Identification of Carboxylesterase Metabolites of Residual Malathion in Wheat Kernels Using Semi-Micro Radio Liquid Chromatography

Kimihiko Yoshii,∗,a Yasuhide Tonogai,b Jun’ichi Katakawa,c Hitoshi Ueno,c and Katsuhiko Nakamuroc

aOsaka Prefectural Government, Pharmaceutical Affairs Division, Department of Public Health and Welfare, Otemae 2, Chuo-ku, Osaka 540–8570, Japan, bOsaka Pharmaceutical Association, 1–3–8 Izumi-cho, Chuo-ku, Osaka 540–0019, Japan, and cFaculty of Pharmaceutical Sciences, Setsunan University, 45–1 Nagaojoge-cho, Hirakata, Osaka 573–0101, Japan

(Received September 16, 2006; Accepted September 30, 2006; Published oneline November 10, 2006)

Since residual malathion in wheat kernels is shown to be enzymatically decomposed during sample preparation using the Japanese official method for pesticide analysis, the decomposition products were identified by semi-micro radio liquid chromatography (LC). The reaction mixture of the supernatant of wheat kernel homogenate incubated with [14C] malathion for 0–8 hr was fractionated, and the radioactive decomposition products in the fractions were identified by comparison with the retention time of fifteen reference standards. Twelve of these products occurring in the environment, diethyl fumarate, diethyl maleate, diethyl malate and diethyl succinate, and their hydrolysate and desmethyl malathion dicarboxylic acid were not detected in the reaction mixture, indicating no contribution of organophosphorus hydrolase or methyl transferase. A trace amount of desmethyl malathion was observed, however, this seemed to be non-enzymatically produced. Malathion monocarboxylic acid and malathion dicarboxylic acid were identified as the major products by the LC condition with and without an ion pair reagent, respectively. The half life of malathion decomposition was 2.1 hr, and the reaction time course of these products demonstrated that malathion was decomposed to malathion monocarboxylic acid followed by malathion dicarboxylic acid. These results suggested that residual malathion in wheat kernels was mainly hydrolyzed to malathion carboxylic acids by wheat carboxylesterase during the sample preparation.

Key words —– wheat kernel carboxylesterase, malathion degradation, malathion dicarboxylic acid, malathion monocarboxylic acid

INTRODUCTION

Malathion is relatively safe to mammals because of its low toxicity and is often used for pest control in agriculture,1) food processing such as a post-harvest pesticide in wheat,2) gardening,3) and for malaria vector control.4) In Japan, malathion residue in wheat kernels is analyzed by the official method in the Food Sanitation Law,5) in which samples are swelled by adding water as the pretreatment before acetone extraction of the pesticide. However, we previously showed that the amount of residual malathion could not be accurately determined because the insecticide was decomposed by wheat kernel enzyme eluted into the additional water.6)

Residual malathion in the environment can be biologically metabolized.7) Of these metabolites, those with a phosphorus atom have been measured by gas chromatography with a flame photometric detector (GC-FPD).8) Semi-micro radio liquid chromatography (LC) with [14C] radioactive malathion is useful to identify the other [14C] decomposition products, because those derived from the insecticide can be distinguished from wheat element by their radioactivity. Cleavages on sites A, B, C and D as shown in Fig. 1 will produce radioactive desmethyl malathion, diethyl thiomale, diethyl maleate and malathion carboxylic acids, respectively. Site A is hydrolyzed by methyl transferase,9) sites B and C are hydrolyzed by organophosphorus hydrolase,10,11) site D is hy-
Fig. 1. Chemical Structure of [14C] Malathion and the Cleavage Sites

Hydrolyzing site A produces desmethyl malathion. Cleavage of sites B and C lead to dimethyl thiomalate and dimethyl maleate, respectively. Hydrolyzing site D produces malathion α and β monocarboxylic acid and malathion dicarboxylic acid. C*: Carbon atoms with asterisk are the radioactive atoms of [14C] malathion used in the present study.

drolyzed by carboxylesterase. However, it is not clear which hydrolyzation enzyme causes degradation of malathion residue in wheat kernels during sample preparation. The other metabolites, diethyl fumarate and maleate that are produced from diethyl maleate by dehydration, diethyl succinate that is produced from diethyl maleate by deoxygenation, and their ester hydrolysates have been shown as decomposition products from malathion in the environment. Thus, these compounds should also be analyzed as target compounds of malathion metabolites in supernatant of wheat kernel homogenate.

