土壌中の3種のヒドロラーゼの性質とそれらの活性に及ぼす数種農薬の影響

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Original Article

Enzymological Properties of Three Soil Hydrolases and Effects of Several Pesticides on Their Activities*

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Effects of pesticides on the activities of acid phosphatase, arylesterase and aryl acylamidase in soil were examined under upland field and/or laboratory conditions. We established methods to assay the activities of arylesterase and aryl acylamidase, while a known method was applied for acid phosphatase. Fenitrothion EC, chlorothalonil WP and paraquat SL were the main pesticides used and trichlorfon was additional for laboratory tests. Effects of the pesticides on the activities of acid phosphatase and arylesterase in soil were small or moderate when they were applied at conventional and 5-fold rates. Trichlorfon and fenitrothion EC inhibited the activity of aryl acylamidase, but the effect was temporary and the activity seemed to easily recover with the degradation of the pesticides or the proliferation of microorganisms.

INTRODUCTION

Biodegradation of pesticides is one of the critical factors not only for their persistence in soil but also for the preservation of soil environment. Although microbial or enzymatic degradation of pesticides has been widely investigated,[1-4] studies on the effects of pesticides on related enzymes are not sufficient yet.[3,5,6] We examined the effects of several pesticides on the activities of hydrolases in soils. From various hydrolases originated from soil microorganisms, acid phosphatase (phosphomonoesterase), arylesterase and aryl acylamidase were chosen because they might be correlated with the first step of degradation of many pesticides. The pesticides chosen were fenitrothion EC, chlorothalonil WP and paraquat SL, and trichlorfon was additionally used in laboratory tests. Their effects on the activities of soil enzymes were assayed under upland field and/or laboratory conditions.

MATERIALS AND METHODS

1. *Pesticides and Chemicals*

Fenitrothion EC (Sumithion® emulsifiable concentrate, 50%, Hokko Chemical Industry Co., Ltd.), chlorothalonil WP (Daconil® wettable powder, 75%, Kumiai Chemical Industry Co., Ltd.) and paraquat SL (Gramoxone S® soluble concentrate, 24%, Takeda Chemical Industries, Ltd.) were obtained commercially. Trichlorfon (>99%) was extracted from Dipterex® SP-80 (soluble power, 80%, Nihon Tokushu Noyaku Seizo K. K.) with *n*-hexane–

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chloroform (8: 5) at 50°C, treated with charcoal and recrystallized by cooling at −20°C. Other chemicals were purchased from Wako Pure Chemical Industries, Ltd. and recrystallized or redistilled when necessary.

2. Soil Treatment and Sampling

Field tests: Pesticide formulations were applied to separate plots (each 1.5×2 m) in an upland field (sandy loam soil) of Shimane University at conventional and 5-fold doses on April 26 and October 8, 1986. The conventional rates: fenitrothion EC, 20 *l* of emulsion diluted 1000 times with water/a; chlorothalonil WP, 3 *l* of suspension diluted 1000 times with water/m²; paraquat SL, 20 *l* of solution diluted 500 times with water/a. As untreated check plots, a plot neither treated with pesticides nor weeded (control NW) and a plot hand-weeded but not treated with pesticides (control HW) were prepared to see rhizospherical effect. Soils were sampled from five points in each plot immediately after application and at appropriate time intervals. Soil samples of each plot were mixed thoroughly, sieved with a 2-mm screen and submitted to enzyme activity assay.

Laboratory tests: Sandy loam soil in the upland field of Shimane University was air-dried and sieved with a 2-mm screen. The soil (800 g) was spread out in a rectangular vessel (25×30 cm). Eight ml of a diluted preparation, containing a pesticide formulation or trichlorfon corresponding to the conventional or 5-fold dose, was applied separately to the vessel and mixed thoroughly with the soil. The conventional dose of trichlorfon was 0.6 *l* of solution diluted 2000 times with water/m². The same amount of distilled water was applied to the control vessel. The soils were allowed to stand at 20°C under saturated humidity in the dark. The soils were sampled for assay after mixing thoroughly, immediately after application and at appropriate time intervals thereafter.

3. Assay of the Enzyme Activities

All activities were expressed as U or mU/g of dry soil. A unit of enzyme activity was defined as the amount of enzyme releasing hydrolysis product at the rate of 1.0 μmole/min under defined reaction conditions.

