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Enzymatic Reduction of Pesticides by Soil Microorganisms

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土壌微生物による農薬類の酵素的還元について

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Introduction

A number of pesticides having a nitro group are extensively used as insecticides or herbicides. The representatives of the pesticides are fenitrothion, CNP, and others. It has been shown that these pesticides are enzymatically reduced to the corresponding amino derivatives in rumen fluid,[1] in soils,[2] in insects,[3] and in fishes, birds, and mammals.[4] Since the resulting amino derivatives are inactive as pesticides, the enzymic reduction is regarded as a pathway of detoxication. On the other hand, since amino-CNP has been shown to form a complex with soil constituents, the enzymic reduction is regarded as a pathway of persistence in soils. Hence, it is important to clarify the functions of soil enzymes catalyzing the reduction.

The present work deals with some properties of a crude enzyme with a nitro group-reducing activity. The enzyme was produced by a microorganism isolated from the soil of paddy fields in Shimane Pref.

Materials and Methods

Isolation of Microorganisms. Soil samples collected from paddy fields in Shimane Pref. were maintained at 30° for 1 week. A weighed mass (0.5 g) of each soil sample was transfered into 10 ml of medium II[2] listed in Table 1. To the mixture, 10 μl of 1% fenitrothion solution (EtOH) and 0.2 ml of 0.25% cycloheximide solution were added. After 3 days of incubation at 30° with mechanical shaking, 1 ml of the culture was inoculated into 50 ml of medium II. Then, 50 μl of 1% fenitrothion solution and 1 ml of 0.25 % cycloheximide solution were added to the medium. The resulting solution was

Table 1. Composition of medium II.

Potassium phosphate	3.4 g
Sodium phosphate	1.0 g
Ammonium sulfate	1.0 g
Yeast extract	1.0 g
Glucose	2.0 g
Mineral stock solution*	10 ml
Distilled water	1 liter
pH	6.8

* Nitrilotriacetic acid 1.5 g, magnesium sulfate 3.0 g, manganese sulfate 0.5 g, sodium chloride 1.0 g, calcium chloride 0.1 g, zinc sulfate 0.1 g, cupric sulfate 10 mg, alminium potassium sulfate 10 mg, boric acid 10 mg and sodium molybdate (VI) 10 mg in 1 liter of distilled water.

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incubated at 30° for 3 days. Bacterial strains were isolated by the plate dilution method, using the culture medium. Some strains were found to catalyze the reduction of the nitro group of fenitrothion. The bacterial strain used in this work, designated as B-1-1, was a short rod.

Cultivation of Microorganisms. The strain B-1-1 was grown on medium II containing 2% agar. A loopful of bacterial cells was inoculated into 10 ml of medium II and was precultivated at 30° for 24 hr with mechanical shaking. The culture was transfered into 1.5 liters of medium II in a 3-l Erlenmeyer flask and was incubated at 30°. Aliquots of the medium (10 ml) were pipetted off at 1-hour intervals, and the optical density of each aliquot was determined at 660 nm to follow the growth of the microorganisms. Then, each aliquot was centrifuged at 4000 rpm for 30 min to remove bacterial cells. The pH and nitro group-reducing activity of the bacteria-free medium were measured. Precipitated cells were washed with 20 ml of 0.9% NaCl solution, were suspended in 10 ml of distilled water, and were subjected to sonic oscillation (Kaijo Denki TA 4201) at 200 mA for 10 min at 1-minute intervals. The centrifugation of the suspension at 8000 rpm for 30 min gave a supernatant, *i. e.*, cell-free extract, the enzyme activity of which was then measured.