The aim of this paper was to investigate the enzymatic reaction of residual malathion in wheat kernels during the sample preparation by adding water. A reaction mixture of [14C] malathion in supernatant of wheat kernel homogenate was analyzed by semi-micro radio LC to identify the decomposition products of the insecticide by comparing it with the retention time of 15 target reference standards. The time course of the identified products was also observed in order to clarify the degradation pathway of the residual malathion.

MATERIAL AND METHODS

Reagents —— A liquid scintillation cocktail, Ultima Gold™ was purchased from Packard Instrument Company (Meriden, CT, U.S.A.). A toluene solution of [14C] malathion as shown in Fig. 1 (13.426 mg/ml) was from Amersham Pharmacia Biotech UK, Ltd. (Buckinghamshire, U.K.) and it was replaced with an acetone solution of 10 mg/ml before usage. Malathion, succinic acid, maleic acid, fumaric acid, malic acid, thiomalic acid, monoethyl succinate and diethyl maleate as reference standards were purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). Diethyl succinate, diethyl fumarate, diethyl maleate, monoethyl fumarate and monoethyl maleate were from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). Malathion monocarboxylic acid was from Chem Service, Inc. (West Chester, PA, U.S.A.).

Chemical Synthesis

Potassium α, α′-dimethyl Phosphorodithioate —— Potassium α, α′-dimethyl phosphorodithioate was prepared according to Fletcher with minor modification in order to synthesize malathion dicarboxylic acid and desmethyl malathion dicarboxylic acid. Phosphorus pentasulfide (6.68 g) was added to 30 ml of benzene. The slurry was stirred and maintained at 80°C and anhydrous methanol (6.0 ml) was added dropwise. The reaction mixture was stirred for 1 hr under 80°C, and refluxed for 4 hr at 120°C. After reaction and cooling, anhydrous potassium carbonate (1.7 g) was added to the obtained solution. The solvent of the mixture was removed by a rotary evaporator, the residue was resolved with a small amount of methanol, and the solution was filtered through filter paper. Diethyl ether was added to the filtrate to separate potassium α, α′-dimethyl phosphorodithioate. This was washed by diethyl ether on filter paper and dried under vacuum condition. The obtained potassium α, α′-dimethyl phosphorodithioate was a white solid (10.2 g, 99%): Infrared absorption (IR) υ (cm⁻¹, KBr); 714, 754 (P=S), 1013 (P-O-C), 1184 (P-O-CH₃), 1436 (-O-CH₃), 1H-NMR (400 MHz, D₂O); δ 3.65 (3H, s, OCH₃), 3.62 (3 H, s, OCH 3), LC/Electrospray Ionization (ESI)-MS; m/z = 157 [M-H]⁻.

Malathion Dicarboxylic Acid and Desmethyl Malathion Dicarboxylic Acid —— Malathion dicarboxylic acid and desmethyl malathion dicarboxylic acid were prepared by the following procedure;

Potassium α, α′-dimethyl phosphorodithioate (1.4 g) and α-bromo succinic acid (2.0 g) in acetone (30 ml) were stirred for 4 hr at room temperature. The reaction mixture was filtered through filter paper to remove by-product KBr and the filtrate was concentrated without deposition. A appropriate quantity of tert-butyl methyl ether was added to the concentrated solution to precipitate desmethyl malathion dicarboxylic acid. The supernatant was concentrated under vacuum, and anhydrous dipotassium carbonate was added to separate malathion dicarboxylic acid as potassium salt. That obtained was resolved with a small amount of
methanol, and the solution was filtered through filter paper. A proper quantity of tert-butyl methyl ether was added to the filtrate for deposition of malathion dicarboxylic acid. Each precipitate of malathion dicarboxylic acid dipotassium salt and desmethyl malathion dicarboxylic acid was washed with tert-butyl methyl ether on filter paper and dried by vacuumed desiccator.

The obtained desmethyl malathion dicarboxylic acid was a hygroscopic white solid (0.7 g, 99%): IR ν (cm⁻¹, KBr): 627 and 768 (P=S), 1033 (P-O-C), 1114 (P-O-CH₃), 1385 and 1408 (O-C=O); δ 2.45–2.79 (2H, m, CCH₂CO), 3.80 (3H, d, J = 8.0 Hz, OCH₃), 3.84 (3H, d, J = 15.2 Hz, OCH₃), LC/ESI-MS; m/z = 259 [M⁻1]⁻.

The obtained malathion dicarboxylic acid dipotassium salt was also a hygroscopic white solid (1.5 g, 99%): IR ν (cm⁻¹, KBr): 662 and 815 (P=S), 1018 (P-O-C), 1174 (P-O-CH₃), 1327 (P-O-C), 1518 (-COO⁻), 1H-NMR (400 MHz, D₂O); δ 2.58–2.79 (2H, m, CCH₂CO), 3.78–3.88 (1H, m, SCHC), 3.63 (3H, d, J = 15.2 Hz, OCH₃), LC/ESI-MS; m/z = 273 [M-1]⁺, 259 [M-C₂H₅]⁻.

