JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Degradation of Malathion, in Aqueous Extracts of Asparagus (*Asparagus officinalis*)

YASUKO OKAMOTO AND TAKAYUKI SHIBAMOTO*

Department of Environmental Toxicology, University of California, Davis, One Shields Avenue, Davis, California 95616

Malathion was incubated in water extracts of vegetables at various temperatures and pH, and the amount of malathion present over time was analyzed by a gas chromatograph with a flame photometric detector. Malathion was degraded to a nondetectable level in a 1% asparagus extract incubated at pH 7.4 and 37 °C for 4 h. Carrot extract showed the second highest rate of malathion degradation (76%), followed by kale extract (23.7%), spinach extract (9.7%), and broccoli extract (1.5%) under the same conditions. The highest degradation rates of malathion were observed at 37 °C, when three different temperatures were tested (5, 25, and 37 °C) at pH 7.4. Rate constants were 0.134 min^{-1} from a 1% asparagus solution and 0.095 min^{-1} from a 0.5% asparagus solution. The highest degradation rate of malathion was achieved at pH 9 among the pHs tested (pH 4, 7.4, and 9) in a 0.5% asparagus solution. The 0.5% asparagus solution degraded dicarboxylic acid esters by almost 100% for dimethyl succinate and diethyl adipate, by 64% for diethyl acetyl succinate, and 30% for diethyl benzyl malonate when incubated at pH 9 for 20 min. The results support the hypothesis that the enzyme that degrades malathion in the asparagus solutions is a carboxylesterase.

KEYWORDS: Asparagus; carboxylesterase; malathion degradation; vegetable extract

INTRODUCTION

Exposure to pesticides is a major concern of the public. The possible adverse effects caused by pesticide residues in foods have been intensively studied. The U.S. Environmental Protection Agency (EPA) has set up tolerance levels of pesticides for each food item and has actively been working on pesticide registration to ensure food safety. Once the safe residue levels are set up for a food item, it is deemed safe as long as the level of pesticides remains below the tolerance level. However, there is still strong concern about the presence of pesticides, even though the level of residues present is lower than the tolerance set by EPA. It is impossible to completely remove all pesticide residues from foods. Therefore, it is ideal if pesticide residues can be degraded into nontoxic materials.

There have been some reports on enzymes catalyzing degradation of various pesticides (*1*). Among pesticides, organophosphorus insecticides have received much attention because these pesticides are less persistent and tend to hydrolyze readily; they have gradually replaced highly persistent organochlorine pesticides such as DDT and toxaphene. Some organophosphorus insecticides, such as malathion, are degraded by enzymes present in animals and plants (*2*). Four distinctly different types of enzymes, which degrade insecticidal organophosphates in the crude supernatant fraction of rat liver, were found using an electrophoresis method (*3*). Some organophosphate degrading enzymes have been discovered in microbacteria (*4*). Certain enzymes that degrade organophosphorus pesticides have been found in aqueous solutions of powder obtained from freeze-dried young barley leaves. When malathion was incubated in a 3% aqueous solution of this powder at 37 °C for 4 h, over 95% of the malathion was degraded. Under the same conditions, chlorpyrifos degraded to a nondetectable level, whereas other pesticides showed lesser degrees of degradation: parathion (75%), diazinon (54%), guthion (41%), and methidathion (23%) (*5*). These reports suggest that there are certain enzymes present in various vegetables, which degrade pesticides.

In the present study, the degradation of an organoposphorus pesticide, malathion, in an aqueous extract of asparagus at various pHs and temperatures was investigated.

MATERIALS AND METHODS

Pesticides. Malathion (purity, 98.2%), chlorpyrifos (99.5%), diazinon (98.7%), methidathion (99.5%), fenitrothion (98.4%), phosmet (97.8%), guthion (99.0%), dimethoate (99.0%), and isofenphos (99.5%) were purchased from Chem Service, Inc. (West Chester, PA). A stock solution of each organophosphorus pesticide (1 mg/mL) was prepared by dissolving the standard pesticide in acetone.

