Bull. Environ. Contam. Toxicol. (1987) 39:982–989

Bulletin of Environmental Contamination and Toxicology

Some Properties of Azoreductase Produced by *Pseudomonas cepacia*

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As for the recent research on azoreductase, (i) the reduction mechanism of azoreductase by hepatic cell-free extracts or rat internal contents (Miller, 1970; Hernandez *et al.* 1967; Gillette, 1967; Roxon, 1967), (ii) the spectrophotometric determination for the enzyme by rat hepatic microsome (Mallett *et al.* 1982), and (iii) the fluorophotometric determination for rat liver cytosol using Methyl Red as a substrate (Huang *et al.* 1978) were studied. Moreover, the enzyme activity in Fusobacterium using Trypan Blue as a substrate (Hartman *et al.* 1978), and effect of flavin and inhibitors on the enzyme with *Ascaris lumbricoides* (Douch, 1975) were carried out. For electron transfer mechanisms, system of electron transfer in the presence of azoreductase was also researched by rat liver microsomes (Fujita and Peisach, 1977). On the other hand, as the research on metabolic intermediates, it was reported that DAM was demethylated, followed by hydroxylated with rat microsome (Levine and Lu, 1982). But there is no research on the purification of the enzyme and also on identification of metabolic intermediates using labelled compounds as substrates. In the previous paper (Idaka *et al.* 1987a, b), the microorganism, which degraded p-aminoazobenzene into aniline and p-phenylenediamine was isolated from wastewaters of dyestuff industries in the Gifu district, Japan, and also reduction, oxidation and acetylation of p-aminoazobenzene were reported. In this paper, we describe partial purification procedure of azoreductase, some properties of the azoreductase and identification of degradative intermediates, p-phenylenediamine and aniline, from p-aminoazobenzene using two types of labelled p-aminoazobenzenes.

MATERIALS AND METHODS

[^{14}C]-p-aminoazobenzene was prepared by coupling diazotized aniline with aniline in a basic solution. When diazotized [U-^{14}C]-aniline was used, [^{14}C]-p-aminoazobenzene labelled in the phenylazo group was produced. When [U-^{14}C]-aniline was used, [^{14}C]-p-aminoazobenzene labelled in the p-aminophenylazo group was prepared. Each [^{14}C]-p-aminoazobenzene was diluted with non-

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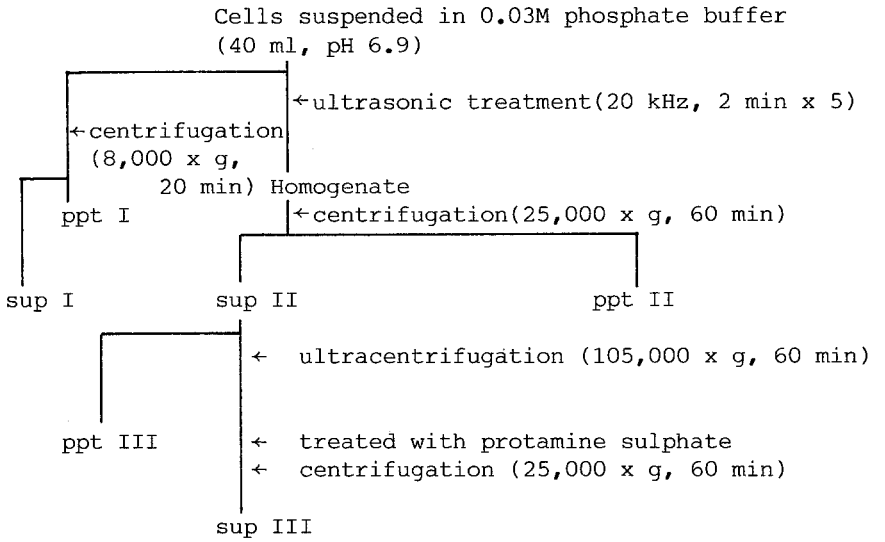


Figure 1. Procedure for the preparation of cell-free extract.

labelled p-aminoazobenzene and the specific activity was prepared to 5.0×10^5 dpm/mmol. Radioactivity was measured with a liquid scintillation counter.

