Kinetic Analysis for Hydrolysis of Malathion by Carboxylesterase in Wheat Kernels

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(Received February 17, 2007; Accepted June 11, 2007; Published online July 18, 2007)

We previously found that malathion residue in wheat kernels was decomposed by wheat kernel carboxylesterase (CE) during sample preparation for pesticide residue analysis. The degradation of malathion in supernatant of wheat kernel homogenate was enzyme-kinetically analyzed to characterize the CE. Malathion α-monocarboxylic acid (MMCα), malathion β-monocarboxylic acid (MMCβ), malathion dicarboxylic acid (MDC) and desmethyl malathion (DMal) were identified by analysis using liquid chromatography/electrospray ionization mass spectrometry (LC/ESI-MS) after incubation at 36°C of the reaction mixture of malathion in the supernatant of wheat kernel homogenate. Since small amounts of DMal and MMCβ were formed in boiled supernatant of wheat kernel homogenate, these compounds were nonenzymatically produced. Examination of the time course of enzymatic malathion degradation indicated that malathion was decomposed into MDC through both MMCα and MMCβ, and the amount of MMCα formed was greater than that of MMCβ at maximum concentrations of both MMC isomers. MMCα and MMCβ were enzymatically decomposed into only MDC, which was the final metabolite of malathion because there was no further decomposition product. The order of degradation half-lives by wheat kernel CE (malathion < MMCβ < MMCα) is consistent with the intensity of their hydrophobicity. These results suggest that when malathion is degraded *via* MMC to MDC by wheat kernel CE, its enzymatic degradability and the decomposition products depends on their hydrophobicity.

Key words —— wheat kernel carboxylesterase, malathion, malathion monocarboxylic acid, liquid chromatography/electrospray ionization mass spectrometry

INTRODUCTION

Malathion is relatively safe for mammals because of its low toxicity and is often used in agricultural applications[1] as an insecticide and postharvest pesticide for wheat kernels.[2] In Japan, malathion residue in crops has been analyzed to confirm its compliance with residual standards of the Food Sanitation Law officially established by the Ministry of Health, Labour and Welfare.[3] However, we previously showed that the malathion residue in wheat kernels could not be accurately determined, because this substance was enzymatically decomposed by homogenization of the samples with water as a pretreatment to the official method prior to acetone-extraction of the pesticide.[4] One of the malathion-degradable enzymes, carboxylesterase (CE) hydrolyzes malathion into malathion monocarboxylic acid (MMC) and malathion dicarboxylic acid (*O*,*O*-dimethyl *S*-[1,2-dicarboxyethyl] phosphorodithioate, MDC).[5–7] This type of CE is ubiquitous in various organisms.[8] Indeed, malathion degradation by CE was observed in rice (*Oryza sativa*)[9] and asparagus (*Asparagus spears*)[10] plants. We previously identified MMC and MDC as malathion metabolites in the supernatant of wheat kernel homogenate, suggesting that there was at least malathion-degradable CE.[11]

Malathion is hydrolyzed into both malathion α-monocarboxylic acid (*O*,*O*-dimethyl *S*-[2-carboethoxy-1-carboxy] phosphorodithioate, MMCα) and malathion β-monocarboxylic acid (*O*,*O*-dimethyl *S*-[1-carboethoxy-2-carboxy] phos-

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phorodithioate, MMCβ) by CE of fly,[12] rabbit,[13] rat[14] and human.[15] Formation of only MMCα is recognized in cotton (*Gossypium barbadense*), broad bean (*Vicia faba*)[16] and rice.[9] However, we were previously unable to separately measure MMCα and MMCβ in a mixture of malathion and the supernatant of wheat kernel homogenate, although MMC formation had been observed in the mixture.[11]

In this paper, we attempt to identify both MMC isomers as the metabolites of malathion in supernatant of wheat kernel homogenate by liquid chromatography/electrospray ionization-mass spectrometry (LC/ESI-MS). The degradations of malathion and the identified products were enzyme-kinetically analyzed to characterize the wheat kernel CE.

MATERIALS AND METHODS

Reagents —— Malathion standard material and porcine pancreatic lipase were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Dibutyl amine acetate as ion pair reagent for liquid chromatography (LC) was from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). MDC, desmethyl malathion (DMal), and desmethyl malathion dicarboxylic acid, which were synthesized in our previous study,[11] and MMCα and MMCβ, which were newly synthesized, were used as standard reference materials of malathion metabolites.

