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Malathion Residue in Wheat Kernels is Degraded by Thion Organophosphorus Pesticide-Specific Carboxylesterase

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We earlier reported that malathion residue in wheat kernels is enzymatically eliminated by adding water to swell the sample as pretreatment in the official method set forth in the Food Sanitation Law of Japan. We examined the activities of monooxygenase, organophosphorus hydrolase, methyltransferase, and carboxylesterase as possible malathion-degradable enzymes in wheat kernels. GC/MS analysis resulted in no activity of monooxygenase in wheat kernels, because malaoxon was not produced in the reaction mixture of the homogenate incubated with malathion for 4 hr. When five organophosphorus pesticides that have a thioether group (P-S-C) were reacted with the wheat kernel homogenate, no formation of thiol groups was detected with the 5,5′-Dithiobis(2-nitro-benzoic acid) (DTNB) reagent, indicating that there was no activity of organophosphorus hydrolase in the kernels. Pesticides that do not have a carboxylester group but do have a dimethyl thio- or dimethyl dithio-phosphate group were not decomposed by the homogenate, suggesting that there is no contribution of methyltransferase to malathion degradation. Among the organophosphorus pesticides with a carboxylester group, only the thion compounds malathion, phenthoate, and methacrifos were enzymatically decomposed by the homogenate. Malathion was also demonstrated to be the substrate of carboxylesterase, as the pesticide competitively inhibited the activity with *p*-nitrophenyl acetate as the typical substrate. Conversely, oxon organophosphorus pesticides were not degraded in the homogenate, the carboxylesterase of which was noncompetitively inhibited. These results suggest that malathion degradation in wheat kernels is caused by the thion organophosphorus pesticide-specific carboxylesterase.

Key words —— malathion residue, wheat kernel, carboxylesterase, malathion degradation, thion organophosphorus pesticide

INTRODUCTION

Since the first report on parathion was published in 1944,[1] many types of organophosphorus pesticides have been developed and widely used. Malathion is one of the most common of these pesticides, because of its selective toxicity to insects and low toxicity to mammals.[1] Malathion is also used for pest control in agriculture as a postharvest pesticide and for malaria vector control in place of the organochlorine pesticide DDT.[2] In Japan, malathion is used for various crops, and its maximum residue limits (MRLs) in the Food Sanitation Law were set for applied crops on the basis of the acceptable daily intake (ADI) value. According to the official method referred to in the Law for the Measurement of Residual Pesticides, the powdered cereals used as samples are generally allowed to swell with water for 2 hr as a pretreatment for extraction with organic solvent to improve the extraction efficiency.[3] However, we previously found that malathion residue is eliminated by this pretreatment.[4] The elimination appeared to be caused by enzymatic reaction in the kernel homogenate, because the reaction is stopped by boiling the homogenate.

Many investigators have reported the enzymatic degradation of malathion[5] by microorganisms,[6,7] insects,[8,9] mammals,[10,11] and humans.[12,13] In those reports, four major metabolites, malaoxon, diethyl

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Table 1. Structures of Organophosphorus Pesticides Examined

$(R)_2P(=X)—Z—R'$

Pesticide	X	Z	R	R′
Malathion	S	S	CH_3O	$-CH(CH_2COOC_2H_5)COOC_2H_5$
Phenthoate	S	S	CH_3O	$-CH(COOC_2H_5)C_6H_5$
Methacrifos	S	O	CH_3O	$-CH_2CH(CH_3)COOCH_3$
Crotoxyphos	O	O	CH_3O	$-C(CH_3)=CHCOOCH(CH_3)C_6H_5$
Mevinphos	O	O	CH_3O	$-C(CH_3)=CHCOOCH_3$
Malaoxon	O	S	CH_3O	$-CH(CH_2COOC_2H_5)COOC_2H_5$
Methidathion	S	S	CH_3O	$—CH_2—N—N$; O, S, OCH_3
Chlorpyrifos-methyl	S	O	CH_3O	Cl, Cl, N, Cl
Dimethoate	S	S	CH_3O	$-CH_2CONHCH_3$

thiomalate, malathion monocarboxylic acid, and desmethyl malathion were shown to be produced by monooxygenase,[2,13] organophosphorus hydrolase,[7] carboxylesterase,[6,8,10,12] and methyltransferase,[11] respectively. Mostafa *et al.* estimated that the degradation of malathion sprayed on cotton and broad beans in field experiments was mainly caused by the carboxylesterases in the crops, judging from the formation of malathion monocarboxylic acid.[14] Reports of such enzymatic degradation by plants are fewer, however, than reports of that by microorganisms, insects, and mammals. It is known that wheat has oxidase, phosphatase, glutathione S-transferase, and lipase activities,[15] and there is a possibility of corresponding to monooxygenase, organophosphate hydrolase, methyltransferase, and carboxylesterase activities as malathion-degrading enzymes, respectively.