Desmethyl Malathion — Desmethyl malathion was prepared by the following procedure:

Potassium ethyl xanthate was added to a solution of malathion (3.0 g) dissolved in anhydrous ethanol (100 ml) and the mixture was refluxed at 80°C for 4 hr. After reaction and cooling, ethanol in the reaction mixture was removed by rotary evaporator. The crude desmethyl malathion dissolved in water (200 ml) was partitioned with hexane (100 ml) in a separatory funnel, and the hexane layer was discarded. The separation was repeated with hexane (100 ml). HCl (35.5%, 5 ml) was added to the water layer and the water layer was partitioned with hexane (200 ml). The hexane layer was taken and anhydrous disodium sulfate was added to remove moisture. The dried solution was filtered through filter paper and evaporated to remove hexane. The obtained desmethyl malathion was a viscous pale yellow liquid, (2.5 g, 97%): IR ν (cm⁻¹, Nujor): 659 and 810 (P=S), 1031 (P-O-C), 1177 (P-O-CH₃), 1727 (C=O), 1H-NMR [400 MHz, (CD₃)₂CO]; δ 1.21 (3H, t, J = 6.0 Hz, CCH₃), 1.26 (3H, t, J = 6.8 Hz, CCH₃), 2.74–3.00 (2H, m, CCH₂CO), 3.68 (3H, d, J = 16.8 Hz, OCH₃), 4.05–4.16 (1H, m, SCHC), 4.11 (2H, q, J = 6.8, COCH₂), 4.16 (2H, q, J = 6.8, COCH₂), LC/ESI-MS; m/z = 315 [M-H]⁻.

Enzymatic Reaction of Malathion in Supernatant of Wheat Kernel Homogenate — The supernatant of wheat kernel homogenate was prepared as follows: Five g of Canadian wheat (Canada Western Red Spring wheat, Triticum aestivum) kernels were homogenized in 10 ml of 0.1 mM sodium phosphate buffer (pH 7.6) including 0.5 mM EDTA, with an Ika Ultra Turrax homogenizer. The homogenate was centrifuged at 6000 × g for 10 min, and was referred to as the “wheat supernatant.” The mixture of wheat supernatant (1.479 ml) was preincubated at 36°C for 2 min and 21 µl of 10 mg/ml [¹⁴C] malathion in acetone was added to the mixture to initiate the reaction. An aliquot of 100 µl was sequentially taken and mixed with 0.1 ml of 2-propanol. The mixture was centrifuged at 6000 × g for 3 min, freeze-dried for 10 min, and then centrifuged again. The supernatant was fractionated by LC with UV monitoring. A liquid scintillation cocktail of 0.9 ml was added to each fraction to count C-dpm by scintillation counter.

Semi-micro Radio Liquid Chromatography — Shimadzu Class VP series LC system (Kyoto, Japan) attached to a Wakosil-II 3C18RS column (2.0 mm id × 150 mm, Wako Pure Chemicals Industries, Ltd.) was used for LC fractionation. The LC fractionation was performed at a total flow rate of 0.2 ml/min, oven temperature of 40°C, an injection volume of 10 µl and two gradient programs of condition I and II. Solvent A was 0.5 M dibutylamine acetate in water and solvent B was acetonitrile under condition I. The gradient elution condition was initially 97% A–3% B, holding 97% A–3% B for 5 min, programming to 20% A–80% B over 20 min, holding 20% A–80% B for 10 min (30 min analysis time). Solvent A was water and solvent B was acetonitrile under condition II. The gradient elution condition was initially 100% A–0% B, holding 100% A–0% B for 5 min, programming to 0% A–100% B over 15 min, holding 0% A–100% B for 10 min (25 min total analysis time). The eluate was collected at intervals of 1 min. The column equilibration was accomplished by using both initial conditions for 10 min prior to the next injection.

