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Mercury and Organomercurial Degrading Enzymes in a Broad-Spectrum Hg-Resistant Strain of *Bacillus pasteurii*

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Hg-resistant bacteria are known to possess plasmids which code for the induced synthesis of Hg^{2+}-reductase (MR) and organomercurial lyase (OL) (Summers and Silver 1978, Silver and Misra 1988). Bacteria sensitive to Hg-compounds lack these enzymes. OL breaks up the C-Hg bonds in organomercurials liberating Hg^{2+} and the MR reduces the liberated Hg^{2+} to Hg°. Both MR and OL have been purified from plasmid bearing *E. coli* (Schottel 1978) and also from *Pseudomonas* K62 (Tezuka and Tonomura 1976). But less attention has been focussed on the mercury volatilizing enzymes from gram positive bacteria (Izaki 1981, Silver and Kinscherf 1982). Some differences in physicochemical properties in the mercury detoxifying enzymes have been reported (Silver and Kinscherf 1982). Here we report the purification and properties of both MR and OL from a broad-spectrum Hg-resistant gram positive *Bacillus pasteurii* strain DR_2.

MATERIALS AND METHODS

Broad-spectrum Hg-resistant *B. pasteurii* strain DR_2 was reported earlier to volatilize Hg-compounds (Pahan *et al.* 1990). All the chemicals and reagents used in this study were of analytical grade (E. Merck, U.K.). $HgCl_2$, phenylmercuric acetate (PMA), thiomersol, merbromine, p-hydroxymercuribenzoate (PHMB), fluorescein mercuric acetate (FMA) and NADPH (tetrasodium salt) were purchased from Sigma Chemical Co., USA. Methoxyethylmercuric chloride (MEMC) was purchased from local market as Emisan-6 containing 6% mercury as MEMC (Excel Industries Ltd., Bombay 400 102, India). Methyl mercuric chloride (MMC) was collected from Wako Chemicals, Japan.

Cell free extracts were prepared to study the effects of different inducers following the method of Summers and Silver (1972) inducing three times with graded concentrations of different Hg-compounds. Concentrations were 1 μM, 10 μM, 20 μM and 50 μM for $HgCl_2$ and 1 μM, 10 μM and 20 μM for organomercurials like PMA, thiomersol, MEMC, FMA and

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merbromine. MR activity was determined by monitoring Hg^{2+}-dependent NADPH oxidation spectrophotometrically at 340 nm. To determine the activity of OL, a suitable volume of the cell-free extract was first incubated for 5 min with PMA or thiomersol and other ingredients in the assay mixture except $HgCl_2$ and NADPH (Pahan *et al.* 1990). One unit of MR activity was defined as the amount of enzyme that oxidised 1 µmole of NADPH per min in the presence of $HgCl_2$ and one unit of OL activity was defined as the amount of enzyme that oxidised 1 nmole of NADPH per min in the presence of organomercurials (Pahan *et al.* 1990). Protein was determined by the method of Lowry *et al* (1951). For purification of MR and OL the bacterial strain was induced thrice with 10 µM PMA in nutrient broth. Cells were grown and harvested as mentioned by Summers and Silver (1972). 13 g of bacterial cells were broken mechanically in a mortar with pestle using sea-sand and suspended in 50 mM sodium phosphate buffer (pH 7.4) containing 0.25 mM GSH, 0.1 mM FAD and 0.2% NaN_3. This buffer was used throughout the purification procedure as the enzymes were reasonably stable in this buffer. Suspended cells were then centrifuged at 13,000 x g for 30 min. Supernatant obtained is then heated at 70°C for 10 min, chilled to 4°C and centrifuged at 10,000 x g for 10 min. Supernatant was then ultracentrifuged at 1,60,000 x g for 90 min. Supernatant so obtained was used as the crude cell-free extract for Hg^{2+}-reductase and OL.

