Degradation of Malathion and Phenthoate by Glutathione Reductase in Wheat Germ

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Residual malathion in wheat was estimated at a lower value when analysis was performed by extraction with acetone after addition of water to swell the wheat, according to the Japanese Bulletin Method. The supernatant of the wheat homogenate showed degradation not only of malathion but also of phenthoate. Malathion and phenthoate were not degraded by the boiled supernatant of the wheat homogenate. It was presumed for this reason that glutathione reductase (GR; EC 1.6.4.2) in the wheat degraded malathion. The following results were obtained: (1) GR originating in wheat could degrade malathion and phenthoate. (2) The degradation of malathion by the GR was inhibited by excessive GSSG. (3) There was a high correlation between GR activity and malathion degradation activity of the supernatant of wheat homogenates. It is likely that GR acted on the specific structure of malathion and phenthoate, the S═P−S bond, and the blanch structure bonding with the sulfur atom. Following the above, extraction with acetone after addition of water (the Japanese Bulletin Method) should be replaced by extraction with pure organic solvent and without addition of water for swelling.

Keywords: *Phenthoate; glutathione reductase (GR; EC 1.6.4.2); degradation; SFE*

INTRODUCTION

There are many kinds of widely used organophosphorus insecticides (Shibuya and Shimazaki, 1996; Tomlin, 1997). Many investigators have explained the effects of exposure to pesticides on humans and the effects on the ecosystem (Roberts and Hutson, 1999).

Changes in pesticide structures in the environment should also be considered (Wolfe and Seiber, 1993). For example, phenthoate is degraded to various metabolites, such as phenthoate acid, dimethyl phenthoate, and dimethyl phenthoate oxon, by exposure to air and sunlight (Takade et al., 1976).

Some investigators have shown that bacterial enzymes cause degradations of several organophosphorothioates such as malathion (Lai et al., 1998). Although many cases of enzymatic degradation of organophosphorus pesticides by mammal (Menn et al., 1996; Miyata and Matsumura, 1972) or insect (Severini et al., 1997; Campbell et al., 1997; Wood et al., 1986; Wool et al., 1982) enzymes have been studied, those by plant enzyme have not been as extensively studied (Bedi and Roy, 1979).

As stated in the Japanese Bulletin Method of pesticide residue analysis, the procedure using extraction of plant tissue by organic solvent after water had been added and the sample had been allowed to stand for 2 h was generally practiced. The explanation that the purpose of this procedure is to improve extraction efficiency through the addition of water and swelling of the cereals is accepted. However, it is possible that activation of plant enzymes may cause degradation of residual pesticides. We found that the measurable residues of malathion in wheat using the Japanese Bulletin Method were much lower than those measured by supercritical fluid extraction (SFE) (Yoshii et al., 1998). In this paper, we elucidate the cause of this phenomenon and propose an improvement to the Japanese Bulletin Method for pesticide residue analysis.

EXPERIMENTAL PROCEDURES

Materials. Inorganic reagents, organic solvents, pesticide standards, reduced glutathione, and oxidized glutathione were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Glutathione reductase from wheat germ (GR; EC 1.6.4.2) and β-nicotinamide adenine dinucleotide phosphate reduced form (NADPH) tetrasodium salt were from Sigma Chemical Co. (St. Louis, MO). Filter paper, No. 5A, was purchased from Toyo Roshi Kaisha, Ltd. (Osaka, Japan). A buffer of 0.1 M potassium dihydrogenphosphate−sodium hydroxide (pH 7.6) including 0.5 mM EDTA was prepared. Malathion and phenthoate were dissolved in water including 10% acetone (100 μg/mL).

Methods (Bedi and Roy, 1979). *(1) Apparatus and Analytical Conditions. (a) The supercritical fluid extractor* used was a JASCO Super-201 (Hachioji, Japan) operating under the following conditions; pressure, 300 kg/cm^2; extraction temperature, 40 °C; flow rate of CO_2 as extraction fluid, 4.9 mL/min; flow rate of acetone as modifier for supercritical CO_2, 0.1 mL/min; extraction time, 30 min; extraction tube volume, 10 mL; trap nozzle temperature, 75 °C; prepackaged column as trap, Extrelut 3; sample size, 5 g.