Chemicals and Reagents. Triphenyl phosphate, acetyl succinic acid, diethyl acetyl succinate, diethyl benzylmalonate, and diethyl adipate were bought from Aldrich Chemical Co. (Milwaukee, WI). Trizma buffer solutions (pH 7.4 and 9) were prepared by combining appropriate amounts of Trizma HCl [tris (hydroxymethyl)aminomethane hydrochloride (tris HCl)] and Trizma base [tris (hydroxymethyl)aminomethane (tris base)] (both purchased from Sigma Chemical Co., St.

* To whom correspondence should be addressed. Tel: (530) 752-4523. Fax: (530) 752-3394. E-mail: tshibamoto@ucdavis.edu.

10.1021/jf049158j CCC: $27.50
Published on Web 08/21/2004

Louis, MO). Potassium biphthalate buffer (pH 4) was obtained from Fisher Scientific (Pittsburgh, PA).

Preparation of Aqueous Extracts from Vegetables. Fresh vegetables—asparagus, carrot, kale, spinach, onion, broccoli, and garlic—were obtained from a local market. After the vegetable (100 g) was washed thoroughly with deionized water, it was cut into approximately 0.5 × 0.5 cm pieces and then blended with a 100 mL of Trizma buffer solution (pH 7.4) for 1 min at room temperature. The solution was filtered through a 4-fold cheesecloth to remove plant tissues. After the aqueous solution was stirred with three spoonfuls of talc for 10 min, it was filtered with a Buchner funnel lined with a talc-spread filter paper. The filtrate (aqueous vegetable extract) was stored in a freezer (−5 °C) until use.

Sample Preparations for Pesticide Degradation Studies in Aqueous Vegetable Extracts. The testing solutions were prepared as follows:

I. Fifty milliliters of Trizma buffer solution (pH 7.4) containing 1% of various vegetable extract (w/w) and malathion (0.2 μg/mL) was incubated at 37 °C for 4 h (results shown in **Figure 1**). The experiment was repeated two times.

II. Fifty milliliters of Trizma buffer solution (pH 7.4) containing 1% of asparagus extract (w/w) and 0.2 μg/mL each of various organophosphorus pesticides (malathion, chlorpyrifos, diazinon, methidathion, fenitrothion, phosmet, guthion, dimethoate, and isofenphos) were incubated at 37 °C for 4 h. The experiment was repeated three times.

III. Fifty milliliters of 1% asparagus extract in Trizma buffer solution (pH 7.4) was heated at 80 °C for 15 min to denature the enzymes, and the solution was then incubated with malathion (0.2 μg/mL) at 37 ° C for 4 h. The experiment was repeated three times.

IV. Fifty milliliters of Trizma buffer solution (pH 7.4) containing either 0.5 or 1% of asparagus extract and 0.2 μg/mL malathion was incubated at 5, 25, or 37 °C for various times (results shown in **Figure 3A−C**). The experiment was repeated three times.

V. Fifty milliliters of buffer solution containing 0.5% asparagus extract and 0.2 μg/mL malathion was incubated at pH 4 for 4 h or at pH 9 for 15 min (results shown in **Figure 5**).

VI. Fifty milliliters of buffer solution containing a pesticide (0.2 μg/mL) alone was incubated under the conditions of each experiment and used as a blank control.

Sample Preparations for Degradation Studies on Carboxylic Esters in Asparagus Solutions. Fifty milliliters of buffer solution (pH 9) containing 0.5% asparagus extract and 50 μg/mL carboxylic ester (dimethyl succinate, diethyl adipate, diethyl benzyl malonate, or diethyl acetyl succinate) was allowed to stand for 20 min at room temperature (25 °C).

Analysis of Pesticides and Other Organic Compounds in Samples. C_{18} solid-phase extraction (SPE) cartridge (1 g) (Varian Corp., Harbor City, CA) with reservoirs (6 mL) secured in an Alltech manifold was used for the removal of pesticides and other organic compounds from an aqueous sample solution. The SPE cartridge was preconditioned with 2 column volumes each of ethyl acetate, methanol, and deionized water in series. A subsample solution (5 mL) was transferred from each sample into a preconditioned SPE cartridge. After eluting a sample at −10 psi using a vacuum manifold (Alltech Associates, Inc., Deerfield, IL), the cartridge was rinsed with 1 column volume of deionized water and dried at −10 psi for 5 min. Pesticides and other organic compounds trapped in the cartridge were eluted with 10 mL of ethyl acetate. The ethyl acetate layer (upper layer) in an eluate was transferred into a 50 mL Erlenmeyer flask with a pipet and then dried over anhydrous sodium sulfate. After removal of sodium sulfate, the sample was condensed to 1 mL under a purified nitrogen stream. One hundred μL of triphenyl phosphate solution (10 μg/mL ethyl acetate) was added as a gas chromatographic (GC) internal standard.