The aim of this paper was to identify the wheat kernel enzymes contributing to malathion elimination when measuring the malathion residue in wheat kernels using the official Japanese method. We examined the activities of monooxygenase, organophosphorus hydrolase, methyltransferase, and carboxylesterase as possible enzymes that decompose malathion in the kernel homogenate. The activities of monooxygenase and organophosphorus hydrolase were detected based on the formation of the metabolites expected from malathion and related compounds. Methyltransferase and carboxylesterase were examined based on the degradability of nine organophosphorus pesticides in the kernel homogenate.

MATERIALS AND METHODS

Materials —— Pesticide standards of phenthoate, methacrifos, and methidathion, sodium dihydrogen phosphate, and disodium hydrogen phosphate were purchased from Wako Pure Chemical Industries Co., Ltd. (Osaka, Japan). 5,5′-Dithiobis(2-nitro-benzoic acid) (DTNB) was from Dojindo Laboratories (Kumamoto, Japan). Pesticide standards of malaoxon, crotoxyphos, mevinphos, malathion, chrolpyrifos methyl, and dimethoate were from Riedel-de Haën Sigma-Aldrich Laborchemikalien GmbH (Seelze, Germany). Paraoxon was from Sigma-Aldrich (St. Louis, MO, U.S.A.). Thiomalic acid and *p*-nitrophenyl acetate were from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). These organophosphorus pesticides are presented in Table 1.

Apparatus and Analytical Conditions —— Pesticides were measured with a Shimadzu GC/MS QP-5050 attached to a DB-5 GC column (5%-phenylmethylsiloxane, 30 m × 0.25 mm i.d., with a

film thickness of 0.25 mm, J&W Scientific, Folsom, CA, U.S.A.). The detector and the injector temperature were maintained at 280°C. The column was held isothermally at an initial temperature of 50°C for 1 min, then temperature-programmed at 25°C/min to 125°C and next programmed at 10°C/min from 125 to 280°C with a final hold time of 10.5 min. Injection volume was 2 μl.

A microplate reader, Tecan (Maennedorf, Switzerland) Sunrise Rainbow Thermo, was used to determine enzyme activities colorimetrically.

Enzyme Assay —— The supernatant of wheat kernel homogenate was prepared as follows: 5 g of wheat kernels was homogenized in 10 ml of sodium phosphate buffer 30 mM, pH 7.6, with an Ika Ultra Turrax homogenizer. The homogenate was centrifuged at 6000 *g* for 10 min. Protein concentration of the obtained supernatant was adjusted to 5.3 mg/ml, and it was referred to as the "wheat kernel homogenate."

Organophosphorus Hydrolase Activity[7] —— Malathion, phenthoate, dimethoate, malaoxon, and methidathion were characterized by quantifying the formation of free thiol groups during hydrolysis, utilizing DTNB reagent, in phosphate buffer 30 mM, pH 7.6, at 36°C. The free thiol groups of 5′-thiol-2-nitrobenzoate-anion produced were detected at 412 nm. The hydrolysis reaction was initiated by addition of 10 μl of organophosphorus pesticide acetone solution 30 mM to the mixture of 10 μl wheat kernel homogenate and 10 μl of DTNB 2 mM in sodium phosphate buffer 30 mM (total volume = 210 μl); the absorbance value was measured with a microplate reader after 10 min. Thiomalic acid was used as the control for detection of the thiol group.

Esterase Activity Using Typical Substrates —— Carboxyl esterase activity using *p*-nitrophenyl acetate as substrate was determined by a microplate reader in sodium phosphate buffer 30 mM, pH 7.6, at 36°C. The mixture of 10 μl of wheat kernel homogenate and 190 μl of sodium phosphate buffer 30 mM was preincubated at 36°C for 2 min and 10 μl of *p*-nitrophenyl acetate 25 mM in acetone was added to the mixture to start the reaction. The liberation of *p*-nitrophenol was monitored for 10 min at 402 nm. One unit of carboxylesterase activity (1 U_{pNP}) was defined as the activity liberating 1 nmol of *p*-nitrophenol per min.