The *Pseudomonas cepacia* (strain 13NA) used in this study was isolated by Idaka *et al.* (1979).

p-Aminoazobenzene reductase activity was measured as follows: The incubation mixture for measuring the loss of the dye contained p-aminoazobenzene (0.25 mmol) in 0.03M phosphate buffer (0.5 ml, pH 6.9) and enzyme preparation (3 ml), reacted at 37°C under an atmosphere of nitrogen and followed for up to 10 min by measuring the change of absorbance at 500 nm.

Pseudomonas cepacia was cultured at 37°C for 24 h in medium containing glucose 1%, polypeptone 0.5%, yeast extract 0.5%, NaCl 0.5% and K_2HPO_4 0.2%, centrifuged at 7,500 x g for 10 min and then cells were collected. The collected cells were washed 3 times in 0.03M phosphate buffer at pH 7.0, suspended and allowed to rest in 0.03M phosphate buffer for 6 h, then centrifuged again and collected.

Resting cells (0.3-2.0 g of dry cell weight) were suspended in 40 ml of 0.03M phosphate buffer in 100 ml beaker at pH 6.8. After cooling the beaker with ice, the cell suspension was treated with 150 W, 20 kHz ultrasonic waves, for 2 min intervals, repeated five times. Nucleic acid and protein were dissolved after the cells were disrupted. Cell homogenates were centrifuged at 25,000 x g for 60 min and the undisrupted cells and coarse debris were removed and then centrifuged at 105,000 x g for 60 min. The soluble fraction was treated with 5% protamine sulphate to remove nucleic acid. The procedure is shown in figure 1.

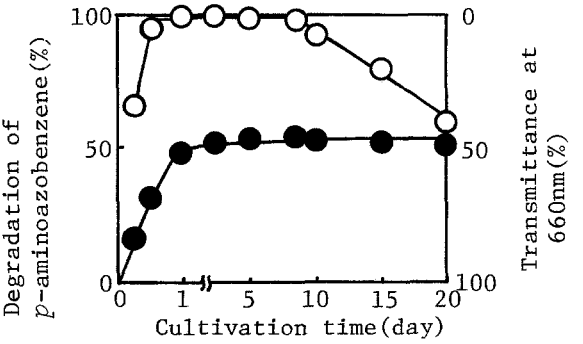


Figure 2. Relationship between the cultivation time of precultured cells and the degradation of p-aminoazobenzene. Preculture of cells was carried out with the shaking culture and the degradation experiment of p-aminoazobenzene was carried out with resting cell incubation [growth of preculture (●) and degradation of p-amionoazobenzene with resting cell incubation (○)]. Resting cell incubation was carried out under the following conditions: cell suspension (3.3 mg/ml) 2 ml + p-aminoazobenzene solution (15 ppm) 3 ml; temperature, 37°C; static culture.

RESULTS

In seed culture, the effects of azoreductase activity on the harvested phase of resting cells are shown in figure 2. The cells harvested between 12 and 200 h of cultivation maintained the highest azoreductase activity. Therefore, cells in the following experiment were harvested after 24 h of seed culture.

Among lysozyme, ultrasonic and Braun's treatments ultrasonic treatment is the most effective to solubilize azoreductase

Table 1. Distribution of azoreductase activity

Homogenate	Color loss (%)
sup I	0
sup II	86
sup III	83
ppt I	42
ppt II	48
ppt III	26
control*	0

Each precipitate (ppt I-III) was suspended in 30 ml of 0.03M phosphate buffer. Each reaction mixture was composed of sup I-III (3 ml) and 0.5 mol of p-aminoazobenzene disolved in 0.03M phosphate buffer, pH 6.9 (1 ml) and incubated with stirring at 37°C under a N_2 atmosphere for 30 min.
*Control was assayed with 0.03M phosphate buffer in place of homogenates.

