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Properties of mercuric reductase from a $HgCl_2$-resistant fungus

Wang Baojun, Li Wenzhong, Yang Huifang

Institute of Microbiology, Chinese Academy of Sciences, Beijing 100080, China

Abstract—Mercuric reductase is detected in *Cephalosporium tabacinum* F2. The enzyme, which is an intracellular one, catalyzes the reduction of mercuric ions to elemental mercury, which requires NADH as an electron donor and added sulfhydryl compound. The optimum temperature and pH of the enzymatic reaction are 30℃ and 7.0—8.0, respectively. The enzyme activity is stable in the range of 25—30℃ for 40min, and stable at pH 7.0 for 2 hours. Metal ions such as Ag^+, Co^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, Ni^{2+} show different degrees of inhibitory effect on the enzyme activity, other compounds such as phenylmercury acetate and potassium ferricyanide also partially inhibit the enzyme activity.

Keywords: *Cephalosporium tabacinum* F2; mercuric reductase; resistant fungus.

1 Introduction

Microbial transformation play an important roles in geochemical cycling of mercury in biosphere. Many bacteria such as *Escherichia coli*, *Pseudomonas*, *Bacillus*, *Thiobacillus*, *Mycobacterium* and so on are found resistant to inorganic and/or organic mercury compounds. The results indicate that the resistance to mercury compounds was due to two different mercury detoxifying enzymes. Mercuric reductase is a key enzyme in detoxification of mercury compounds, the enzyme catalyzes reduction of Hg^{2+} to elemental mercury(Hg^0), which is less toxic than Hg^{2+} and then volatilizes from growth medium (Furukawa, 1972; Izaki, 1974; 1981; Schottel, 1978). Although resistance and detoxification of mercury compounds in some fungi has been reported (Ross, 1975; Gadd, 1986), study on the mercuric reductase in these organisms has not been reported. In recent years. Toshiyuki *et al.* (Toshiyuki, 1990) find that the enzyme has been in a fungal strain, *Penicillium* sp MR -2, which has been isolated from the soil contaminated by organomercury compound and has been able to decompose the compounds. We also isolated a $HgCl_2$-resistant fungus, *Cephalosporium tabacinum* F2 (Wang, 1988), conducted a preliminary investigation on the detoxification of $HgCl_2$ in the strain (Wang, 1992) and demonstrated that the strain has been able to reduce $HgCl_2$ to elemental mercury (Hg^0). This paper describes the detection and properties of the mercuric reductase in the strain.

2 Materials and methods

2.1 Organism

Cephalosporium tabacinum F2, a $HgCl_2$-resistant fungus, is isolated from soil contaminated

by $HgCl_2$.

2.2 Composition of medium for culturing strain F2.

Sucrose 5g, Peptone 10g, NaCl 5g, Potato extract liquid 1000 ml and pH 7.0.

2.3 Cultivation of the strain and preparation of intact cells

The organism is grown aerobically at 30℃ with shaking in the medium with or without $HgCl_2$. After 16—20 hours, cells are harvested by centrifugation (at 5000×g for 30 min), then washed twice with 0.067 mol/L phosphate buffer (pH 7.0) and suspended in the same buffer, to make intact cells suspension and stored at 0—4℃.

2.4 Preparation of cell-free extract

Cells suspended in the above-mentioned buffer containing 2 mmol/L 2-mercaptoethanol are discrupted by a sonic oscillator (Labsonic, 2000. B. Baum, USA) at 19 kHz, 200W, in iced bath for 12 min, cell debris is sedimented by centrifugation at 18000×g for 20 min and the supernatant is diluted with same buffer and used as crude extract (protein of 6 mg/ml).

2.5 Assay of volatilization of ^{203}Hg from $^{203}HgCl_2$ by intact cells

Cells (dry weight 20mg) are incubated at 30℃ with 5 μmol/L $^{203}HgCl_2$, 0.067mol/L phosphate buffer (7.0), 2mmol/L 2-mercaptoethanol in a total volume of 10 ml. Then 0.1ml samples of reaction mixture are withdraw at various time and added into 10ml of toluene-triton×100 counting fluid in standard vial. The radioactivity is measured. At the same time, 0.1 ml of samples are withdrawn, filtrated by a membrane filter (0.45 μm pore size) and washed with the same buffer. Radioactivity of the cells is measured by the above method. Volatilization of ^{203}Hg is estimated from the disappearance of the radioactivity from the reaction mixture.