Analysis of Malathion Residue —— Malathion residue in wheat kernel homogenate was measured using GC/MS. The mixture of 125 μl of wheat kernel homogenate and 392.5 μl of sodium phosphate buffer 30 mM, pH 7.6, was preincubated at 36°C for 2 min and 7.5 μl of malathion 3.0 mM in acetone was added to the mixture to initiate the reaction (final concentration of malathion was 43.3 μM). An aliquot of 10 μl was sequentially taken and mixed with 90 μl of acetonitrile including 4.5 μM of parathion as an internal standard. The mixture was centrifuged at 6000 *g* for 3 min, and the malathion in the supernatant was measured using GC/MS. Other organophosphorus pesticides were determined following the same procedure.

RESULTS

Possible Malathion-Degradable Enzymes in Wheat Kernels

Four types of malathion-degradable enzymes mentioned in the "Introduction" were proposed to be the wheat enzymes that decompose malathion. The enzymes examined were monooxygenase, organophosphorus hydrolase, methyltransferase, and carboxylesterase, and the cleavage patterns of enzymatic degradation of malathion were oxidation of the thion group (P = S) into oxon (P = O), hydrolysis of the P-S bond in the thioether group (P-S-C), demethylation of the dimethyl dithio- or dimethyl thio-phosphate group, and/or hydrolysis of carboxyl ester, respectively.

Monooxygenase: To detect monooxygenase activity against malathion in wheat kernels, the formation of malaoxon, which is the oxidative enzymatic product from malathion in the homogenate, was measured using GC/MS. Figure 1 shows a selected ion monitoring (SIM) chromatogram of the reaction solution of wheat kernel homogenate incubated with malathion for 4 hr. Compared with the SIM chromatogram of the reference standard solution, 70% of the initial malathion amount was eliminated, although there was no formation of malaoxon. The result indicates that there is no activity of monooxygenase in wheat kernels, because malathion is not oxidized to malaoxon in the homogenate.

Organophosphorus Hydrolase: To detect organophosphorus hydrolase activity in wheat kernels, five types of organophosphorus pesticides with a thioether group, malathion, phenthoate, methidathion, dimethoate and malaoxon for which the Z in Table 1 was a sulfur atom, were examined using the method of Lai *et al.*[7] No activity of organophosphorus hydrolase was observed in wheat kernel homogenate, because formations of thiol com-

pounds such as diethyl thiomalic acid from malathion were not detected when using the DTNB reagent in the case of either pesticide (data not shown). Therefore organophosphorus hydrolase does not appear to contribute to malathion degradation.

Methyltransferase and Carboxylesterase: Enzymatic demethylation of the dimethyl dithio- or dimethyl thio-phosphate group of malathion by the methyltransferase and hydrolysis of the carboxylester group by carboxylesterase were examined using nine types of organophosphorus pesticides in GC/MS. As shown in Table 2, the organophosphorus pesticides that do not have a carboxylester group but have a dimethyl dithio- or dimethyl thio-phosphate group, *i.e.*, methidathion, chlorpyrifos-methyl, and dimethoate were not decomposed by the homogenate. This suggests that methyltransferase does not contribute to malathion degradation. Of the oxon and thion organophosphorus pesticides that have a carboxylester group, the former (crotoxyphos, mevinphos, and malaoxon) were not eliminated in the homogenate, but the latter (malathion, phenthoate, and methacrifos) were. This suggests that malathion degradation in the wheat homogenate is caused by thion organophosphorus pesticide-specific carboxylesterase that is inactivated by oxon organophosphorus pesticides.

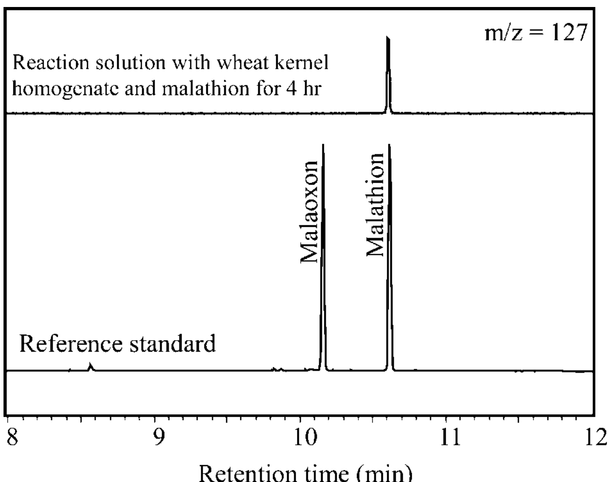

Fig. 1. SIM Chromatogram of Reaction Solution of Malathion in Wheat Kernel Homogenate for 4 hr

The sample was obtained as follows. Malathion was added to wheat kernel homogenate. Four hours later, an aliquot was deproteinized and subjected to GC/MS analysis. Experimental details are described in MATERIALS AND METHODS.