The degradation values per minute (dpm) of [¹⁴C] corrected fractions were counted by an Aloka (Tokyo, Japan) LSC-5100 liquid scintillation counter.
Table 1. Retention Time for Malathion and its Related Decomposition Products by LC

<table>
<thead>
<tr>
<th>Compound</th>
<th>Condition I (with ion pair reagent)</th>
<th>Condition II (without ion pair reagent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malathion</td>
<td>22.4</td>
<td>15.2</td>
</tr>
<tr>
<td>Desmethyl malathion</td>
<td>16.7</td>
<td>8.2</td>
</tr>
<tr>
<td>Malathion monocarboxylic acid</td>
<td>15.4, 15.7</td>
<td>7.5</td>
</tr>
<tr>
<td>Malathion dicarboxylic acid</td>
<td>13.3</td>
<td>2.3</td>
</tr>
<tr>
<td>Desmethyl malathion dicarboxylic acid</td>
<td>13.2</td>
<td>1.5</td>
</tr>
<tr>
<td>Diethyl fumarate</td>
<td>20.1</td>
<td>13.6</td>
</tr>
<tr>
<td>Diethyl maleate</td>
<td>18.4</td>
<td>12.2</td>
</tr>
<tr>
<td>Diethyl malate</td>
<td>22.0</td>
<td>10.1</td>
</tr>
<tr>
<td>Diethyl succinate</td>
<td>21.9</td>
<td>12.3</td>
</tr>
<tr>
<td>Monoethyl fumarate</td>
<td>12.9</td>
<td>6.9</td>
</tr>
<tr>
<td>Monoethyl maleate</td>
<td>12.2</td>
<td>6.9</td>
</tr>
<tr>
<td>Monoethyl succinate</td>
<td>12.3</td>
<td>7.2</td>
</tr>
<tr>
<td>Fumaric acid</td>
<td>12.1</td>
<td>1.6</td>
</tr>
<tr>
<td>Malic acid</td>
<td>12.0</td>
<td>1.9</td>
</tr>
<tr>
<td>Maleic acid</td>
<td>12.0</td>
<td>1.9</td>
</tr>
<tr>
<td>Succinic acid</td>
<td>12.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

These compounds, except for malic acid, maleic acid and succinic acid, could be separated by LC condition I or II, with or without an ion pair reagent, respectively. Condition I: Solvent A was 0.5 M dibutylamine acetate in water and solvent B was acetonitrile. The gradient elution condition was initially 97% A–3% B, holding 97% A–3% B for 5 min, programming to 20% A–80% B over 20 min, holding 20% A–80% B for 10 min (30 min total analysis time). Condition II: Solvent A was water and solvent B was acetonitrile. The gradient elution condition was initially 100% A–0% B, holding 100% A–0% B for 5 min, programming to 0% A–100% B over 15 min, holding 0% A–100% B for 10 min (25 min total analysis time).

RESULTS

Identification of Enzymatic Malathion Decomposition Products of Residual Malathion in Wheat Kernels

Radioactive [14C] malathion, \( o, o'-\)dimethyl S-(1,2-di(ethoxycarbonyl)[1,2,14C]ethyl) phosphorothioate, shown in Fig. 1 was chosen in order to trace the broad range of enzymatic degradation products of residual malathion in wheat kernels. The expected malathion decomposition products and their retention times are shown in Table 1. The labeled malathion was added to the wheat supernatant, an aliquot was separated by semi-micro LC, and the decomposition products were identified by comparing the retention time of the radioactive fraction with that of 15 standards. Figure 2 shows the radioactive fractionation profile at 0 and 8 hr after reaction by LC condition I. Malathion radioactivity was noted in No.45 fraction and weak radioactivity of metabolite in No.31 fraction at the start of the reaction. The insecticide disappeared after 8 hr, and radioactivity was newly detected in No.27, 31, 32 and 34 fractions. Malathion monocarboxylic acid was identified in No.31 and 32 fractions, when compared with the retention time of reference standards. The decomposition product in No.27 fraction was believed to be malathion dicarboxylic acid or desmethyl malathion dicarboxylic acid. Desmethyl malathion was identified in No.34 fraction with weak radioactivity, there was no radioactivity in other fractions. This indicates there is no production of maleic acid, fumaric acid, malic acid, succinic acid or their esters.

To identify the decomposition product in No.27 fraction, the same mixture 8 hr after the reaction was fractionated under LC condition II, and the radioactivity of their fractions was measured (Fig. 3). Radioactivity derived from monocarboxylic acid and desmethyl malathion was detected in No.8 and 9 fractions, respectively. The metabolite found in No.27 fraction under LC condition I was eluted in No.3 fraction under LC condition II, and the metabolite, but was not identified with desmethyl malathion dicarboxylic acid but with malathion dicarboxylic acid. These results identified malathion monocarboxylic acid, malathion dicarboxylic acid and desmethyl malathion as enzymatic malathion decomposition products in wheat supernatant.
**Time Course of the Malathion Decomposition Products**