Acid phosphatase: The activity was assayed with disodium *p*-nitrophenylphosphate hexahydrate (PNP) as the substrate.[7,8] One g of soil sample and 1 ml of 115 mM aqueous PNP solution were added to 4 ml of modified universal buffer[8] (MUB, pH 6.5) and incubated at 37°C for 1 hr. After incubation, 1 ml of 0.5 M $CaCl_2$ and 4 ml of 0.5 M NaOH were added and the mixture was swirled and filtered through a Toyo No. 2 filter paper. The absorbance of the filtrate at 400 nm was measured with a spectrophotometer (Shimadzu UV-120-02) and the amount of released *p*-nitrophenol (NP) was determined colorimetrically.

Arylesterase: *p*-Nitrophenyl acetate (NPA) was used as the substrate after recrystallized from CH_2Cl_2 for removing free NP. One g of soil sample was preincubated at 30°C for 10 min with shaking in 5 ml of MUB (pH 7.0) of 4-fold concentration. After 15 ml of 3.5 mM aqueous NPA solution was added, the soil suspension was incubated at 30°C for 30 min with shaking. Enzyme reaction was stopped by adding 2 ml of 1 N HCl. Five ml of the upper phase was pipetted out and washed twice by partitioning with 10 ml of *n*-hexane to remove residual NPA. One ml of 0.5 M $CaCl_2$ and 4 ml of 0.5 M NaOH were added to the aqueous layer and the mixture was swirled and filtered through a Toyo No. 2 filter paper. The absorbance of the filtrate at 400 nm was measured and the amount of released NP was determined colorimetrically.

Aryl acylamidase: Acetanilide (AAN) was used as the substrate. Four g of soil sample was added to 20 ml of 0.2 M Tris·HCl buffer (pH 8.5) containing 22.2 mM AAN and incubated at 30°C for 3 hr with shaking. Immediately after incubation, the soil suspension was centrifuged at 2°C and 12,000 rpm for 10 min, and 5 ml of the supernatant was pipetted out and acidified with 2 ml of 1 N HCl. Then 20 ml of 0.05 M $CaCl_2$ was added to the soil sediment, stirred and centrifuged. Five ml of the supernatant was pipetted out and combined with the above acidified supernatant. The amount of released aniline (AN) was determined by coupling reaction after diazotization[9] as follows: To the combined solution, *ca.* 15 ml of distilled water and 1 ml of 2%

$NaNO_2$ were added. After standing for 5 min with occasional stirring, 1 ml of 10% sulfamic acid was added and the solution was allowed to stand for 10 min with occasional stirring. After 1 ml of 1% *N*-(1-naphthyl)ethylenediamine dihydrochloride (NED) was added, the solution was allowed to stand for 2 hr and filled up to just 50 ml with distilled water. The released aniline was determined colorimetrically by measuring the absorbance at 565 nm. AN for calibration was used after redistillation.

4. Inhibition Test

Inhibitory effects of pesticides were examined *in vitro* on aryl acylamidase in soil. Four g of soil was preincubated in aqueous solution containing a pesticide of different concentrations at 30°C for 1 hr with shaking. To the soil suspension, 12 ml of 37 mM AAN was added and the activity was assayed as described above. IC_{50} values were calculated as the amount of active ingredients.

RESULTS AND DISCUSSION

1. Assay of Activities and Characteristics of Enzymes in Soil

Many soil hydrolases are intracellular *sensu stricto*, but they are also found as extracellular enzymes associated with cell debris or immobilized on clay or organic colloids.[10] In the activity measurement of soil enzymes, microbial inhibitors (usually toluene, K or Na azide, *etc.*) are used to stop an increase of activity due to the proliferation of microbial cells during incubation,[10] but they may inhibit the activities of enzymes.[11] For this reason, we did not use any microbial inhibitors in this study.

Acid phosphatase: The activity of acid phosphatase in soil was assayed satisfactorily by Tabatabai & Bremner's method[7,8,12] by using disodium *p*-nitrophenyl phosphate (PNP) as the substrate and by determining released *p*-nitrophenol (NP) after incubation at 37°C for 1 hr. The K_m and V_{max} values of the enzyme in sandy loam soil were calculated to be 4.5 mM and 70 mU/g dry soil, respectively, by Hanes-Woolf plot as shown in Fig. 1. The K_m and V_{max} reported by Eivazi & Tabatabai range 1.1 to 3.4 mM and 24 to 75 mU/g soil, respectively, in five kinds of soils.[12]