Chemical Synthesis —— MMCα: MMCα was synthesized enzymatically as follows; Malathion (2 g) was added to porcine pancreatic lipase solution (20 g/150 ml) in 0.02 M sodium phosphate buffer (pH 7.0) and incubated at 36°C for 40 hr. In this reaction, malathion was converted only to MMCα. The reaction mixture was partitioned with hexane (150 ml) and the hexane layer was collected. The partition was repeated with an additional 50 ml of hexane. The collected hexane layer was partitioned with 5% disodium carbonate solution (20 ml) and the hexane layer was discarded. The water layer was acidified with HCl and partitioned with hexane (200 ml). The hexane layer was then evaporated by a rotary evaporator to remove the hexane. Pale yellow liquid (99.7% MMCα, 1.7 g) was obtained and a part of the liquid was purified by LC with YMC-Pack ODS-AQ column (20 mm id. × 250 mm, YMC Co., Ltd., Kyoto, Japan). The purified MMCα was thick pale yellow liquid (160 mg, 99%): Infrared Spectrum (IR) ν (cm^{-1}, nujor); 656, 822 ($P = S$), 1014 (P-O-C), 1178 (P-O-CH_3), 1213, 1298 (C-O), 1375, 1406 (-O-CH_3), 1725 ($C = O$), ^{1}H-NMR [400 MHz, $(CD_3)_2CO$]; δ = 1.22 (3 H, t, J = 7.0 Hz, CCH_3), 2.89–3.05 (2 H, m, CCH_2CO), 3.82 (3 H, d, J = 15.2 Hz, OCH_3), 3.83 (3 H, d, J = 15.2 Hz, OCH_3), 4.05–4.16 (1 H, m, SCHC), 4.12 (2 H, q, J = 6.8 Hz, $COCH_2$), LC/ESI-MS; $m/z = 287[M - H]^-$, found 157 and 141.

MMCβ: The MMCβ was prepared according to Suzuki and Miyamoto[17] with minor modification. Rat liver (40 g) was homogenized with 100 ml of saline and centrifuged at $8000 \times g$. Malathion (300 mg) and Tween 80 (1 ml) were added to the supernatant (70 ml) and the mixture emulsified was incubated at 37°C for 2 hr. In this reaction, malathion was converted to both MMCα and MMCβ. The reaction mixture was partitioned with hexane (70 ml) and the hexane layer was collected. The partition was repeated with 50 ml of hexane. The hexane layer was partitioned with 5% disodium carbonate solution (10 ml) and the hexane layer was discarded. The water layer was acidified with HCl and partitioned with hexane (80 ml). The collected hexane layer was concentrated by a rotary evaporator and subjected to silica gel thin layer chromatography (TLC) using a Whatman flexible plate for TLC (250 μm layer, 20 × 20 cm, UV254, Middlesex, U.K.) and *n*-hexane-diethyl ether-acetic acid (8 : 2 : 1 v/v) as developing solvent. The separated MMC mixture was scraped out from a silica gel plate and extracted with acetone. The obtained mixture was diluted with water and injected to the LC under the conditions mentioned in the synthesis of MMCα for purification. The obtained MMCβ was thick pale yellow liquid (41 mg, 99%): IR ν (cm^{-1}, nujor); 658, 824 (P = S), 1015 (P-O-C), 1178 (P-O-CH_3), 1230, 1299 (C-O), 1373, 1407 (-O-CH_3), 1725 (C = O), ^{1}H-NMR [400 MHz, $(CD_3)_2CO$]; δ = 1.27 (3 H, t, J = 7.4 Hz, CCH_3), 2.89–3.07 (2 H, m, CCH_2CO), 3.82 (3 H, d, J = 15.2 Hz, OCH_3), 3.84 (3 H, d, J = 15.2 Hz, OCH_3), 4.03–4.11 (1 H, m, SCHC), 4.20 (2 H, q, J = 6.9 Hz $COCH_2$), LC/ESI-MS; $m/z = 287[M - H]^-$, found 157.