Effects of Malathion and Malaoxon on Carboxylesterase Activity in Wheat Kernels

To determine why the oxon organophosphorus pesticides were not degraded in the wheat kernel homogenate, the effects of malaoxon on the carboxylesterase activity using *p*-nitrophenyl acetate were examined using the Lineweaver-Burk plot. As shown in Fig. 2, malaoxon noncompetitively inhibited carboxylesterase, because maximal reaction rate (V_{max}) was decreased with the increase in malaoxon concentration, although the michaelis-menten constant (K_m) wvalue, which was an average 0.35 mM, was almost constant. Therefore the oxon organophosphorus pesticides including malaoxon are not degraded because the pesticides themselves can act as noncompetitive inhibitors of wheat kernel carboxylesterase.

To confirm the degradability of malathion by the carboxylesterase in wheat kernels, the effects of malathion on carboxylesterase activity using *p*-

Table 2. Elimination of Organophosphorus Pesticides in Wheat Kernel Homogenate

Organophosphorus pesticide				Elimination rate
Common name	Type			(%/30 min)
Malathion	$(CH_3O)_2$	+ COOR	P = S	33
Phenthoate				16
Methacrifos				26
Crotoxyphos			P = O	0
Mevinphos				0
Malaoxon				0
Methidathion		− COOR	P = S	0
Chlorpyrifos-methyl				0
Dimethoate				0

Elimination activities are expressed as the elimination rates estimated by deducting the residual amount from the initial amount after 30 min. The data were obtained in three trials.

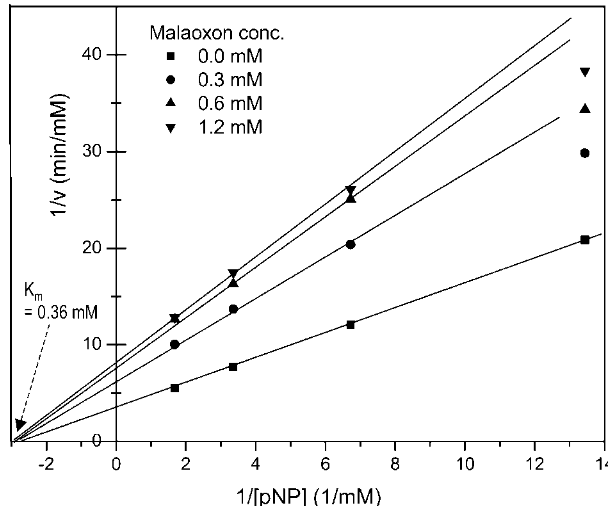

Fig. 2. Lineweaver-Burk Plot for Wheat Carboxylesterase Activity with 0.075–0.6 mM of *p*-Nitrophenyl Acetate as Substrate and 0–1.2 mM of Malathion as Inhibitor

The activity without inhibitor was about 36 U_{pNP} per ml of wheat kernel homogenate.

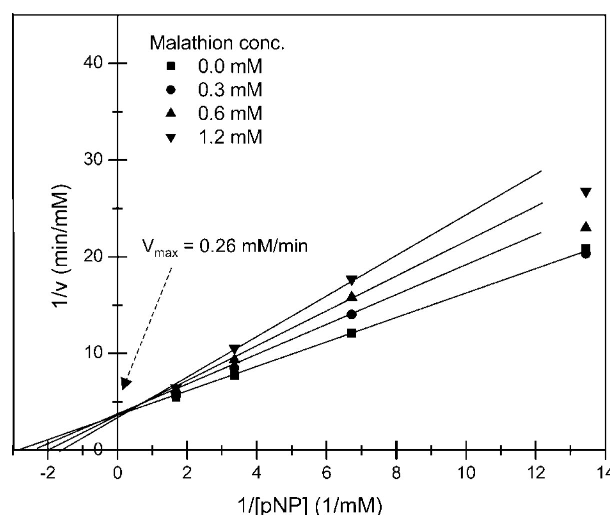

Fig. 3. Lineweaver-Burk Plot for Wheat Carboxylesterase Activity with 0.075–0.6 mM of *p*-Nitrophenyl Acetate as Substrate and 0–1.2 mM of Malaoxon as Inhibitor

The activity without inhibitor was about 36 U_{pNP} per ml of wheat kernel homogenate.

nitrophenyl acetate were also examined in the Lineweaver-Burk plot. Figure 3 shows that the K_m value rose with the increase in malathion concentration, whereas V_{max}, which was an average 0.26 mM/min, was almost constant; thus malathion competitively inhibited the carboxylesterase activity that enzymatically hydrolyzed *p*-nitrophenyl acetate. This result suggests that the malathion residue in wheat kernels can be degraded by thion organophosphorus pesticide-specific carboxylesterase.