(b) GC-MS. A Shimadzu (Kyoto, Japan) QP-5050 was used to determine chlorpyrifos-methyl, malathion, and phenthoate. The GC column was a J&W DB5 (5% phenylmethylsiloxane) (30 m × 0.25 mm i.d.) with a film thickness of 0.25 μm (J&W Scientific, Folsom, CA). Detector and injector temperatures were maintained at 280 °C. The column was held isothermally

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at an initial temperature of 50 °C for 1 min and then programmed at 25 °C/min to 125 °C and finally programmed at 10 °C/min from 125 to 280 °C with a final hold time of 10.5 min.

(c) Spectrophotometer. An Hitachi (Hachioji, Japan) U-3210 was used to determine glutathione reductase activities.

(2) Sample Preparation To Analyze Residual Pesticides. (a) Extraction Method Based on the Japanese Bulletin Method (Official Gazette, 1995). Five grams of ground wheat or barley was weighed into a homogenization cup, and 10 mL of water was added. Samples were allowed to stand for 2 h, and then 100 mL of acetone was added to the cup and blended for 3 min. The homogenate was filtered through filter paper into a round-bottom flask. The cup and the filter paper were rinsed with 50 mL of acetone. Both filtrates were combined and concentrated to 30 mL using a rotary evaporator. To the concentrate was added 100 mL of saturated sodium chloride solution, and the mixture was re-extracted with 100 and 50 mL of hexane in a separatory funnel. Both hexane layers were transferred into a round-bottom flask and concentrated to 5 mL using a rotary evaporator.

(b) Extraction Method with Acetonitrile or Acetone. Five grams of ground wheat or barley was weighed into a homogenization cup, and 100 mL of acetonitrile or acetone was added and blended for 3 min. The homogenate was filtered through filter paper into a round-bottom flask. The cup and the filter paper were rinsed with 50 mL of acetone and evaporated to dryness using a rotary evaporator. The extract was dissolved in 5 mL of hexane.

(c) Extraction Method with Acetone Containing 30% Water. Five grams of ground wheat or barley was weighed into a homogenization cup, and 100 mL of acetone/water (70:30) was added and blended for 3 min. After this, homogenate was prepared according to procedure a.

(d) Extraction Method with SFE. Five grams of ground wheat or barley was weighed into an extraction tube for SFE. The sample was extracted for 20 min, and the extract was collected with an Extrelut column.

(e) Cleaning Up. Each extract or eluate was applied to an Extrelut 3 with Sep-Pak C_{18} and eluted with 20 mL of acetonitrile. The eluate was dried using a rotary evaporator, dissolved in 20 mL of acetone/hexane (1:1), eluted through Sep-Pak Florisil, and collected into a round-bottom flask. After drying using a rotary evaporator, the eluate was dissolved to 2 mL exactly with acetone, and the quantitative and qualitative GC-MS (SIM) analyses were performed.

(3) Degradation of Malathion by Supernatant of Wheat Homogenate with Water. Ten grams of wheat was weighed, and 100 mL of 0.1 M potassium dihydrogen phosphate−sodium hydroxide buffer was added and blended. After 1 h of standing, the supernatant was centrifuged at 40000*g* for 3 min. The supernatant was filtered through a 0.45 μm pore membrane filter, and this filtrate was expressed as the "wheat homogenate" here. Two milliliters of the wheat homogenate was placed in a test tube, and 50 μL of malathion or phenthoate water solution (absolute weight of pesticide was 5 μg) was added and incubated at 30 °C. An aliquot of 0.1 mL was sequentially taken, and 0.9 mL of 2-propanol was added. The mixture was centrifuged at 42000*g* for 3 min, and the supernatant was analyzed by GC-MS for malathion and phenthoate.

(4) Estimation of GR Activity for Wheat. The GR activities of wheat were estimated following the spectrophotometric method by Racker (1955). The rate of oxidation of NADPH by GSSG and GR at 30 °C was used as a standard measure of enzymatic activity. One unit of the enzyme is defined as a change in absorbance (340 nm) of 0.001 per minute under the following conditions. The reaction system of 1 mL contained such compounds as 1 mmol of GSSG and 0.1 mmol of NADPH in 0.25 mL of wheat homogenate in 0.1 M potassium dihydrogenphosphate−sodium hydroxide buffer (pH 7.6) including 0.5 mM EDTA.