To distinguish MMCα with MMCβ, TLC was performed with Merck Kieselgel (250 μm layer, 20 × 20 cm, UV254, Darmstadt, Germany) and developed by 0.5% acetic acid in acetone. Two detected spots with RF values of 0.39 and 0.57 on the TLC plate were confirmed as MMCα and MMCβ,

Table 1. Retention Time, *m/z* Value and Linearity of Calibration Curve of Malathion Decomposition Products on LC/ESI-MS

Metabolite	Retention Time (min)	*m/z*	Linearity of Calibration Curve (R^2) and its Range (μg/ml)[a)]
DMal	17.2	315, 157	0.999 (5–50)
MMCβ	16.7	287, 157	0.975 (5–50)
MMCα	16.5	287, 157, 141	0.995 (5–50)
MDC	14.2	273	0.999 (5–50)
Desmethyl malathion dicarboxylic acid	14.1	259	0.997 (5–50)

a) The linearity was estimated using the injection of 10 μl to LC/ESI-MS.

respectively, referring to a report by Welling and Blaakmeer.[14)]

Enzymatic Reaction of Malathion and Its Metabolites in Supernatant of Wheat Kernel Homogenate —— The wheat supernatant was prepared as follows: Ten g of Canadian wheat (Canada Western Red Spring wheat, *Triticum aestivum*) kernels was homogenized in 20 ml of 0.1 mM sodium phosphate buffer (pH 7.6) including 0.5 mM EDTA, by IKA Ultra Turrax homogenizer (Staufen, Germany). The homogenate was centrifuged at $6000 \times g$ for 10 min, and the obtained supernatant was referred to as "wheat supernatant" The wheat supernatant (4.93 ml) was preincubated at 36°C for 2 min and 0.07 ml of malathion solution (30 mM of acetone solution) was added to it to initiate the reaction. An aliquot of 0.1 ml was sequentially taken and mixed with 0.1 ml of 2-propanol. The mixture was centrifuged at $6000 \times g$ for 3 min and, after freezing (−80°C) for 10 min was centrifuged again. Malathion metabolites in the wheat supernatant were measured by the selected ion monitoring (SIM) mode of LC/ESI-MS, and the sample solutions concentrated by nitrogen gas were measured by the total ion chromatography (TIC) mode. Malathion in the supernatant diluted 20-fold with 2-propanol was quantitatively measured by gas chromatography/mass spectrometry (GC/MS).

Measurement Conditions of LC/ESI-MS and GC/MS —— An LC/ESI-MS instrument, Shimadzu LCMS-QP2010 system (Kyoto, Japan) to which a Wakosil-II 3C18RS column (2.0 mm id. × 150 mm, Wako Pure Chemicals Industries, Ltd.) was attached was used to measure malathion metabolites. The LC separation was performed with a total flow rate of 0.2 ml/min, an oven temperature of 40°C, an injection volume of 10 μl and a gradient elution. The gradient elution was carried out with 0.5 M dibutylamine acetate in water (solvent A) and acetonitrile (solvent B) and the condition was initially 97% A–3% B, holding at 97% A–3% B for 5 min, programming to 20% A–80% B over 20 min, and holding at 20% A–80% B for 10 min (30 min total analysis time). The column equilibration was accomplished using the initial conditions for 10 min prior to the next injection.

The mass-spectrometry was performed using the TIC mode and SIM mode. Retention times and *m/z* values of the target compounds and the linearity of the calibration curves are shown in Table 1. A Shimadzu GCMS-QP5050 with a DB-5 ms column (0.25 μm film thickness, 0.25 mm id. × 30 m length, J&W Scientific, Folsom, CA, U.S.A.) attached was used to determine malathion. Detector and injector temperatures were maintained at 200°C. The column was held isothermally at an initial temperature of 50°C and temperature-programmed at 20°C/min to 270°C with a final hold time of 5 min. Injection volume was 2 μl and measurement mode was the SIM mode with $m/z = 173$ as the target ion. Recovery of sample preparation and measurement was almost 100%.

RESULTS

Identification of Malathion Metabolites in Wheat Supernatant

Standard reference materials of malathion metabolites were synthesized for qualitative and quantitative determination by LC/ESI-MS. MMCα and MMCβ were enzymatically synthesized, and MDC, DMal and desmethyl malathion dicarboxylic acid were chemically synthesized.