The following protocol gave the best result in this system. The crude cell-free extract was then passed through a Sephadex G-75 column (1.2 cm x 68 cm). The flow rate was 9 mL/hr and 1.5 mL fractions were collected in each tube. MR was eluted immediately after void volume of the Sephadex G-75 column and OL was eluted within fraction number 29 to 37 (Fig. 1). MR was assayed as mentioned earlier (Pahan *et al.* 1990). To assay OL activity, a suitable volume of the enzyme source was first incubated for 5 min with 30 mM PMA, MEMC, MMC or thimersol and other ingredients of the assay mixture except for $HgCl_2$ and NADPH. Then 25 µL of semipurified Hg^{2+}-reductase enzyme devoid of any OL activity isolated from narrow-spectrum Hg-resistant bacterial strain *Acinetobacter* sp AR_2 (Pahan *et al.* 1990) was added and the oxidation reaction was started by adding 0.15 mM NADPH.

Fractions containing MR activity (Fig. 1) were pooled and lyophilised and then passed through a Sephadex G-200 column (1.2 cm x 65 cm). The flow-rate was 9 mL/hr and each fraction volume was 1.5 mL.

Fractions of Sephadex G-200 column containing MR activity were pooled, lyophilised and passed through DEAE-cellulose column (1.8 cm x 15 cm). The flow-rate was 20 mL/hr and the volume of each fraction was 4 mL. The enzyme was eluted using a KCL-gradient (0-0.5 M).

Fractions of DEAE-cellulose containing MR activity were pooled, lyophilised and passed through the same Sephadex G-200 column as mentioned earlier.

Fractions containing OL activity of Sephadex G-75 column (Fig. 1) were pooled and lyophilised and then passed through a DEAE-cellulose column (1.8 cm x 10 cm). Enzyme was eluted using a salt gradient (KCL 0-0.5 M). The flow-rate was 20 mL/hr and the volume of each fraction was 4 mL.

Fractions of DEAE-cellulose column containing OL activity were pooled, lyophilised and passed into the same Sephadex G-75 column under similar condition as mentioned earlier. Polyacrylamide gel electrophoresis (PAGE) of proteins was done under nondenaturing conditions. Bromophenol blue was used as the tracking dye. The gels were stained in 0.1% Coomassie blue in methanol : water : acetic acid (50:50:10) for two hours and destained by repeated washing with a solution containing 7.5% acetic acid and 5% methanol in water. To assay enzyme activity unstained gels were sliced into 20 mm pieces and incubated at 4°C for at least 12 hr in 0.2 mL of 50 mM sodium phosphate buffer (pH 7.35). The eluted protein was then assayed. The molecular weights of the enzymes were determined by gel filtration through Sephadex G-200 (1.2 x 65 cm) column. For detecting the elution volume of molecular weight standards, each fraction was assayed for protein concentration spectrophotometrically; the elution volume of the reductase was determined by assaying each fraction for enzyme activity. Molecular weight markers were catalase (230 kD), yeast alcohol dehydrogenase (150 kD), tubulin (subunit) (55 kD) and lactic dehydrogenase (33 kD). The number of moles of FAD per mole of MR was determined fluorometrically following the method of Fader and Seigel (1973). K_m, V_{max}, optimum temperature and optimum pH of both MR and OL were also determined.

RESULTS AND DISCUSSION

Both MR and OL were inducible in this bacterial strain. Among the Hg-compounds used, PMA was the best inducer in inducing these Hg-volatilizing enzymes. FMA and merbromine also induced these enzymes at 10 to 20 µM concentration (Table 1). Therefore, in this study, during purification of mercury volatilizing enzymes PMA was used as inducer. MR was purified up to 57 fold (Table 2). Purified enzymes showed a single band on polyacrylamide gel electrophoresis (PAGE) (Fig. 2). Eluted proteins from similar unstained gels showed MR activity.