2.6 Assay of volatilization of ^{203}Hg from $^{203}HgCl_2$ by the crude extract

The crude extract (6mg protein) is incubated at 30℃ with 10 μmol/L $^{203}HgCl_2$, 0.067mol/L phosphate buffer (pH 7.0), 200 μmol/L NADH, 2mmol/L 2-mercaptoethanol in a total volume of 10ml. Radioactivity in the reaction mixture is measured at various time by using the above-mentioned method. Volatilization of ^{203}Hg is estimated from the disappearance of the radioactivity from the reaction mixture.

2.7 Spectrophotometric assay of mercuric reductase activity

The reaction mixture consists of a crude extract (3mg protein), 0.067mol/L phosphate buffer (pH 7.0), 100 μmol/L $HgCl_2$, 200μmol/L NADH or NADPH, and 2mmol/L 2-mercaptoethanol in a total volume of 3 ml. The reaction is started by adding the crude enzyme extract to the rest of reaction mixture which has been preincubated for 5 min at 30℃. $HgCl_2$-dependent oxidation of NADH or NADPH is measured by following the decrease in absorbance at 340 nm using a spectrophotometer (DU-7, Beckman, USA). One unit of mercuric reductase activity is defined as the amount of enzyme causing an initial decrease of 0.01/min in the absorbance at 340 nm at 30℃.

2.8 Determination of protein

Protein is determined by method of Bradford (Bradford, 1976).

2.9 Analysis of Hg^{2+}

Mercuric ion concentration is routinely monitored spectrophotometrically by the dithizone as-

say as described previously (Johnson, 1965).

2.10 Chemicals

$^{203}HgCl_2$ is a product of Radiochemical Centre, Amersham, England, with a specific activity of 205 mCi/mmol (1 Ci = 37 GBq). NADH and NADPH are the products of Sigma Chemical Company.

3 Results

3.1 Removal of $HgCl_2$ by the intact cells

Cells (dry weight 40mg) are incubated at 30℃ with shaking, with 0.067mol/L phosphate buffer (pH 7.0), 30 mg/L $HgCl_2$, 2mmol/L 2-mercaproethanol in a total volume of 20 ml. After incubation at various time, samples are withdrawn, centrifugated at 18000×g for 20 min, mercury content of the supernatant is measured. As shown in Fig. 1, after reaction for 40 min, the removal rate of Hg^{2+} by the intact cells that have grown in the presence of $HgCl_2$ are higher than that in the absence of $HgCl_2$. It is obvious that the removal ability for mercury by the intact cells is facilitated through adaptive growth in the medium containing $HgCl_2$.

3.2 Volatilization of ^{203}Hg from $^{203}HgCl_2$ by the intact cells

The results indicated that the radioactivity in the reaction mixture decreased gradually during the reaction; after 80 min, about 26% $^{203}HgCl_2$ in the reaction mixture remained. Simultaneously, the radioactivity in the cells is only 18%, while that in the reaction mixture without cells does not change nearly as much (Fig. 2). We presumed from the results that the loss of radioactivity is due to volatilization of ^{203}Hg in the presence of the cells. It is thought that the mercury volatilized is elemental mercury (Summers, 1972). It is , therefore, possible that mercuric reductase activity has been present in the cells of stain F2.

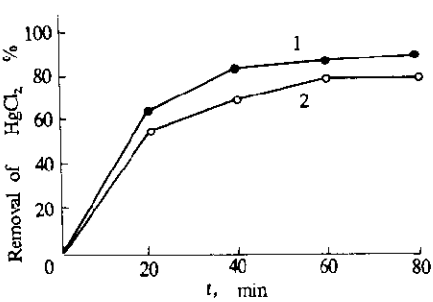

Fig. 1 Removal of $HgCl_2$ by the intact cells
1. Cells from the cultivation with mercury;
2. Cells from the cultivation without mercury

