between that of the two soils above. The changeable Cu had greater bioactivity, while the residue had smaller activity. Therefore, Cu inhibited the enzyme greatly in acid soil, but in lime soil, the damage action was considerable lighter than that of the three soils (Tu, 1993).

Table 1 shows the physical and chemical properties of the soil samples tested. Of the three soils, Soil 1, with minimum organic content (only 6.4 g/kg) and lowest pH, was damaged badly by heavy metal. Soil 3, with higher organic content and pH, could protect the enzymes to some extent. From the relation between the enzyme activity and the change of contact time, it was found that the activity of soil ACPase

tended to increase with extension of the culture time, except Soil 3. This result is in according with He *et al*.(2001) who found analogous behaviour of soil urase and invertase. This may be attributed to two factors: (1) The proliferation of soil microorganism induced increase of soil enzyme; (2) The change in the form and quantity of heavy metals reduced the toxicity to soil enzyme (Banerjee *et al*., 1997). The effect of heavy metal on the ecosphere and environment are based on its effective proportion, but intermixing with soil components could decrease the effect of heavy metal. Zhu *et al*.(1996) considered that after $HgCl_2$ enters the soil, active Hg, acidic Hg and alkalinic Hg tended to transform to residue Hg as the contact time extended. It is suggested that active Hg is the most unstable form and would change into a stable form easily. Though acidic Hg and alkalinic Hg are stable for a short time, both of them would change into residue Hg as time passed. In a word, the form distribution of Hg was mostly influenced by time (Zhu *et al*., 1996). In various form of heavy metal, the biological availability in residue form was lowest, thus the effect on the activity of soil enzyme was lowest, as the culture time extended, the inhibition of Cu^{2+} on soil ACPase was mitigated to a certain extent.

Linear fitting was conducted for the concentration of heavy metal and the activities of soil ACPase. As shown in Table 2, enzyme activities and the concentration of heavy metal Hg are significantly correlated ($P<0.05$) in Soil 1 with 60 d culture time and Soil 2 with 40 d culture time, whereas in seven other soil samples, are very significantly correlated ($P<0.01$).

Table 1 Physical and chemical properties of the soil samples tested

Soil No.	pH	*OM* (g/kg)	*TN* (g/kg)	*TP* (g/kg)	Cation exchange capacity (cmol/kg)	Particle size distributions (%)			Cu (mg/kg)	Hg (mg/kg)
						Clay	Silt	Sand		
1	4.14	6.4	0.53	0.56	15.7	49.3	31.0	15.6	30.33	0.052
2	5.91	18.0	1.15	0.83	21.0	23.7	34.6	35.8	22.45	0.069
3	6.40	28.0	1.72	1.16	16.5	14.0	68.2	13.4	18.72	0.065

Table 2 Relationship between activities of soil ACPase and concentrations of Hg and Cu

Heavy metal	Soil samples	Culture time (d)	Matching equation	Correlation coefficient
Hg	1	20	$U=0.3023-0.01003\times C_{Hg^{2+}}$	−0.888[**]
		40	$U=0.3384-0.0113\times C_{Hg^{2+}}$	−0.918[**]
		60	$U=0.3580-0.01116\times C_{Hg^{2+}}$	−0.857[*]
	2	20	$U=0.4552-0.00734\times C_{Hg^{2+}}$	−0.929[**]
		40	$U=0.4615-0.00721\times C_{Hg^{2+}}$	−0.927[**]
		60	$U=0.4731-0.00708\times C_{Hg^{2+}}$	−0.940[**]
	3	20	$U=0.5626-0.00355\times C_{Hg^{2+}}$	−0.928[**]
		40	$U=0.5420-0.00294\times C_{Hg^{2+}}$	−0.853[*]
		60	$U=0.5615-0.00372\times C_{Hg^{2+}}$	−0.940[**]
Cu	1	20	$U=0.2897-0.0003340\times C_{Cu^{2+}}$	−0.889[**]
		40	$U=0.3150-0.0003564\times C_{Cu^{2+}}$	−0.912[**]
		60	$U=0.3743-0.0004142\times C_{Cu^{2+}}$	−0.947[**]
	2	20	$U=0.4225-0.0004052\times C_{Cu^{2+}}$	−0.934[**]
		40	$U=0.4255-0.0003961\times C_{Cu^{2+}}$	−0.918[**]
		60	$U=0.4408-0.0004112\times C_{Cu^{2+}}$	−0.929[**]
	3	20	$U=0.5271-0.0004383\times C_{Cu^{2+}}$	−0.952[**]
		40	$U=0.5387-0.0003335\times C_{Cu^{2+}}$	−0.988[**]
		60	$U=0.5607-0.0003261\times C_{Cu^{2+}}$	−0.995[**]