The mixture of malathion in wheat supernatant was incubated for 8 hr, deproteinized and concentrated, and the malathion metabolites were measured by LC/ESI-MS using the TIC mode. Three

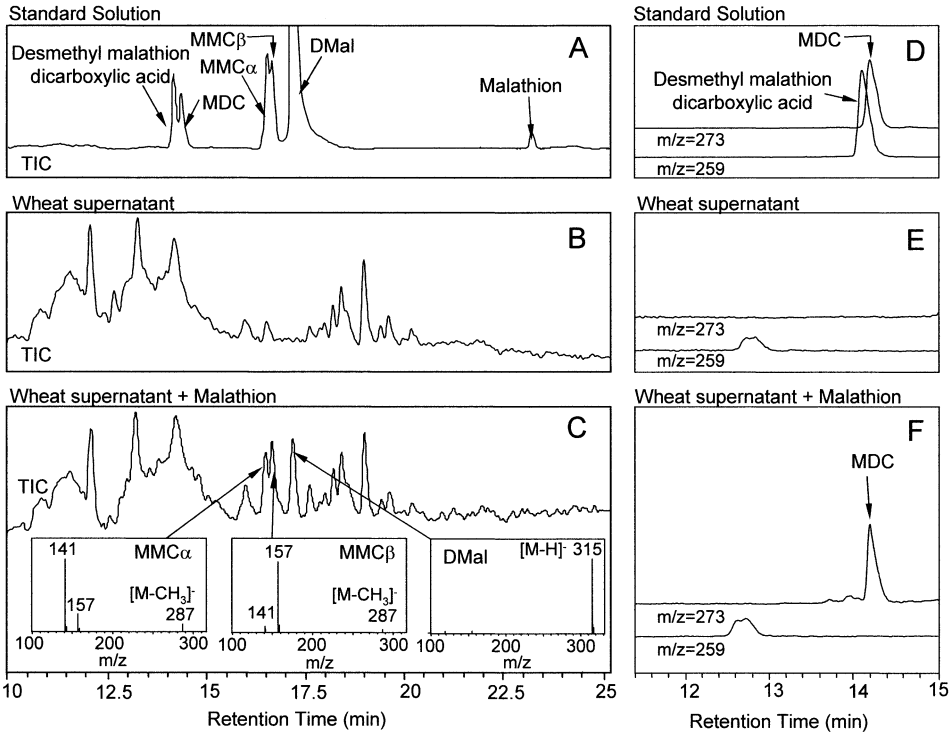

Fig. 1. Total Ion (A–C) and Selected Ion Monitoring (D–F) Chromatograms by LC/ESI-MS for Reaction Mixture of Malathion and Wheat Supernatant

new peaks appeared on the chromatogram when malathion was added to the wheat supernatant (Fig. 1C). From the spectrum patterns and the retention times of the reference standards, the metabolites were identified as MMCα, MMCβ and DMal. However, MDC and desmethyl malathion dicarboxylic acid could not be detected using the TIC mode, since there were interfering peaks which had originated from the wheat supernatant. Both metabolites were then measured using SIM mode as shown in Table 1. Desmethyl malathion dicarboxylic acid was not formed, because there was no peak on the SIM chromatogram of m/z = 259 at 14.1 min (Fig. 1E). The metabolite at 14.2 min on the SIM chromatogram of m/z = 273 was identified as MDC (Fig. 1F).

Enzyme-kinetic Analysis of Malathion Degradation in Wheat Supernatant

Time course of degradation of malathion, and formation of MMCα and MMCβ, MDC and DMal that were identified as metabolites of malathion in the wheat supernatant were examined. Small amounts of DMal and MMCβ were formed as nonenzymatic decomposition products when malathion was added to the boiled wheat supernatant (Fig. 2A). Since such nonenzymatic decomposition was recognized, the enzymatic degradation was determined by subtracting the amount of nonenzymatic degradation products in the boiled supernatant from that of the total decomposition products in the supernatant. As shown in Fig. 2B, the concentrations of both MMCα and MMCβ were increased with decrease of malathion. The concentrations of both MMC isomers were maximum after incubation for 8 hr, and the concentration of MMCα (260 μM) was 2.6 times higher than that of MMCβ (100 μM). MDC concentration increased with decrease of both MMC isomers. With incubation for