Analytical Method. Aminofenitrothion, a reduction product of fenitrothion, in the culture medium was determined as follows. One loopful of cells of the isolated bacteria was inoculated into 50 ml of medium II containing 50 μl of 1% fenitrothion and was incubated at 30° for 1 week with shaking. The mixture was extracted twice with 20 ml of *n*-hexane. After the *n*-hexane phase was dried over sodium sulfate, it was concentrated to 0.5 ml. The reduction product was identified by tlc. Thin-layer chromatography was carried out on a silica gel G (Type 60) plate. The solvent system used was a mixture of benzene, *n*-hexane, and ethanol (20/5/1, v/v/v). The chromogenic reagents were 0.2% ninhydrin solution and 0.5% palladium chloride in 1 *N* HCl (for S-containing compounds). Authentic aminofenitrothion was prepared by the reduction of fenitrothion with zinc dust/HCl according to the method of Averell and Norris.[5]

The colorimetric determination of the reduced species was also carried out by the method of Averell and Norris.[5] Thus, to 1 ml of the reaction mixture containing amino derivatives were added 0.2 ml of 6 *N* HCl, 3.4 ml of distilled water, and 0.1 ml of 0.25% sodium nitrite. The solution was mixed well and left at room temperature for 10 min. Then, 0.1 ml of 2.5% ammonium sulfamate was added. The solution was again mixed and left at room temperature for 10 min. Finally, 0.2 ml of 1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride was added. After incubation at room temperature for 10 min and, if necessary, centrifugation at 4000 rpm for 15 min, the absorbance at 550 nm was measured. A blank and a standard were set up in the same way.

Enzyme Assay. Enzyme activity was assayed by the colorimetric determination of an amino derivative. To a 1-ml aliquot of the crude enzyme, 2 ml of a buffer and 2 ml of a substrate solution were added, and the mixture was incubated at 40° for 24 hr. At the end of the reaction, the amount of an amino derivative formed was determined according to the colorimetric method described above.

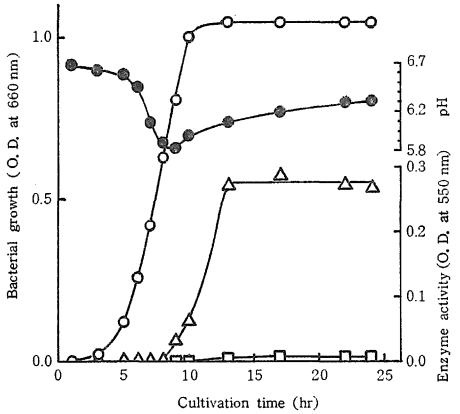

Fig. 1. Time course of bacterial growth and enzyme production.
Enzyme activity was measured by the incubation of 1 ml of a enzyme solution with 1 ml of 44.4 mM sodium *p*-nitrobenzenesulfonate at 30° for 24 hr, followed by the colorimetric analysis. Other experimental conditions are described in **Materials and Methods**. ○—○, bacterial growth ; ●—●, pH ; △—△, enzyme activity in culture filtrate ; □—□, enzyme activity in cell-free extract.

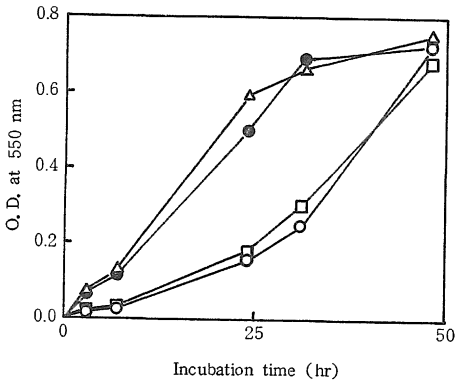

Fig. 2. Substrate specificity.
The mixture of 5 ml of a crude enzyme solution and 5 ml of a substrate solution was incubated at 30°. The amino derivative formed was measured by the colorimetric method as described in **Materials and Methods**.
○—○, parathion (49 μM) ; □—□, methyl parathion (54 μM) ; △—△, sodium *p*-nitrobenzoate (1.06 mM) ; ●—●, sodium *p*-nitrobenzenesulfonate (4.44 mM).

Results

Reduction of Fenitrothion by Soil Microorganisms.

Many strains of bacteria isolated from paddy soils by the enrichment culture technique were found to reduce fenitrothion to aminofenitrothion. The *n*-hexane-extract from the culture medium contained two species which were separated by silica gel tlc. One of them (Rf 0.33) was identified as aminofenitrothion by comparison with an authentic compound. This compound gave an intense yellow color with palladium chloride and a purple color with ninhydrin. An upper compound (Rf 0.86) was assigned to unchanged fenitrothion.

Time Course of Bacterial Growth and Enzyme Production.