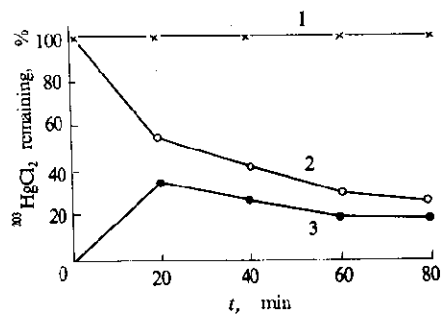

Fig. 2 Volatilization of ^{203}Hg from $^{203}HgCl_2$ by the intact cells
1. control (no cell);
2. radioactive mercury in the reaction mixture with cells;
3. radioactive mercury in the cells

3.3 Detection of mercuric reductase activity

As shown in Fig. 3, after reaction, NADH is rapidly oxidized by the crude extract in the presence of Hg^{2+}, whereas, NADPH is oxidized very slowly, and oxidation of NADH in the absence of Hg^{2+} is very little. It is obvious that mercuric reductase activity is present in the crude extract; NADH acted as an electron donor in the enzymatic reaction. Then, the changes of

NADH absorbance at 340 nm in the course of reaction are measured. The results shown in Fig. 4 indicate that the absorbance of NADH at 340 nm decreases gradually with prolongation of the reaction time. This further demonstrates that the reaction is an enzymatic reaction requiring NADH.

3.4 Volatilization of ^{203}Hg from $^{203}HgCl_2$ by the crude extract

The crude extract is incubated with $^{203}HgCl_2$ in the presence of NADH and 2—mercaptoethanol. Rapid volatilization is observed in the reaction, while no volatilization of ^{203}Hg without either enzyme or NADH occurs (Fig. 5). Volatilized ^{203}Hg is presumably elemental mercury (Hg^0).

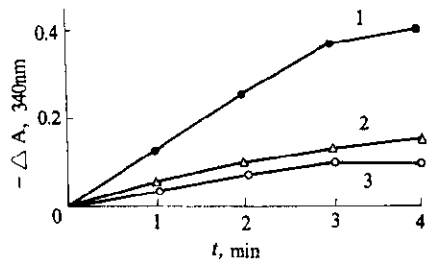

Fig. 3 Detection of mercuric reductase activity in the crude extract
1. added NADH; 2. added NADPH;
3. control (no mercury)

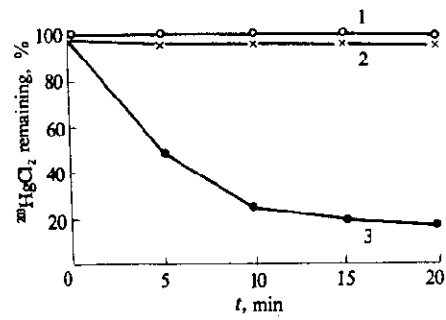

Fig. 5 Volatilization of ^{203}Hg form $^{203}HgCl_2$ by the crude extract
1. control (no crude extract); 2. control(no NADH);
3. complete reaction mixture

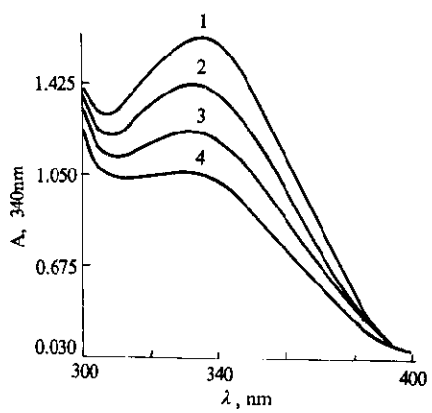

Fig. 4 The changes of absorbance of DADH at 340nm in the presence of Hg^{2+}
1. 0 min; 2. 2 min; 3. 4 min; 4. 6 min

3.5 Properties of mercuric reductase

3.5.1 Substrate specificity of the enzyme

The crude extract rapidly oxidizes NADH in the presence of $HgCl_2$ or mercuric acetate whereas no significant oxidation of NADH occurs either in the absence of $HgCl_2$ or in the presence of phenylmercury acetate as shown in Fig. 6.

Optimum pH of enzyme reaction is 7.0—8.0. Optimum temperature on enzyme reaction is 30℃.

The results indicate that the enzyme is stable after incubation at pH 7.0 for 2 hours (Fig. 7a), and also stable after treatment at 25—30℃ for 40 min, but only 40% enzyme activity remained after treatment at 45℃ for 40min (Fig. 7b).