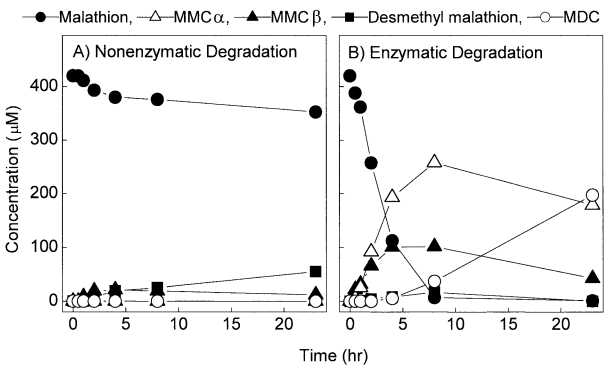

Fig. 2. Timecourse of Malathion Metabolites on Nonenzymatic (A) and Enzymatic Degradations (B)

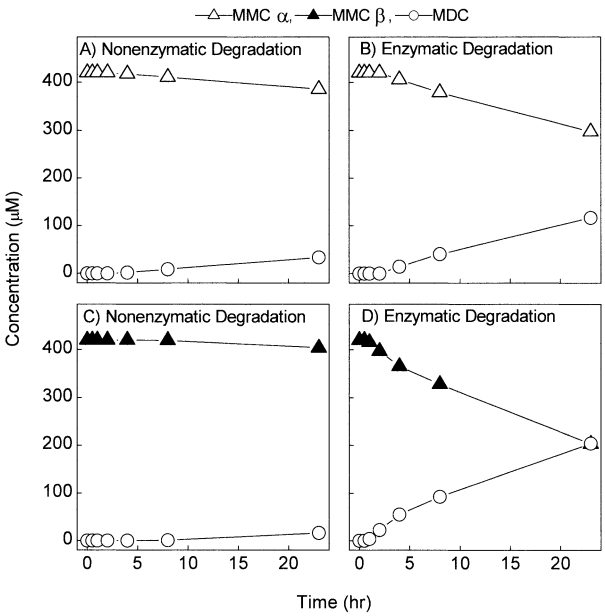

Fig. 3. Time course of MMCα (A, B) and MMCβ (C, D) Metabolites on Nonenzymatic (A, C) and Enzymatic Degradations (B, D)

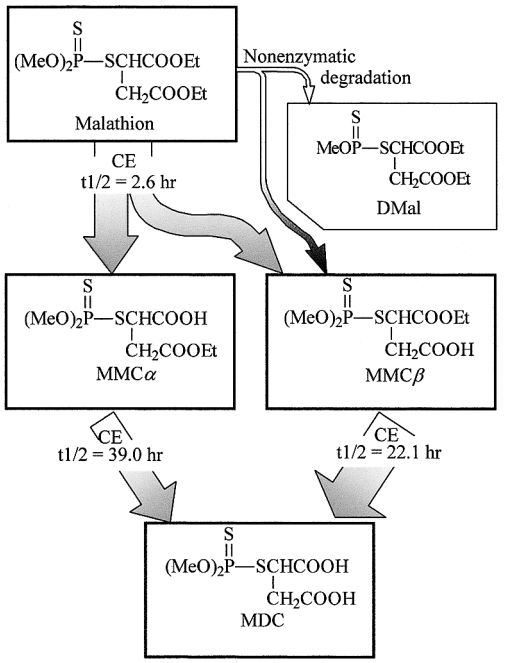

Fig. 4. Proposed Metabolic Pathway of Malathion in Wheat Supernatant

23 hr, the concentration of MMCα (180 μM) was about 3.6 times as high as that of MMCβ (50 μM). The enzymatic half-life of malathion in the supernatant was regarded as 2.6 hr based on a linear decrease at the initiation of the reaction.

Enzyme-kinetic Analysis of Degradation of MMCα, MMCβ and MDC in Wheat Supernatant

Investigation of the enzyme kinetics of MMCα and MMCβ as the metabolic intermediates showed that both MMC isomers were nonenzymatically hydrolyzed to a small amount of MDC (Fig. 3A and 3C). Enzymatic degradation amounts of MMC isomers and amounts of the products are shown in Fig. 3B and 3D by subtracting the amounts of the nonenzymatic decomposition products from those of the total decomposition products in wheat supernatant. The concentrations of both MMC isomers were linearly decreased with increase of MDC concentration, indicating that both were hydrolyzed to MDC in wheat supernatant. The half-lives of MMCα and MMCβ were 39 and 22.1 hr, respectively. Thus, the decomposition rate of MMCβ was higher than that of MMCα. No other product was found when MDC was added to the wheat supernatant.