[**] $P\leq0.01$; [*] $P\leq0.05$; $C_{Cu^{2+}}$, $C_{Hg^{2+}}$ is Hg^{2+} and Cu^{2+} concentration (mg/kg) respectively; U is the activity of soil acid phosphatase

Enzyme activities of the three soils and the concentration of heavy metal Cu are very significantly correlated ($P<0.01$). The above results suggested that the activity changes of soil ACPase could indicate the pollution level of heavy metal Hg and Cu in soil.

Effect of Hg and Cu on soil ACPase kinetic property

Table 3 shows the effects of Hg and Cu on kinetic parameters of soil ACPase in the three studied soils. V_{max} decreased with increasing Hg concentration, especially in Soil 1. In the three soils the effect of Hg and Cu on ACPase was more complex. The K_m values of Soil 1 changed with the varied Hg concentration varied; for Soil 2, the K_m values decreased by increasing Hg concentration, but K_m values increased when Hg concentration reached 5 mg/kg. The K_m values of Soil 3 increased when the Hg concentration was 0~10 mg/kg, but over 10 mg/kg, K_m began to decrease. Affected by Cu, the K_m values of Soil 1 and Soil 2 changed unregularly with the Cu concentration; for Soil 3, the K_m values decreased with increasing the Cu concentration, but when the Cu concentration reached 500 mg/kg, the K_m values began to increase.

From a statistical viewpoint, heavy metals had small effect on enzyme K_m. When [Hg^{2+}] increased, the K_m changes of Soil 1, Soil 2, Soil 3 were (0.627±0.0957), (0.707±0.0346), (0.799±0.0206) mmol/(dm³·g) respectively. For Cu^{2+}, the value was (0.719±0.139), (0.727±0.0373), (0.737±0.0432) mmol/(dm³·g) respectively. It indicated that Hg and Cu had stronger effect on the relationship between ACPase and substrate of Soil 1, compared with Soil 2 and Soil 3, in which the effect was weaker. From the changes of V_{max} and K_m values affected by Hg and Cu, we concluded that inhibition of Cu for soil ACPase was similar to that of Hg, it may be a type of compound of non-competitive and anti-competitive inhibition.

Table 4 shows the linear fitting results of the relationship between dynamic parameters of soil ACPase and the concentration of Hg and Cu. For Soil 1, the concentration of Hg and Cu was not significantly correlated with the V_{max} values. For Soils 2 and 3, the V_{max} values were significantly or very significantly correlated with concentration of Hg and Cu, which suggested that the V_{max} values of soil ACPase could express the soil pollution degree by heavy metal Hg and Cu as an auxiliary method.