A strain, B-1-1, which showed the highest fenitrothion-reducing activity was used in this work. As shown in Fig. 1, the stationary phase in growth was attained after approximately 12 hr. The pH of the medium lowered to 5.8 after 9 hr and, thereafter, rised gradually. The enzyme activity appeared at the middle period of the logarithmic growth phase, then rapidly increased, and reached maximum at 13 hr. Most of the enzyme activity (95.5%) was detected in the culture filtrate. After 24 hr of cultivation, the bacterial cells were removed by centrifugation at 8000 rpm for 60 min. In subsequent experiments, the supernatant was used as a crude enzyme solution.

Properties of the Crude Enzyme.

Substrate Specificity. The time courses for the reaction of the crude enzyme with parathion, methyl parathion, sodium *p*-nitrobenzoate, and sodium *p*-nitrobenzenesulfo-

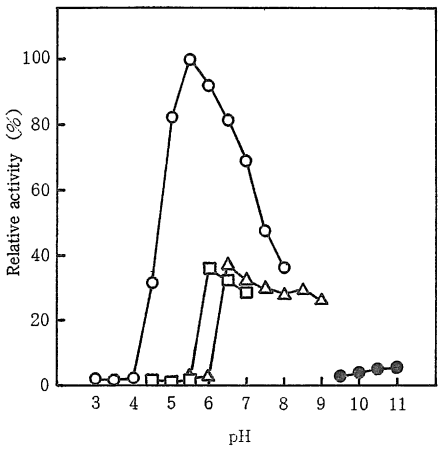

Fig. 3. Optimal pH for enzyme activity.
After the mixture of 1 ml of the crude enzyme, 2 ml of a buffer, and 2 ml of 44.4 mM sodium *p*-nitrobenzenesulfonate was incubated at 30° for 24 hr, the amino derivative formed was measured by the colorimetric method. ○—○, McIlvaine buffer ; □—□, H_3PO_4-K_2HPO_4 buffer ; △—△, KH_2PO_4-Na_2HPO_4 buffer ; ●—●, $Na_2B_4O_7$-NaOH buffer.

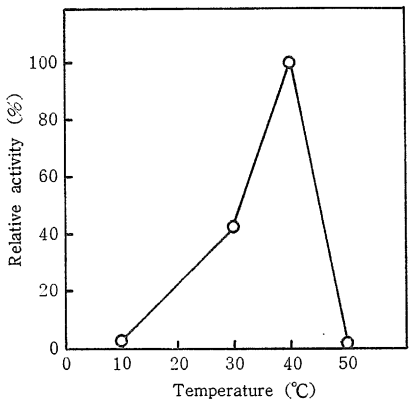

Fig. 4. Optimal temperature for enzyme activity.
The mixture of 1 ml of the crude enzyme, 2 ml of McIlvaine buffer at pH 5.5, and 2 ml of 44.4 mM sodium *p*-nitrobenzenesulfonate was incubated for 24 hr. The amino derivative formed was measured by the colorimetric method.

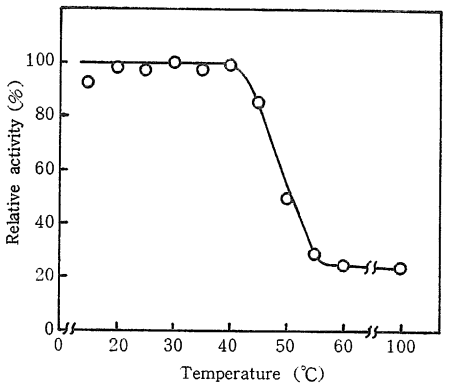

Fig. 5. Thermal stability of the enzyme.
One ml of a crude enzyme solution was incubated for 30 min. After the addition of 2 ml of McIlvaine buffer and 2 ml of 44.4 mM sodium *p*-nitrobenzenesulfonate to the solution, the mixture was incubated at 30° for 24 hr. The amino derivative formed was measured by the colorimetric method.

nate were illustrated in Fig. 2. These nitro compounds were able to serve as substrates. *p*-Nitrophenol was also reduced, although at very slow rate. *p*-Aminophenol formed was visualized by spraying a ninhydrin reagent on a tlc plate.