Molecular weight of this enzyme was 62 kD. It is optimum temperature and optimum pH were 50°C and 7.4 respectively. K_m and V_{max} values of MR of $HgCl_2$ were 12.5 µM and 26.3µM/min/mg protein. Fluorometric analyssis for FAD confirmed that MR contained 2.05, 1.96, 2.0 and 2.02 mole of FAD per mole

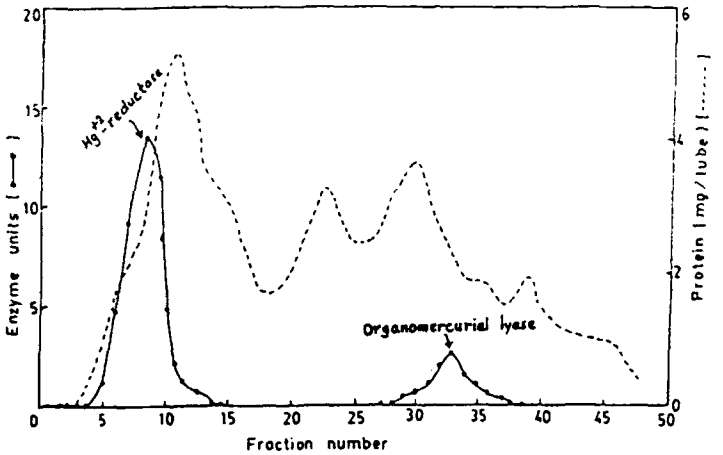

Figure 1. Gel-filtration through Sephadex G-75 column. Unit of MR is defined in the text. One unit of OL is defined here as the amount of enzyme protein that oxidised 1 µmole of NADPH per min in presence of PMA.

of the enzyme. $Zn(NO_3)_2$ and $PbCl_2$ inhibited this enzyme competitively (Fig. 3). But when NEM was used as inhibitor same K_m value but different V_{max} values were obtained (Fig. 3) indicating non-competitive inhibition.

A purification of 22 fold of OL was achieved (Table 3). Lyophilised OL after PAGE and Coomassie blue staining showed three separate bands. As the enzyme was not purified to homogeneity its molecular weight was not determined. Optimum temperature and optimum pH of this partially purified enzymes were also 50°C and 7.4 respectively. Its K_m values were 9.5 µM for PMA, 15.3 µM for thiomersol, 20 µM for MEMC and 24.4 µM for MMC. PMA was found to be the best inducer for OL also in *Bacillus pasteurii* strain DR_2. The inducer patterns of the MR of *Bacillus pasteurii* DR_2 differed significantly from those found in case of gram-negative bacteria. No OL from gram-positive bacteria has been studied biochemically (Summers 1986). $HgCl_2$ was the best inducer of MR and OL activities in a number of gram-negative bacteria (Izaki *et al.* 1974, Schottel 1978) whereas PMA was the best inducer for both MR and OL activities in *B. pasteurii* DR_2. Merbromine which was a good inducer for MR of *Pseudomonas aeruginosa* (Fox and Walsh 1982) was found to be less effective in case of *B. pasteurii* DR_2. Also the molecular weight and FAD content per mole of

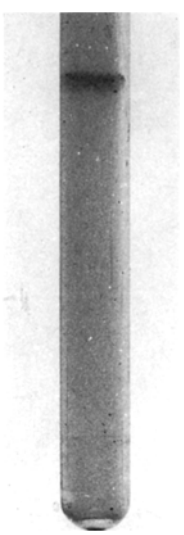

Figure 2. PAGE of purified MR. 15 µg protein was applied.

Table 1. Effect of graded concentrations of different inducers on Hg^{2+} reductase and organomercurial lyase isolated from broad-spectrum Hg-resistant *B. pasteurii* DR_2.