(5) Degradation of Malathion by GR Originating in Wheat. The reaction system contained such compounds as 106.7 units of GR, 0.75 mmol of NADPH, and 2.5 μg of malathion in 1 mL of 0.1 M potassium dihydrogen phosphate−sodium hydroxide buffer. This system was incubated at 30 °C. Aliquots of 0.1 mL were sequentially taken, 0.9 mL of 2-propanol was added, and the mixture was centrifuged at 42000*g* for 3 min. The supernatant was analyzed by GC-MS for malathion and phenthoate.

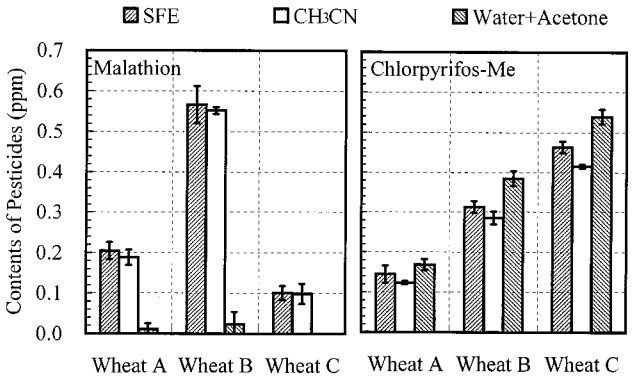

Figure 1. Comparison among values of residual pesticides obtained by three different extraction methods, with SFE, acetonitrile, or acetone after additional water for swelling. The mean ± SD from three separate experiments is presented.

RESULT AND DISCUSSION

(1) Comparison among Values of Residual Pesticides Obtained by Three Different Extraction Methods, with SFE, Acetonitrile, or Acetone. Wheats A−C included residual malathion and chlorpyrifos-methyl as postharvest pesticides. Figure 1 shows a comparison among the values of residual malathion and chlorpyrifos-methyl in wheats A−C using three different extraction methods, with SFE, acetonitrile, or acetone. Extractions with SFE or with acetonitrile were single-step extractions from wheat. In contrast, extraction with acetone was a two-step extraction; first, water was added to allow the wheat to swell, and, second, extraction with acetone, based on the Japanese Bulletin Method, was performed after 2 h. The important observation to note is that only the value of malathion using the method based on the Japanese Bulletin Method was remarkably lower, in contrast to similar values for chlorpyrifos-methyl. These pesticides might be used in postharvest application.

(2) Comparison among Values of Residual Pesticides Obtained by Three Extraction Methods, with Acetone after Additional Water for Swelling, with Acetone Only, or with a Mixture of Acetone and Water (7:3). To clarify the phenomenon regarding whether it was caused by the addition of water or acetone, the same sample of wheat B was extracted according to three different extraction methods, based on the Bulletin method or the methods using pure acetone or a mixture of acetone and water (7:3) (Figure 2). The two methods other than the method based on the Bulletin Method were performed without the addition of water and without allowing the sample to stand for 2 h but with immediate extraction. Only the value of malathion using the method based on the Bulletin Method was considerably lower than the others. These results suggested that adding water and allowing the mixture to stand for 2 h caused a lower malathion value and allowed something to decompose the malathion eluted from wheat into water.

(3) Degradation of Malathion and Phenthoate by Homogenates of Rice, Barley, and Wheat. Figure

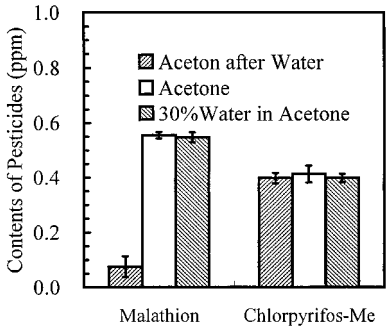

Figure 2. Comparison among values of residual pesticides obtained by three extraction methods, with acetone after additional water for swelling, with acetone, or with acetone containing 30% water in acetone. The mean ± SD from three separate experiments is presented.