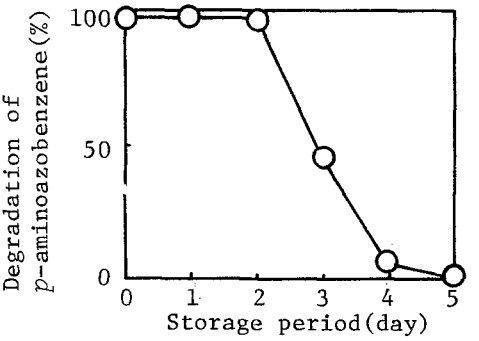


Figure 3. Stability of azoreductase

activity in cells. The procedure of the ultrasonic treatment is shown in figure 1. Distribution of azoreductase activity of each fraction (sup I-III, ppt I-III) is shown in table 1. In an assay of azoreduction, color loss (%) of p-aminoazobenzene at 500 nm was used. Azoreductase activity appears in the soluble fractions (sups II and III).

The effects of various kinds of electron-donating systems are shown in table 2. The non-enzymic reduction of p-aminoazobenzene by NADPH itself was partially observed; however, in the case of adding 0.05-0.1 mmol of NADPH, azoreductase activity was increased. Azoreductase activity was also partially observed by incubation under air.

Azoreductase activity of sup III, which was kept under a nitrogen atmosphere at 5°C, was examined every 24 h. The relation between its storage period and its activity is shown in figure 3. After 48 h azoreductase activity was lowered, so the storage period of

Table 2. Effect of cofactor system on azoreductase system

Azoreductase system	Color loss (%)
Sup III	54
+NADPH 0.01 mmol	60
0.05 mmol	95
0.1 mmol	97
+NADH 0.05 mmol	75
NADPH 0.01 mmol	0
0.05 mmol	7
0.1 mmol	9
NADH 0.05 mmol	2

Each reaction mixture was composed of sup III (3 ml), which contained about 25 mg of protein, 0.5 mol of p-aminoazobenzene dissolved in 0.03M phosphate buffer, pH 6.9 (0.5 ml) and cofactor solution (0.5 ml), and was incubated with stirring at 37°C under an atmosphere of nitrogen for 10 min.

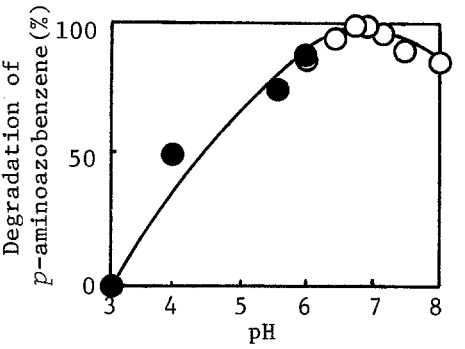


Figure 4. Effect of pH on the degradation of p-aminoazobenzene (●) phosphate-citrate buffer, (○) phosphate buffer

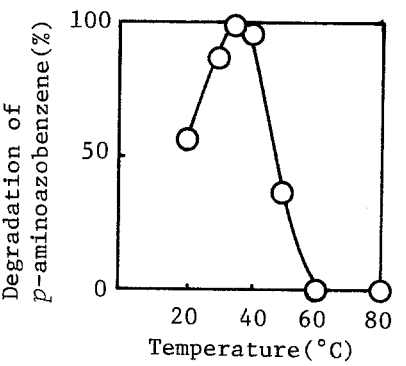


Figure 5. Effect of temperature on the degradation of p-aminoazobenzene

enzyme preparation was limited within 48 h.

Dependence of the azoreductase activity of sup III on pH is shown in figure 4. 0.03M phosphate buffer (pH 6-8) and 0.03M phosphate-citrate buffer (pH 3-6), were used respectively. As a result, azoreductase activity appeared highest in pH 6.8-7.0.

The temperature dependence of azoreductase activity of sup III is shown in figure 5. It can be seen that the optimum temperature was 37°C and that azoreductase activity decreased rapidly above 40°C.