Table 3 Soil acid phosphatase kinetics affected by Hg and Cu pollution

Soil samples	Hg²⁺				Cu²⁺			
	C (mg/kg)	K_m [mmol/(L·g)]	V_{max} [mmol/(L·g·h)]	Correlation coefficient	C (mg/kg)	K_m [mmol/(L·g)]	V_{max} [mmol/(L·g·h)]	Correlation coefficient
1	0	0.614	0.00620	−0.660[**]	0	0.614	0.00620	−0.660[**]
	0.5	0.570	0.00542	−0.706[**]	10.0	0.682	0.00646	−0.609[**]
	1.0	0.544	0.00484	−0.737[**]	20.0	0.649	0.00571	−0.694[**]
	2.0	0.566	0.00453	−0.688[**]	50.0	0.695	0.00514	−0.637[**]
	5.0	0.577	0.00430	−0.629[**]	100.0	0.609	0.00401	−0.659[**]
	10.0	0.801	0.00441	−0.597[**]	200.0	0.728	0.00369	−0.531[**]
	20.0	0.717	0.00380	−0.635[**]	500.0	1.013	0.00378	−0.514[**]
2	0	0.714	0.00792	−0.622[**]	0	0.714	0.00792	−0.622[**]
	0.5	0.699	0.00748	−0.667[**]	10.0	0.689	0.00744	−0.636[**]
	1.0	0.679	0.00693	−0.691[**]	20.0	0.682	0.00689	−0.654[**]
	2.0	0.661	0.00641	−0.741[**]	50.0	0.722	0.00638	−0.670[**]
	5.0	0.707	0.00608	−0.740[**]	100.0	0.781	0.00581	−0.654[**]
	10.0	0.716	0.00566	−0.749[**]	200.0	0.732	0.00480	−0.667[**]
	20.0	0.771	0.00555	−0.751[**]	500.0	0.769	0.00429	−0.592[**]
3	0	0.770	0.00932	−0.692[**]	0	0.770	0.00932	−0.692[**]
	0.5	0.774	0.00906	−0.755[**]	10.0	0.730	0.00920	−0.799[**]
	1.0	0.809	0.00906	−0.769[**]	20.0	0.724	0.00869	−0.718[**]
	2.0	0.815	0.00888	−0.790[**]	50.0	0.715	0.00786	−0.728[**]
	5.0	0.821	0.00862	−0.806[**]	100.0	0.701	0.00694	−0.773[**]
	10.0	0.792	0.00816	−0.834[**]	200.0	0.702	0.00624	−0.785[**]
	20.0	0.813	0.00797	−0.811[**]	500.0	0.820	0.00605	−0.742[**]

[**] $P \leq 0.01$

Table 4 Relationship between dynamic parameters of soil ACPase and concentration of Hg and Cu

Heavy metal	Soil samples	Dynamics parameter	Matching equation	Correlation coefficient
Hg	1	V_{max}	$V_{max}=0.00524-8.1758\times10^{-5}\times C_{Hg^{2+}}$	-0.746
	2	V_{max}	$V_{max}=0.00712-9.9450\times10^{-5}\times C_{Hg^{2+}}$	-0.801^*
	3	V_{max}	$V_{max}=0.00908-6.4160\times10^{-5}\times C_{Hg^{2+}}$	-0.934^{**}
Cu	1	V_{max}	$V_{max}=0.00561-4.8549\times10^{-6}\times C_{Cu^{2+}}$	-0.740
	2	V_{max}	$V_{max}=0.00704-6.5246\times10^{-6}\times C_{Cu^{2+}}$	-0.872^*
	3	V_{max}	$V_{max}=0.00854-6.2405\times10^{-6}\times C_{Cu^{2+}}$	-0.815^*

** $P\leq0.01$; * $P\leq0.05$

CONCLUSION

Both Hg and Cu have remarkably negative effects on soil ACPase activity and the enzyme reaction kinetic properties. At the same concentration, the effect of Hg on soil ACPase activities was stronger than that of Cu. The enzyme activities of soil affected by Hg and Cu are significantly or very significantly correlated with Hg and Cu concentrations. Except Soil 1, the V_{max} values of Soils 2 and 3 were significantly correlated with heavy metal; it was suggested that the changes of soil ACPase activities and the V_{max} values could indicate the level of heavy metals pollution in the soil. The inhibition effect of Cu on soil ACPase was similar to that of Hg, it may be a type of non-competitive and anti-competitive inhibition compound.

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