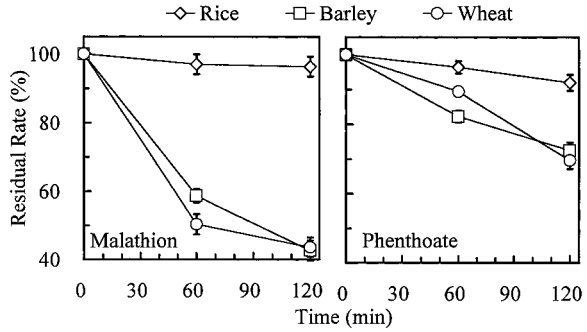

Figure 3. Degradation of malathion and phenthoate by homogenates of rice, barley, and wheat. The mean ± SD from five separate experiments is presented.

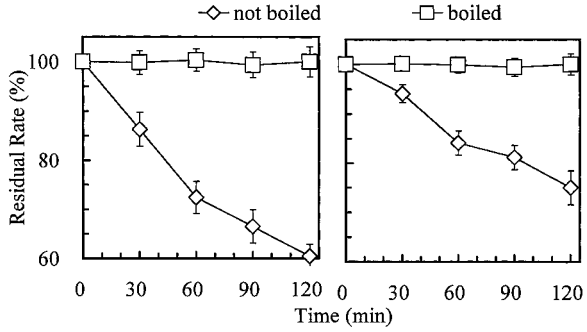

Figure 4. Effect of boiling wheat homogenate on degradation of malathion and phenthoate. The mean ± SD from five separate experiments is presented.

3 shows the sequential degradation of malathion added to wheat homogenate. At the same time, phenthoate, having a structure similar to that of malathion, and the supernatant of barley or rice homogenate were examined. Whereas the rice homogenate caused a slight degradation of malathion and phenthoate, those of wheat and barley caused extensive degradation. Phenthoate was decomposed less than malathion. It follows from the above results that the supernatants of wheat and barley homogenate do not decompose chlorpyrifos-methyl, but they decompose specifically malathion and phenthoate.

(4) Effect of Boiling Wheat Homogenate on Degradation of Malathion and Phenthoate. We assumed that wheat contains an enzyme or a water soluble agent which decomposes malathion. Figure 4 shows the sequential change in malathion and phenthoate by the supernatant of wheat homogenate boiled at 100 °C. The boiled supernatants from wheat and

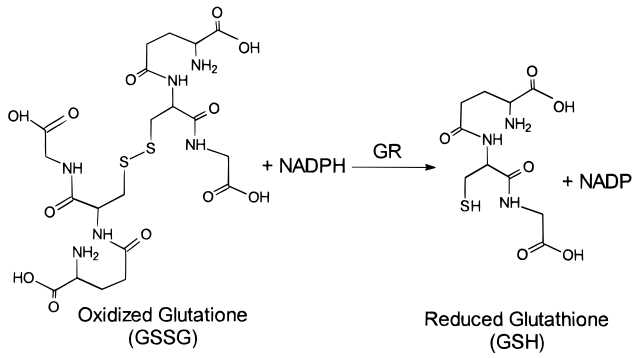

Figure 5. Reduction of GSSG to GSH by glutathione reductase.

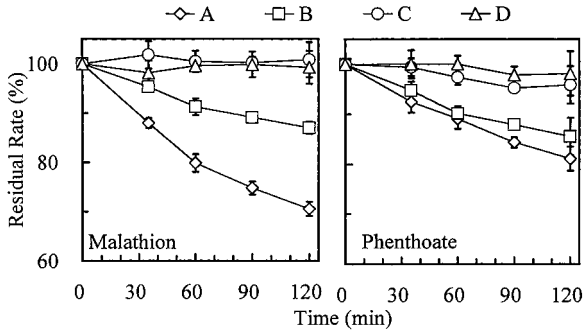

Figure 6. Degradation of malathion and phenthoate by glutathione reductase with or without NADPH: (A) presence of GR and NADPH; (B) GR alone; (C) NADPH alone; (D) absence of both GR and NADPH. The mean ± SD from five separate experiments is presented.

barley did not decompose malathion and phenthoate. Following this result, it is likely that malathion degradation by the supernatant of the wheat homogenate is an enzymatic reaction and that the contents of the enzyme in wheat and barley are much higher than in rice in Figure 3.