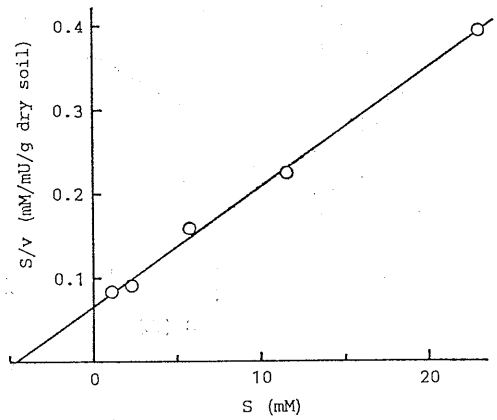

Fig. 1 Hanes-Woolf plots of acid phosphatase in a sandy loam soil.

K_m: 4.5 mM, V_{max}: 70 mU/g of dry soil.

Arylesterase: *p*-Nitrophenyl acetate (NPA) was used as the substrate. Its concentration in reaction mixture for activity assay was limited to 2.6 mM because of its low water solubility. The activity of arylesterase was assayed by determining released NP after incubation at 30°C for 30 min in the same manner as for acid phosphatase. It was necessary in this case, however, to remove residual NPA from the reaction mixture before coloring with alkaline, because ester was unstable at pH 8.0 or over. NPA was sucessfully removed by extracting twice with *n*-hexane after acidification with HCl, by which the enzyme reaction was completely stopped.

The activity of arylesterase in soil was linear to the soil amount of 0 to 1.5 g and there was no activity in sterilized soil (120°C, 50 min). The optimal pH and temperature of the enzyme reaction were about 7.0 and 50°C, respectively, but the enzyme was unstable in incubation at over 40°C for 1 hr. The K_m and V_{max} values of the enzyme were calculated to be 1.6 mM and 720 mU/g dry soil, respectively, by Hanes-Woolf plot as shown in Fig. 2.

Aryl acylamidase: Acetanilide (AAN) was used as the substrate and the activity of aryl acylamidase was assayed by determining aniline (AN) released after incubation at 30°C for 3 hr. The enzyme reaction, however, must have been stopped by centrifugation at

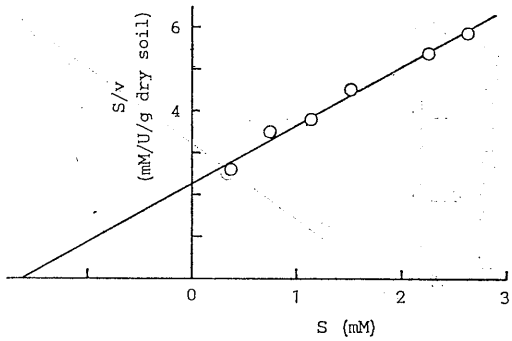

Fig. 2 Hanes-Woolf plots of arylesterase in a sandy loam soil.

K_m: 1.6 mM, V_{max}: 0.72 U/g of dry soil.

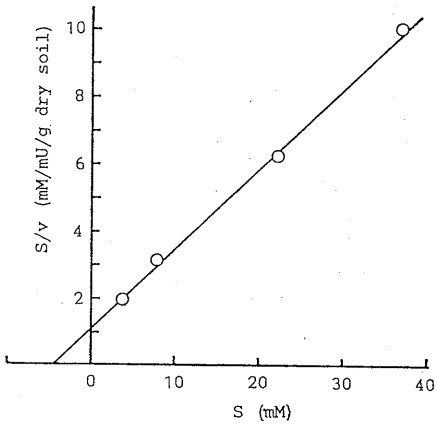

Fig. 3 Hanes-Woolf plots of aryl acylamidase in a sandy loam soil.

K_m: 4.5 mM, V_{max}: 4.2 mU/g of dry soil.

2°C immediately after incubation, because AN was easily adsorbed on soil when acidified and an appropriate stopper for the enzyme reaction was not found.

The activity of aryl acylamidase in soil was linear to the soil amount of 0 to 4.0 g and there was no activity in sterilized soil (120°C, 20 min). The optimal pH and temperature of the enzyme reaction were about 8.5 and 40°C, respectively, but the enzyme was unstable in incubation at over 30°C for 3 hr. The K_m and V_{max} values of the enzyme were calculated to be 4.5 mM and 4.2 mU/g dry soil, respectively, by Hanes-Woolf plot as shown in Fig. 3.