Name of inducers	Concentration of inducer in µM	Sp. activity of Hg^{2+}-reductase	Sp. activity of organomercurial lyase using			
			PMA as substrate	Thimersol as substrate	MEMC as substrate	MMC as substrate
No Hg-compounds	0	0	0	0	0	0
$HgCl_2$	1	0.052	12.35	10.93	8.75	8.75
	10	0.197	34.18	29.30	19.20	16.86
	20	0.198	32.74	27.83	18.32	16.00
	50	0.196	32.10	26.48	19.58	16.62
PMA	1	0.210	36.60	34.92	22.00	18.70
	10	0.222	37.50	35.09	23.10	19.65
	20	0.224	37.10	33.45	22.80	20.40
Thimersol	1	0.045	7.46	7.46	5.22	5.22
	10	0.088	19.63	17.40	12.65	10.35
	20	0.088	18.75	18.75	13.55	11.95
FMA	1	0.017	0	0	0	0
	10	0.046	14.20	12.75	10.96	10.96
	20	0.047	14.42	12.92	11.12	11.12
Merbromine	1	0.030	6.30	5.42	4.57	4.57
	10	0.086	17.39	15.04	14.23	12.65
	20	0.088	18.25	16.12	14.68	12.96
MEMC	1	0.155	20.09	17.22	13.20	13.20
	10	0.182	26.33	22.20	17.80	15.12
MMC	1	0.176	27.78	23.72	18.50	18.50
	10	0.177	30.64	24.90	19.20	19.20
	20	0.175	29.70	23.82	17.45	19.35

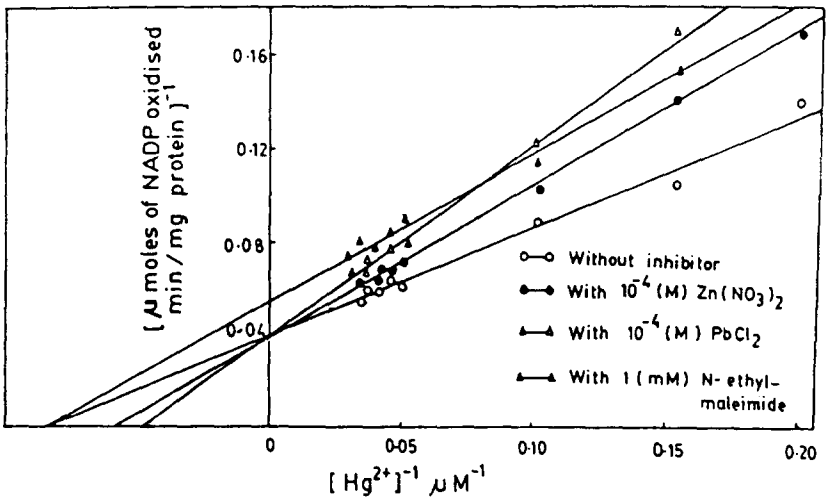

Figure 3. Double reciprocal (Lineweaver Burk) plot : Effect of $Zn(NO_3)_2$, $PbCl_2$ and N-ethylmaleimide (NEM) as inhibitors the reaction velocity of purified Hg^{2+}-reductase.

Table 2. Purification of Hg^{2+} reductase

Step	Total activity (units)	Protein (mg)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Crude extract	42	110	0.381	-	100
Sephadex G-75	34	50	0.7	1.83	80.9
Sephadex G-200	24	13	1.8	4.72	57.1
DEAE-cellulose	13	4	3.2	8.39	30.9
Sephadex G-200	8	0.4	20	52.49	19

One unit of MR activity was defined as the amount of enzyme that oxidised 1 μmole of NADPH per min in the presence of $HgCl_2$.

MR of this bacterial strain were lower than those reported for the MR from gram-negative *E. coli* (Schottel 1978).

It appears from our results that the MR and OL from a gram-positive bacterium, *Bacillus pasteurii* DR_2 differ in many respects from those of a gram-negative bacterial system.

Table 3. Purification of organomercurial lyase

Step	Total activity (units)	Protein (mg)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Crude extract	9.0	110	0.081	-	100
Sephadex G-75	5.8	14	0.414	5	64.44
DEAE-cellulose	3.2	5	0.64	8	35.55
Sephadex G-75	1.5	0.8	1.875	32	16.66

One unit of OL activity was defined as the amount of enzyme that oxidised 1 nmole of NADPH per min in the presence of organomercurials.

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