(5) Degradation of Malathion and Phenthoate by Glutathione Reductase. Malathion and phenthoate have two sulfur atoms and branch structure. Wheat is rich in reduced glutathione (Weber and Grosch, 1978). We noticed glutathione reductase, an enzyme, can reduce oxidized glutathione (GSSG), which has two sulfurs and a branch structure, to reduced glutathione (GSH) in the presence of NADPH (Figure 5). Figure 6 shows a sequential degradation of malathion and phenthoate by glutathione reductase and NADPH. The presence of both GR and NADPH led to the strongest degradation of malathion and phenthoate. Lack of GR led to little degradation of them, and the lack of NADPH also led to decreasing degradation.

Inhibition of malathion degradation by GSSG was observed when 8.3 μmol of GSSG was added to the reaction system including 14.2 units of GR, 0.075 mmol of NADPH, and 0.5 μg of malathion (Figure 7) in 1 mL. In this reaction system, the concentration of GR and NADPH was lower than that of an ordinary system mentioned under (3) Degradation of Malathion by Supernatant of Wheat Homogenate with Water under Experimental Procedures. In the ordinary system, GR and NADPH were too concentrated to observe the inhibition of malathion degradation by GSSG. This result shows that malathion degradation activity was competitively inhibited by excessive GSSG. In other words, this suggested the possibility of the same activity site for malathion degradation and GR activity.

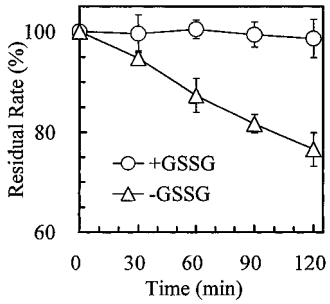

Figure 7. Inhibition of malathion degradation by oxidized glutathione. The mean ± SD from five separate experiments is presented.

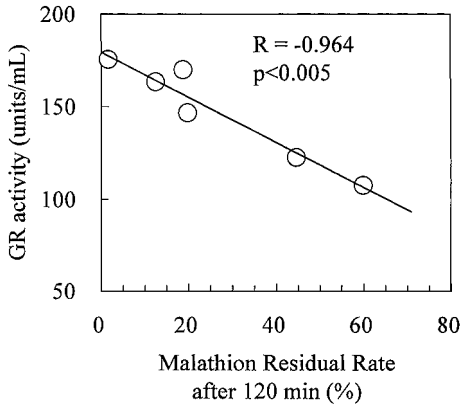

Figure 8. Relationship between malathion residual rate at incubation after 120 min and GR activity. Each point is the mean of three replicates.

Figure 8 shows a comparison between GR activity and the degradation activity of wheat homogenate. The GR activity of wheat homogenate was defined as GR units per milliliter of wheat homogenate. One GR unit of wheat homogenate is defined as a change in absorbance of 0.001 per minute. There was a high correlation between them (correlation coefficient: $R = -0.964$, $p < 0.005$). Judging from the above results concerned with GR, we can be fairly certain that malathion degradation by the supernatant of the wheat homogenate was mainly caused by GR.

Conclusion. We found that residual malathion in wheat was estimated at a lower value when the analysis was conducted by extraction with acetone after the addition of water for swelling. In contrast, a phenomenon like this was not observed for residual chlorpyrifos-methyl in wheat. It is presumed for this reason that glutathione reductase (GR) was activated by the added water. GR in wheat, which reduces oxidized glutathione to reduced glutathione, degrades not only malathion but also phenthoate. Malathion and phenthoate have a similar chemical structure, an S=P−S bond and a branch structure bonding with the sulfur atom. It is likely that GR specifically acted on such structures of malathion and phenthoate in the degradation of both pesticides. Thus, extraction with acetone after the addition of water (the Japanese Bulletin Method) should be replaced by extraction with only acetone without the addition of water for swelling.

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