Separation of the metabolites from p-aminoazobenzene with sup III by HPLC is shown in figure 6. The extraction method was the same as indicated in the previous paper (Idaka *et al.* 1987a, b). After 2 min, 80% of the color loss was indicated and aniline, p-

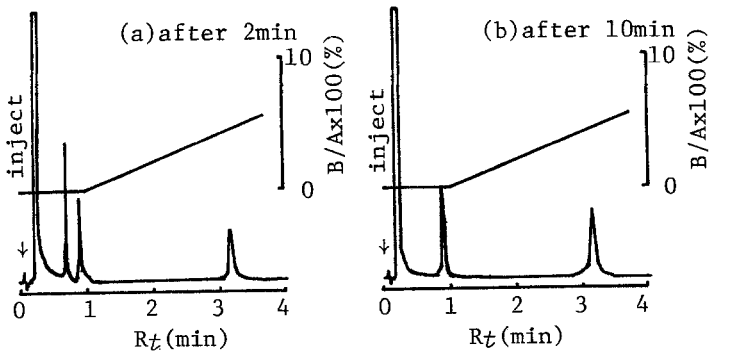
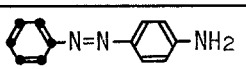
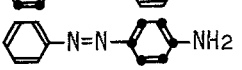


Figure 6. HPLC of degrarative compounds from p-aminoazobenzene by sup III.
Conditions: column, DuPont Permaphase ETH 500 mm x ϕ2.1 mm I. D.; elution solvent system, (A solution) n-hexane, (B solution), n-hexane/CH_2Cl_2/EtOH (1 : 1 : 1); gradient rate, 2%(B/A)/min; pressure, 30 kg/cm^2; flow rate, 2.0 ml/min; and detection, UV 254 nm.

Table 3. Metabolism of [^{14}C]-p-aminoazobenzene by sup III

Substrate	Distribution of specific activity (%) Aniline	p-Phenylenediamine
	96	0
	0	94

●:[^{14}C]-labelled position

aminoazobenzene and p-phenylenediamine were separated at R_t 0.7, 0.9 and 3.2 min (figure 6a). After 10 min, 100% of the color loss was indicated and aniline and p-phenylenediamine were only separated at R_t 0.7 and 3.2 min. The peak of p-aminoazobenzene at R_t 0.9 min disappeared (figure 6b).

Next, the experiment using [^{14}C]-p-aminoazobenzene labelled in the phenylazo group and in the p-aminophenylazo group as substrate was carried out under similar conditions. After 10 min, the mixture was extracted and aniline and p-phenylenediamine were

Table 4. Degradation of azo dye by sup III

Substrate	Color loss (%)
p-Aminoazobenzene	95
p-Acetamidoazobenzene	93
P-Hydroxyazobenzene	94
2,4-Diaminoazobenzene	96
4,4'-Diaminoazobenzene	97

collected by HPLC. The specific activities were measured by a liquid scintillation counter as shown in table 3. When [^{14}C]-p-aminoazobenzene labelled in the phenylazo group was added as a substrate, radioactivity was specifically incorporated into aniline. On other hand, when [^{14}C]-p-aminoazobenzene labelled in the p-aminophenylazo group was added as a substrate, radioactivity was specifically incorporated into p-phenylenediamine. From this fact, it was confirmed that p-aminoazobenzene was reduced quantitatively by sup III and, aniline and p-phenylenediamine were formed.

Color loss of azo dyes by sup III was measured under similar conditions and the results are shown in table 4. Within 10 min, each azo dye was reduced quantitatively by sup III.

DISCUSSION

By ultrasonic treatment azoreductase was effectively solubilized but partially inactivated by lysozyme and Braun's mechanical treatments. Solubilized enzyme preparation showed an optimum temperature of 37^{o}C and an optimum pH of 6.8-7.0. This coincided with those of azoreductase from hepatoma cells (Autrup and Warwick, 1975). Azoreductase preparation required NADPH or NADH. NADPH was more effective cofactor than NADH and others. By the NADPH enzyme system, the reduction of [^{14}C]-p-aminoazobenzene was catalyzed and the recovery of radioactivity in [^{14}C]-aniline and [^{14}C]-p-phenylenediamine was quantitative. The enzyme system quantitatively reduced p-acetamidoazobenzene, p-hydroxyazobenzene, 2,4-diaminoazobenzene and 4,4'-diaminoazobenzene.

Acknowledgments. This investigation was supported by funds from the Ministry of Education, Japan.

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Received August 4, 1987; accepted September 16, 1987.