Effect of pH. As shown in Fig. 3, the optimal pH was attained at pH 5.5 in citrate-phosphate buffer (McIlvaine buffer). In H_3PO_4-K_2HPO_4 buffer and KH_2PO_4-Na_2HPO_4 buffer, however, the enzyme was inactive at pH 5.5. The optimal pH in H_3PO_4-K_2HPO_4 buffer and KH_2PO_4-Na_2HPO_4 buffer shifted to pH 6.0 and 6.5, respectively. Moreover, their maximal activities were only 35% of that in McIlvaine buffer. The reason for the differences in optimal pH and in activity between the buffers was not yet studied. McIlvaine buffer at pH 5.5 was used in a standard enzyme assay system.

Effect of Temperature and Thermal Stability. The optimal temperature was found to be around 40° (Fig. 4). The enzyme was stable up to 40° and 25% of the original activity was retained even after heating at 100° for 30 min (Fig. 5).

Effect of Dialysis. The crude enzyme solution was separated into an inner solution and an outer solution by dialysis against water at 5° for 24 hr. The dialysis caused

Table 2. Effect of dialysis on enzyme activity.

The crude enzyme was dialyzed against water (5°) for 24 hr. The outer solution was concentrated at 30° under reduced pressure. The amino derivative formed in each incubation system (40°, 24 hr) was measured.

p-Aminobenzenesulfonate formed (μmole/ml)			
Crude enzyme	Inner soln. (1)	Outer soln. (2)	(1) + (2)
0.143	0.003	0.020	0.087

Table 3. Effects of EDTA and metal ions on enzyme activity.

After the incubation was carried out at 40° for 24 hr, the amino derivative formed was measured.

Additive	Concn. (mM)	*p*-Aminobenzenesulfonate formed (μmole/ml)		
		Crude enzyme	Inner soln.	Outer soln.
None		0.141	0.003	0.021
EDTA	2	0.007	—	0.001
	20	0.002	—	0.001
$FeSO_4$	10	—	0.030	0.033*
$ZnSO_4$	10	—	0.001	0.001*
$CuSO_4$	10	—	0.001	0.001*
Na_2MoO_4	10	—	0.002	0.009*
$MgSO_4$	10	—	0.002	0.001*
$MnSO_4$	10	—	0.001	0.001*

* Incubation mixture contained 2 mM EDTA.

Table 4. Effect of cofactors on the activity of the dialyzed enzyme.

The preincubation of the dialyzed enzyme with each coenzyme was carried out at 40° for 24 hr in McIlvaine buffer at pH 5.5. Then, to the mixture (3 ml) was added 2 ml of 44.4 mM *p*-nitrobenzenesulfonate. After the incubation at 40° for 24 hr, the amino derivative formed was measured by the colorimetric method.

Cofactor	*p*-Aminobenzenesulfonate formed (μmole/ml)
None	0.003
NAD(0.3 mM)	0.006
NADH(0.3 mM)	0.014
NADP(0.3 mM)	0.005
NADPH(0.3 mM)	0.016
NADH(0.5 mM) + riboflavin(0.015 mM)	0.015
NADH(0.5 mM) + FMN(1 mM)	0.012
NADH(0.5 mM) + NAD(0.01 mM)	0.019
NADPH(0.5 mM) + riboflavin(0.015 mM)	0.014
NADPH(0.5 mM) + FMN(1 mM)	0.013
NADPH(0.5 mM) + FAD(0.01 mM)	0.022

almost complete inactivation. Only 14% of the initial activity was found in the outer solution. However, about 60% of the initial enzyme activity was restored by the addition of the outer solution to the inner solution (Table 2).

Effect of EDTA and Metal Ions. The effect of EDTA on the enzyme activity was investigated. As shown in Table 3, significant inhibition was noted when EDTA was added to the reaction mixture. Poor activity in the outer solution obtained by dialysis was also almost lost. The dialyzed enzyme was not reactivated by the incubation with various metal ions tested.

Effect of Coenzymes on the Activity of the Dialyzed Enzyme. Although slight reactivation was accomplished by the incubation of the dialysis-inactivated enzyme solution with several coenzymes, the initial activity was not completely restored (Table 4). Among coenzyme systems tested, NADPH-FAD system was the most effective for the reactivation of the dialyzed enzyme.