Pesticides in the samples were analyzed by GC with a flame photometric detector (FPD) at phosphorus mode. The quantitative analysis of pesticides (mainly malathion) was conducted according to an internal standard method (*6*).

Instruments. A Hewlett-Packard (HP) model 5890 series gas chromatograph equipped with a 30 m × 0.25 mm i.d. (d_f = 0.25 μm) DB-5 bonded phase fused silica capillary column (J & W Scientific, Folsom, CA) and a flame photometric detector (FPD) was used for quantitative analysis of pesticides. An HP model 6890 GC interfaced to an HP 5791A mass selective detector (GC/MS) was used for mass spectral identification of pesticides in the samples at MS ionization voltage of 70 eV.

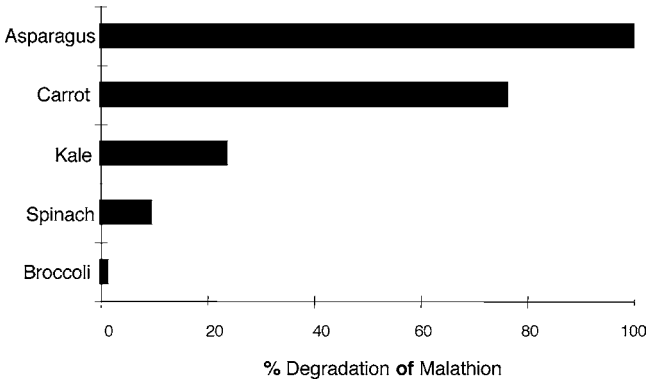

Figure 1. Degradation of malathion (%) in various vegetable extracts.

RESULTS AND DISCUSSION

Figure 1 shows the results of malathion degradation in various vegetable extracts. The values are the average of two experiments. This preliminary experiment was conducted to find the vegetable extract with the highest level of malathion degradation. Among the vegetables tested, asparagus exhibited the highest level of malathion degradation. In the asparagus extract, malathion degraded to a nondetectable level within 4 h. Carrot extract showed the second highest malathion degradation (76%), followed by kale extract (23.7%), spinach extract (9.7%), and broccoli extract (1.5%). In a preliminary experiment, nine different organophosphorus pesticides (malathion, chlorpyrifos, diazinon, methidathion, fenitrothion, phosmet, guthion, dimethoate, and isofenphos) were incubated at 37 °C for 4 h in 1% asparagus solution. Malathion degraded to a nondetectable level within 4 h, whereas no appreciable degradation was observed in the other organophosphorus pesticides under the conditions tested. Therefore, further experiments were conducted using asparagus extract and malathion.

Figure 2A shows a typical gas chromatogram of ethyl acetate extract obtained from an asparagus essence (1%) immediately after malathion (0.2 μg/mL) spiked at pH 7.4 and 25 °C. **Figure 2B** shows a typical gas chromatogram of an ethyl acetate extract obtained from asparagus essence with malathion incubated at pH 7.4 and 25 °C for 50 min. **Figure 2B** is a very similar as a chromatogram of an ethyl acetate extract obtained from an asparagus blank sample. It should be noted that when a sample was analyzed immediately after the addition of 0.2 μg/mL pesticide, greater than 90% recovery was obtained.

Figure 3 (A−C) shows the degradation of malathion in asparagus solutions (0.5 and 1%) under different conditions. The values are mean ± SD (n = 3). Three different temperatures were chosen to use, 5 °C (the temperature in a refrigerator), 25 °C (room temperature), and 37 °C (the temperature commonly used for in vitro studies to simulate in vivo temperature). Also, pH 7.4 was used to simulate in vivo pH. Malathion degradation was not observed when the heat-treated asparagus (80 °C) extract was used, suggesting that malathion degradation occurring in the extract was likely due to enzymatic action. Also, no enzymatic activity was observed after the extract was stored at −5°C (the extract had been stored for no more than 1 h). The same phenomenon has been reported in the case of lipoxygenase and peroxidase in fresh asparagus tips (*7*). The peroxidase activity of green asparagus was also inactivated when it was heated to between 90 and 125 °C (*8*, *9*).