2. Effects of Pesticides on the Activities of Enzymes in Soil

Fenitrothion EC, chlorothalonil WP and paraquat SL were the pesticides mainly used. In addition, trichlorfon was used in laboratory tests because it was soluble in water and might be inhibitory to aryl acylamidase. The effects of pesticides on acid phosphatase, arylesterase and aryl acylamidase were evaluated under upland field and/or laboratory conditions. A plot neither treated with pesticides nor weeded (control NW) and a plot hand-weeded but untreated with pesticides (control HW) were prepared to see rhizospherical effect in upland field tests.

Acid phosphatase: Effects of fenitrothion EC, chlorothalonil WP and paraquat SL on the activity were assayed at the conventional and 5-fold rates in an upland field of Shimane University, twice in spring and autumn as shown in Figs. 4 and 5, respectively. The activity in control NW was always larger than that in control HW, showing a rhizospherical effect. The activities in treated plots were approximately in the range of those in the controls, suggesting that the effect was small or moderate.

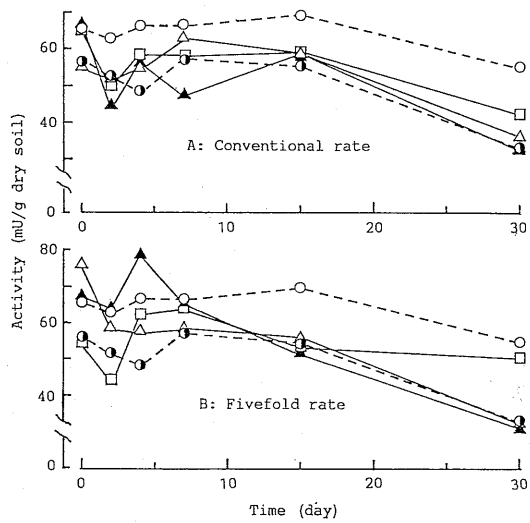

Fig. 4 Effects of pesticides on the activity of acid phosphatase in an upland field soil (spring).

○: Control NW, ◑: Control HW, △: Fenitrothion EC, ▲: Chlorothalonil WP, □: Paraquat SL.

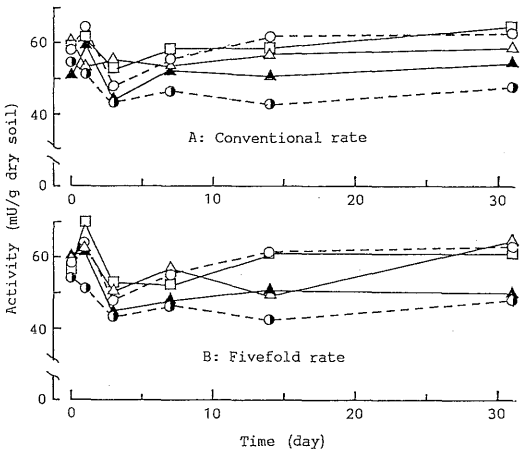

Fig. 5 Effects of pesticides on the activity of acid phosphatase in an upland field soil (autumn).
Symbols: see Fig. 4.

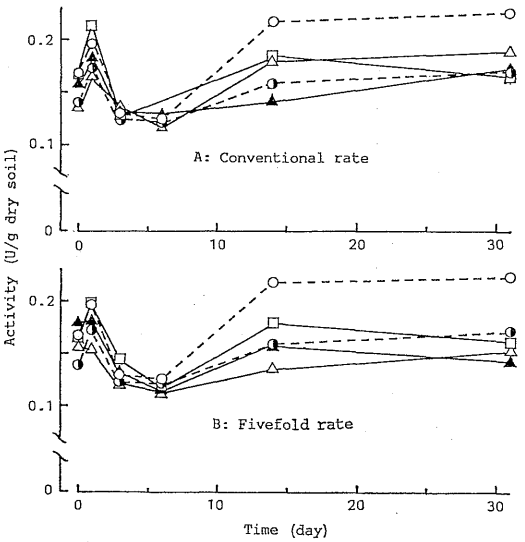

Fig. 6 Effects of pesticides on the activity of arylesterase in an upland field soil (autumn).
Symbols: see Fig. 4.

Arylesterase: Effects of the above three pesticide formulations on the activity were assayed in the upland field in autumn, as shown in Fig. 6. Similar tendencies described above were observed, although the activities in plots applied at the 5-fold rate were comparable to that in control HW. Moreover, assay was done under laboratory condition in order to find the effects of the pesticides more precisely. The laboratory assay also showed small or moderate effects of the pesticides as shown in Fig. 7. All the results suggest that the effects are small or moderate.