DISCUSSION

We examined the degradability of malathion by monooxygenase, organophosphorus hydrolase, methyltransferase, and carboxylesterase in wheat kernel homogenate to identify the enzymes that decompose the pesticide during the analytical operation of their swelling with water. It was believed that monooxygenase was not involved in the elimination of malathion residue in wheat kernels, as malaoxon is not generated in wheat kernel homogenate with malathion added. In humans, the production of malaoxon from malathion has been shown due to an enzyme in the liver microsome.[13] However, monooxygenase activity and malaoxon residue have not been detected in wheat kernels containing malathion residue.[16,17] Our results support the previous reports showing that monooxygenase does not contribute to the elimination of malathion residue in wheat kernels.

If there were organophosphorus hydrolase activity toward malathion in wheat kernel homogenate, the pesticide would be degraded into diethyl thiomalic acid by the cleavage of the thioether group. However, such degradation did not occur in the homogenate when malathion was added. Furthermore, when we examined the other four organophosphorus pesticides with a thioether group using the same method, no thiol compounds were produced, as in the case of malathion. These results indicate that wheat kernels have no organophosphorus hydrolase activity against the pesticides investigated, and that the enzyme is not involved in the elimination of malathion residue. For example, malathion degradation by organophosphorus hydrolase of some microorganisms is known, whereas hydrolase activity in plants has not been reported.[7] As shown by our results, no organophosphorus hydrolase activity involved in the elimination of malathion residue occurs in wheat kernels.

We found that enzymatic elimination of organophosphorus pesticides with carboxylester including malathion occurred in wheat kernel homogenate, but that none of those without this group but with a dimethyl dithio- or dimethyl thio-phosphate group was enzymatically eliminated in this homogenate. This suggests that methyltransferase, which converts malathion into desmethyl malathion, is not involved

in the elimination of malathion residue in wheat kernels, but that carboxylesterase is. Enzymatic desmethylation of organophosphorus pesticide by methyltransferase was observed in the mouse and rat.[11] Although desmethyl malathion was also detected in malathion-sprayed kernels of rice, whether the conversion was enzymatic or nonenzymatic was not clarified.[18] However, from an enzymologic viewpoint, we showed that there was no methyltransferase activity against malathion in wheat kernels.

In this study, it was clarified for the first time that malathion residue is degraded by the action of carboxylesterase in wheat kernels. Malathion-degradable carboxylesterase has been reported in insects[8] and mammals.[10] In cotton and broad bean plants sprayed with malathion in agricultural fields, the involvement of carboxylesterase is indicated by the production of malathion monocarboxylic acid.[14] In wheat kernels containing malathion residue following postharvest treatment, however, malathion mono- and di-carboxylic acid were also detected by GC measurement.[16,17] Judging from our results, both malathion metabolites were produced by carboxylesterase of the wheat kernels.

Furthermore, this carboxylesterase was specific for thion organophosphorus pesticides and noncompetitively inhibited by oxon organophosphorus pesticides. Such noncompetitive inhibition was also observed in rat liver carboxylesterase and caused by the phosphorylating serine residue of the active site with malaoxon.[19] The carboxylesterase in wheat kernels appears to be inhibited by the same mechanism.

A similar phenomenon of malathion degradation was observed in the homogenates of edible oats, barley, rye, and other species of wheat (data not shown). As oats have unique isozymes of carboxylesterase, the electrophoresis of isozymes is performed when a breed is identified.[20–22] Therefore the degradation of malathion residue in the homogenate of the oats must be caused by their carboxylesterase. Malathion degradation was not observed in the homogenates of corn or rice kernels, however (data not shown). In these cereals, there may be either no carboxylesterase activity or only weak activity in its activity with malathion as a substrate.

In conclusion, the reason for malathion degradation during the preparation of samples when using the official method to determine malathion residue in wheat kernels is the carboxylesterase that dissolves when water is added to wheat as a pretreatment for extraction. Therefore the official method should be revised so that water is not added as pretreatment for the extraction;[4] or supercritical fluid extraction is used without swelling;[23] or extraction is performed with the addition of a noncompetitive inhibitor of carboxylesterase such as an oxon organophosphorus pesticide. Further detailed study such as the identification of metabolites is necessary to clarify the entire picture of degradation.

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