DISCUSSION

We previously demonstrated by analysis using semi-micro radio-LC that malathion was degraded to MDC through MMC by wheat kernel CE.[11] However, it could not be elucidated whether this MMC was MMCα, MMCβ or a mixture of the two. The aim of this paper is to characterize wheat kernel CE by identification of the MMC isomers formed from malathion and to investigate the degradability of malathion and each product by the enzyme.

The present study proved for the first time that small amounts of DMal and MMCβ were nonenzymatically formed and both MMCα and MMCβ as enzymatic metabolites were predominantly formed from malathion which had been added to the wheat supernatant (Fig. 2). These MMC isomers were further enzymatically degraded to MDC (Fig. 3B and 3D). MDC was the final metabolite because there was no further decomposition product. These results are summarized in Fig. 4 as the proposed metabolic pathway of malathion by wheat kernel CE. Such enzymatic degradation behavior is observed in mouse[18] and human.[15, 18] Since there is no other enzymatic metabolite, such as *O*,*O*′-dimethyl phosphorodithioic acid or *O*,*O*′-dimethyl phosphorothioic acid, which are produced in mouse[19] and human,[20] CE is the only

malathion-degradable enzyme in wheat kernels.

The amount of MMCα formed in wheat supernatant was greater than that of MMCβ (Fig. 2B). Such formation is recognized in rice kernels,[9] cotton and broad beans,[16] suggesting that plant CE generally generates a great deal of MMCα from malathion.

The order of degradation half-lives by wheat kernel CE (malathion < MMCβ < MMCα) is consistent with the intensity of the hydrophobicity of these metabolites as estimated by their retention times using a Octadecyl silyi silica gel (ODS) column shown in Table 1. This indicates that wheat kernel CE hydrolyzes the substrates depending upon their hydrophobicity. Such a property is indeed recognized in human CE.[18]

The hydrophobicity can be electro-chemically predicted from their chemical structures as shown in Fig. 4. Malathion was seen to be high hydrophobic because of its nonionicity. MMC whose carboxyl group (-COOH) dissociates into carboxylate ion (-COO^-) in aqueous solution is relatively hydrophilic. The distance between the carboxyl group and the phosphorothionyl ($S = P$) as an electron-withdrawing group of MMCβ is greater than that of MMCα. The electron-withdrawing effect against the carboxyl group of MMCβ is weaker than that of MMCα. Thus, hydrophobicity of MMCβ is electrochemically predicted to be higher than that of MMCα.

These considerations indicate that malathion in wheat supernatant is enzymatically metabolized by two stages of ester hydrolyses: malathion is hydrolyzed to MMCα or MMCβ, and then MMCβ being more hydrophobic than MMCα is more rapidly hydrolyzed to MDC than MMCα. CE in wheat kernels might not always act to decompose malathion. When malathion is applied to stored wheat kernels as a post-harvest pesticide, the pesticide remains for a long period with little decomposition.[21] This may be due to the malathion residue adsorbed on the surface of the intact kernels barely permeating inside the kernels with CE.

MMC and MDC were enzymatically formed from residual malathion in wheat kernels by crushing the kernels in additional water relatively rapidly.[4] The estimation of toxicity for such newly-formed decomposition products is important. Only malathion has been evaluated for its toxicity,[22] while neither metabolite has been assessed. Nevertheless, the United States Environmental Protection Agency (EPA) regarded these metabolites as low toxic compounds,[22] because humans rapidly excrete their hydrophilic metabolites into the urine.[20] Any health risk of residual malathion in wheat kernels may be decreased by hydrolysis of the pesticide to MMC and MDC when the kernels are processed by adding water during cooking.

In conclusion, malathion in wheat supernatant is mainly metabolized *via* MMCα and MMCβ into MDC by wheat kernel CE, though small amounts of DMal and MMCβ are non-enzymatically formed (Fig. 4). The degradation rate of malathion, MMCα and MMCβ by CE in the kernels tends to depend upon their hydrophobicity. Since residual malathion in wheat kernels is decomposed by CE during the pesticide analysis when the official Japanese method is used, the method requires improvement.

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