The time course of the decomposition products identified in the reaction mixture of malathion in wheat supernatant at 0, 1, 2, 4 and 8 hr after reaction was determined to clarify the degradation pathway (Fig. 4). Malathion decomposed relatively quickly, and the half life was about 2.1 hr. Until all malathion was decomposed at 8 hr after reaction, production of malathion monocarboxylic acid increased. A small amount of malathion dicarboxylic acid and a trace amount of desmethyl malathion were formed when malathion was almost completely eliminated after 8 hr. The total radioactivity of the malathion and identified decomposition products was almost constant during these reactions.
DISCUSSION

Though the presence of $^{14}$C malathion decomposition products can be confirmed by measurement of radioactivity in the LC fractions, their identification requires comparison with a reference standard material corresponding to each product. Therefore, fifteen possible compounds as shown in Table 1 were selected as target compounds and their reference standards were prepared. The reference standards for malathion dicarboxylic acid, desmethyl malathion and desmethyl malathion dicarboxylic acid were not available, however, and thus were synthesized for this study.

Malathion can be decomposed by hydrolytic cleavage from site A to D as shown in Fig. 1. Cleavage on site A leads to production of desmethyl malathion, which is reported as an environmental degradation product.\(^7\) Organophosphorus hydrolase cleaves site B of malathion into diethyl thiomalate.\(^{10}\) However, thiomalic acid and its ethyl esters were not covered as targets in this study, because there was no production of these sulfhydryl compounds from malathion added to wheat supernatant in our previous report.\(^{17}\) Some organophosphorus hydrolase also produces diethyl malate by hydrolysis of site C.\(^{11}\) Hydrolysis of site D by carboxylesterase leads to malathion monocarboxylic acid and malathion dicarboxylic acid. We previously demonstrated that malathion could be a substrate of carboxylesterase, since the pesticide competitively inhibits the activity with \(p\)-nitrophenyl acetate as the typical substrate.\(^{17}\) However, the decomposition products by the carboxylesterase were not clarified. Diethyl fumarate, diethyl maleate and diethyl succinate were detected in the environment,\(^7\) and also measured as other possible degradation products. We identified malathion decomposition products to clarify which enzymes degrade malathion in wheat supernatant using semimicro radio LC. Using two LC conditions, with and without an ion pair reagent, comparatively large amounts of malathion monocarboxylic acid and malathion dicarboxylic acid and a trace amount of desmethyl malathion were detected as the malathion decomposition products, whereas no production of maleic acid, fumaric acid, malic acid, succinic acid and their esters or desmethyl malathion dicarboxylic acid was observed. Accordingly, these results suggest that malathion in wheat supernatant is at least not hydrolyzed on site B or C in Fig. 1. Desmethylation of malathion does not proceed enzymatically in wheat supernatant as we previously reported. Malathion monocarboxylic acid and malathion dicarboxylic acid were major products, because the total radioactivity of the malathion and both metabolites was almost constant during the reaction. These results therefore indicate that enzymatic malathion decomposition in wheat supernatant is mainly due to wheat carboxylesterase. This is corresponding to the possibility as previously mentioned that malathion is degraded by carboxylesterase in wheat supernatant because the pesticide competitively inhibits against carboxylesterase activity in the supernatant.\(^{17}\) Therefore, the present results strongly support the possibility.

Carboxylesterases may be responsible for malathion metabolism in several species of plants. Indeed, malathion carboxylic acids as possible metabolites have been detected in the roots, stem, and leaves, when malathion was applied to the growing plants of cotton (\textit{Gossypium barbadense}) and broad beans (\textit{Vicia faba}).\(^{18}\) However, there was almost no enzymatic degradation in wheat kernels that were sprayed with malathion as a postharvest pesticide when the metabolites were analyzed with a direct organic solvent-extraction.\(^{19}\) This may be due to the carboxylesterase in the intact kernels being inactive or malathion residue adsorbed on the surface hardly permeating inside. Such carboxylesterases also seem to be present in other grains of \textit{Tribe Triticeae} such as barley (\textit{Hordeum vulgare}) and rye (\textit{Secale cereale}).\(^{20–22}\) We previously demonstrated that most malathion residue in their kernels could be detected by analysis with direct organic solvent-extraction, and that homogenizing the kernels with water before the extraction resulted in degradation of the residue.\(^6,17\)

In conclusion, malathion residue in cereal kernels such as wheat, barley and rye, on which this insecticide was sprayed as a postharvest pesticide, is expected to be hydrolyzed by their carboxylesterases when their homogenates are prepared with water for the analysis according to the Japanese official method. Therefore, the official method for the analysis of such residue in cereal kernels should immediately be improved.

REFERENCES