Aryl acylamidase: The effects were evaluated only at the conventional rate of application under laboratory condition and by an additional inhibition test *in vitro*. With aryl acylamidase, trichlorfon and fenitrothion EC affected the activity as shown in Fig. 8. The IC_{50} values were approximately 5×10^{-4} M for trichlorfon, 2×10^{-3} M for fenitrothion EC and >0.1 M for chlorothalonil WP and paraquat SL. The order of effects under laboratory condition almost agreed with that of their IC_{50} values, although the inhibitory effect of fenitrothion EC might be due to the formulation because the IC_{50} value was beyond the water solubility of fenitrothion. It has been known that some organophosphorus insecticides inhibit aryl acylamidase in animals[13,14] (*e.g.*, IC_{50} for aryl acylamidase from chicken liver: trichlorfon, 2.5×10^{-8} M; fenitrothion, 3.2×10^{-6} M) and in plants[15] (*e.g.*, IC_{50} for aryl acylamidase from rice plant: fenitrothion, 3.4×10^{-4} M). Aryl

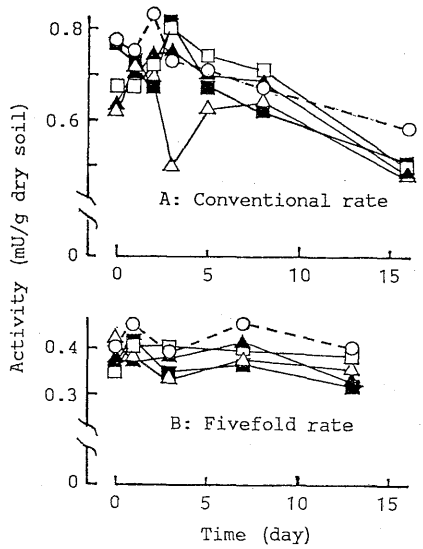

Fig. 7 Effects of pesticides on the activity of arylesterase in a sandy loam soil (laboratory condition).
○: Control, △: Fenitrothion EC, ▲: Chlorothalonil WP, □: Paraquat SL, ■: Trichlorfon.

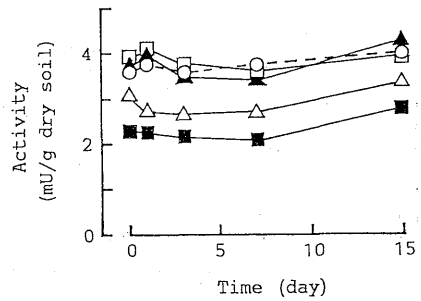

Fig. 8 Effects of pesticides on the activity of aryl acylamidase in a sandy loam soil (laboratory condition).

Symbols: see Fig. 7.

acylamidase in soil was comparatively low in sensitivity compared with aryl acylamidases in animals and plants.

Moreover, the inhibitory effect on the extracellular enzymes in soil may be temporary. It seems to be nonpersistent, because both fenitrothion and trichlorfon are unstable in soil and have little effect on soil microorganisms. The activity in soil seemed to be easily restored with the degradation of the pesticides and the proliferation of soil microorganisms.

In conclusion, the effects of the pesticides examined in this study on the activities of acid phosphatase, arylesterase and aryl acylamidase in soil were small and temporary.

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要　　約

土壌中の3種のヒドロラーゼの性質とそれらの活性に及ぼす数種農薬の影響

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酒井まどか，貢　尚吾

土壌中での化合物分解に関与するヒドロラーゼ類の中から，酸性ホスファターゼ，アリルエステラーゼおよびアリルアシルアミダーゼを選び，これらの酵素活性に及ぼすフェニトロチオン乳剤，クロロタロニル水和剤，パラコート液剤およびトリクロルホンの影響を畑地圃場または室内条件下で評価した．アリルエステラーゼとアリルアシルアミダーゼについては活性測定法を確立し，各酵素について若干の性質を明らかにした．酸性ホスファターゼおよびアリルエステラーゼ活性には，各農薬とも常用量および5倍量処理でほとんど影響を示さなかった．アリルアシルアミダーゼ活性には，フェニトロチオン乳剤およびトリクロルホンのみが若干影響し，*in vitro* での阻害性と同傾向であったが，これも一時的な酵素阻害であり，化合物分解や微生物増殖につれて容易に回復するものと思われた．