Discussion

Microorganisms responsible for the reduction of the nitro group of fenitrothion in soil was isolated from paddy soils in Shimane Pref. A major metabolite soluble in *n*-

hexane gave a single spot on a tlc plate and was identified as aminofenitrothion by cochromatography with an authentic compound.[2] The result agrees well with those in *Bacillus subtilis*,[6] soil microorganisms,[2] and bacteria in polluted water.[7] It has been already shown that the reduction occurs also in insects[3] and vertebrates,[4] although the reductive degradation pathway is minor. It was also reported that, in the degradation of diphenyl ether herbicides such as CNP and nitrofen in paddy soils, the corresponding amino derivatives are predominant products.[8]

The activity of an enzyme which catalyzes the reduction of nitro groups was detected in the bacteria-free culture filtrate after the middle period of the logarithmic growth phase, in contrast to a report that the activity was associated with the presence of living cells.[7]

The enzyme showed different pH-activity curves in different buffers used in this work. The activity in McIlvaine buffer was the highest of all buffers examined. The pH optima were also different from one another. No reasonable explanation of the phenomena has been obtained.

The enzyme was stable up to 40° and gradually lost the activity at a temperature above 40°. However, 25% of the original activity was retained even after heating at 100° for 30 min. The crude enzyme solution probably contains a heat-stable component which catalyzes the reduction. When the crude enzyme was separated into an outer solution and an inner solution by dialysis, only very weak activity was observed in the outer solution at the level of that of the heat-stable component, as shown in Table 2. It remains to be solved whether these activities arise from the same component.

Although the enzyme lost the activity by dialysis as described above, 60% of the initial activity was restored by adding the outer solution to the inner solution. These findings suggest the possibility that the component in the outer solution is a cofactor of the enzyme. Since the activity in the crude enzyme and the outer solution was inhibited by the addition of EDTA, the cofactor may include a metal ion. The effect of metal ions on the activity of the inner solution and the outer solution was investigated. However, the metal ions tested did not substantially affect the activity. Nitroreductases in rat liver[4] and houseflies[3] have been shown to require NADPH as a cofactor. Therefore, the effect of several coenzymes on the dialyzed enzyme was examined. Table 4 shows that NADPH and FAD are the most effective for the reactivation of the inactivated enzyme in the inner solution, but the activity was not restored up to the initial level.

Further enzymological studies are required to clarify the degradation pathway of pesticides and are now under way.

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Summary

Decomposition of fenitrothion by microorganisms isolated from paddy soils in Shimane Pref. was investigated. Fenitrothion was enzymatically converted to aminofenitro-

thion by the organisms. The nitro group-reducing activity was detected in the bacteria-free filtrate. The enzyme also acted upon some aromatic nitro compounds other than fenitrothion. The enzyme was the most active at pH 5.5, 40°, and was gradually inactivated at a temperature above 40° in McIlvaine buffer (pH 5.5). Twenty five percent of the initial activity was retained even after heating at 100° for 30 min. Although dialysis caused almost complete inactivation of the enzyme, poor activity was detected in the outer solution. Sixty percent of the original activity was restored by the addition of the outer solution to the inner solution. The enzyme activity was inhibited by EDTA. The effect of metal ions and coenzymes on the activity of the inner solution was also examined. Coenzymes such as NADPH and FAD slightly reactivated the inner solution but the original activity was hardly restored.

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摘　　要

Fenitrothion を aminofenitrothion に還元する細菌を水田土壌から単離し，その菌が生産する還元酵素の性質を調べた。この酵素は fenitrothion のほか，いくつかの芳香族ニトロ化合物を基質とすることができ，反応の至適 pH 及び温度はそれぞれ 5.5 (McIlvaine buffer 中)，40°付近であった．また40°までは安定であったが，それ以上の温度では徐々に不活性化された．しかし100°，30分の加熱によっても25%の活性が残った．透析によっても殆んど活性が失なわれたが，外液に弱い活性が見られた．これが前述の熱に安定な還元因子と同一であるかどうかは不明であった．透析内液に外液を加えると元の活性の60%が回復することから補因子の存在が考えられた．そこで内液に対する金属イオン及び補酵素の影響を調べた．NADPH などで若干活性が回復するものの，顕著な効果は見られなかった．