3. 5. 2 Effects of various sulfhydryl compounds on enzyme activity

As shown in Table 1, sulfhydryl compounds tested are all effective in producing enzyme activity although the degree of effectiveness varied. Then, the effects of various concentration of 2-mercaptoethanol on enzyme activity are tested. Optimum concentration is 1 mmol/L. As for the action of sulfhydryl compound in the enzyme reaction, Izaki (Izaki, 1981) suggested that it was not only necessary for the formation of mercaptide complex with Hg^{2+}, but also for the activation of SH group in the enzyme.

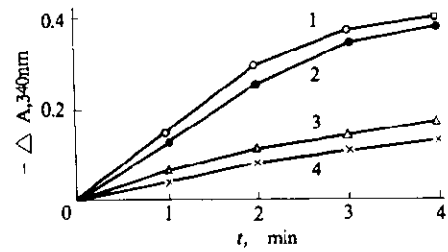

Fig. 6 Substrate specificity of mercuric reductase
1. complete reaction mixture containing $HgCl_2$; 2. complete reaction mixture containing mercuric acetate; 3. complete reaction mixture containing phenylmercury acetate; 4. control (no mercury).

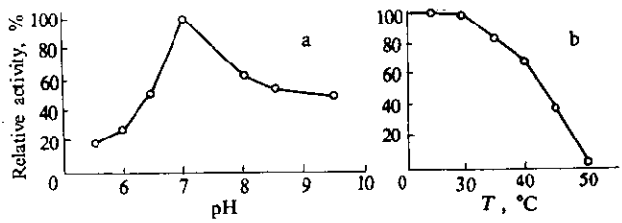

Fig. 7 Effects of pH(a) and temperature (b) on enzyme stability

Table 1 Effects of various sulfhydryl compounds on enzyme activity

Sulfhydryl compounds	Relative activity, %
2-mercaptoethanol	100
L-cysteine	91.64
Thioglycollic acid	84.8
Dithiothreitol	74.0
None	62.8

3. 5. 3 Effects of various metal ions on enzyme activity

The effects of various metal ions on enzyme activity are examined. As shown in Table 2, metal ions such as Ag^+, Mn^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+}, Ni^{2+} in the range of 200—1000 μmol/L are different degree of inhibitory effects on enzyme activity.

3. 5. 4 Effects of some inhibitors on enzyme activity

Effects of some inhibitors on enzyme activity are tested at a concentration of 1000 μmol/L. Phenylmercury acetate and potassium ferricyanide partially inhibite enzyme activity with inhibition 65% and 27%, respectively (Table 3).

Table 2 Effects of various metal ions on enzyme activity

Metal compounds	Concentration, μmol/L	Relative activity, %
None		100
$CaCl_2$	100	100
	1000	100
$MgCl_2$	100	100
	1000	100
$ZnCl_2$	100	100
		83
$MnCl_2$	100	100
	1000	58
$Cd(NO_3)_2$	100	100
	1000	94
$AgNO_3$	100	58
	200	43
$NiSO_4$	100	93
	1000	70
$CuSO_4$	100	97
	400	55

Table 3 Effects of some inhibitors of enzyme activity

Inhibitor	Concentration, μmol/L	Inhibition, %
None		0
As_2O_3	100	0
	1000	0
Iodoacetic acid	100	0
	1000	0
Potassium ferricyanide	100	5
	1000	27
Phenylmercury acetate	100	6
	1000	65.6

4 Discussion

Mercuric reductase is a key enzyme of mercury detoxification system in mercury-resistant bacteria. Up to now, *Penicillium* sp MR-2, only a fungus decomposing organomercury compounds, was reported to posses this mercuric reductase. In this paper, we have described the detection and properties of the mercuric reductase from strain F2. The mercuric reductase is similar to that from bacteria and *Penicillium* sp MR-2 in various properties, including substrate specificity, requirement of sulfhydryl compounds, optimum pH range, sensitivity to some heavy metal ions and so on. On the other hand, in the enzymatic reaction of Hg^{2+} to elemental mercury, mercuric reductase in strain F2 is dependent on NADH but not on NADPH as an electron donor, while most of the mercuric reductase in bacteria and *Penicillium* sp MR-2 is dependent on NADPH instead of NADH. The results show that mercuric reductase, as an important mercury detoxifying enzyme is not only present in many mercury-resistant bacteria, but also in some mercury-resistant fungi.

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