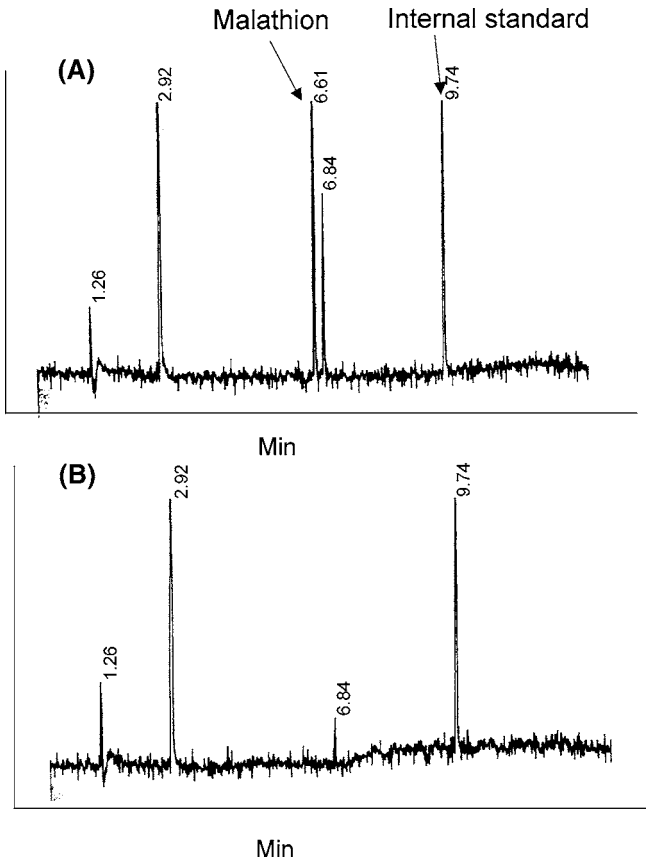

Figure 2. Typical gas chromatograms of asparagus samples: (**A**) ethyl acetate extract from 1% asparagus extract immediately after malathion (0.2 μg/mL) spiked at pH 7.4 and 15 °C. (**B**) Ethyl acetate extract from 1% asparagus extract with malathion incubated at pH 7.4 and 25 °C for 50 min.

Figure 3A shows the degradation of malathion in asparagus solutions (0.5 and 1%) at pH 7.4 and 5 °C for various times. Malathion degraded to the nondetectable level after 2 h in a 1% asparagus solution, whereas it took 4 h to degrade 95% in a 0.5% asparagus solution. The plot of log [malathion concentration] versus time gave a linear relationship, $R^2 = 0.978$ (0.5% solution) and $R^2 = 0.999$ (1% solution).

Figure 3B shows the degradation of malathion at 25 °C under the same conditions as the experiment at 5 °C. Malathion degraded to the nondetectable level after 50 min in a 1% solution, while 95% degradation was achieved by a 0.5% solution after 80 min. The plot of log [malathion concentration] versus time gave a linear relationship, $R^2 = 0.995$ (0.5% solution) and $R^2 = 0.998$ (1% solution). The results suggest that degradation occurs at room temperature.

Figure 3C shows the degradation of malathion at 37 °C under the same conditions as the experiment at 5 °C. Malathion degraded to the nondetectable level after 20 min in a 1% solution, while 95% degradation was achieved by a 0.5% solution after 30 min. The plot of log [malathion concentration] versus time gave a linear relationship, $R^2 = 0.998$ (0.5% solution) and $R^2 = 0.993$ (1% solution). The results suggest that degradation occurs quickly under in vivo temperature conditions.

Even though the malathion degraded by nearly 40% within 5 min (during sample preparation), the high linear relationship seen in the plotted results suggest that only a few (or only one) mechanisms are involved.

Figure 4 shows the rate constant of malathion degradation obtained from each sample. The highest degradation rates were

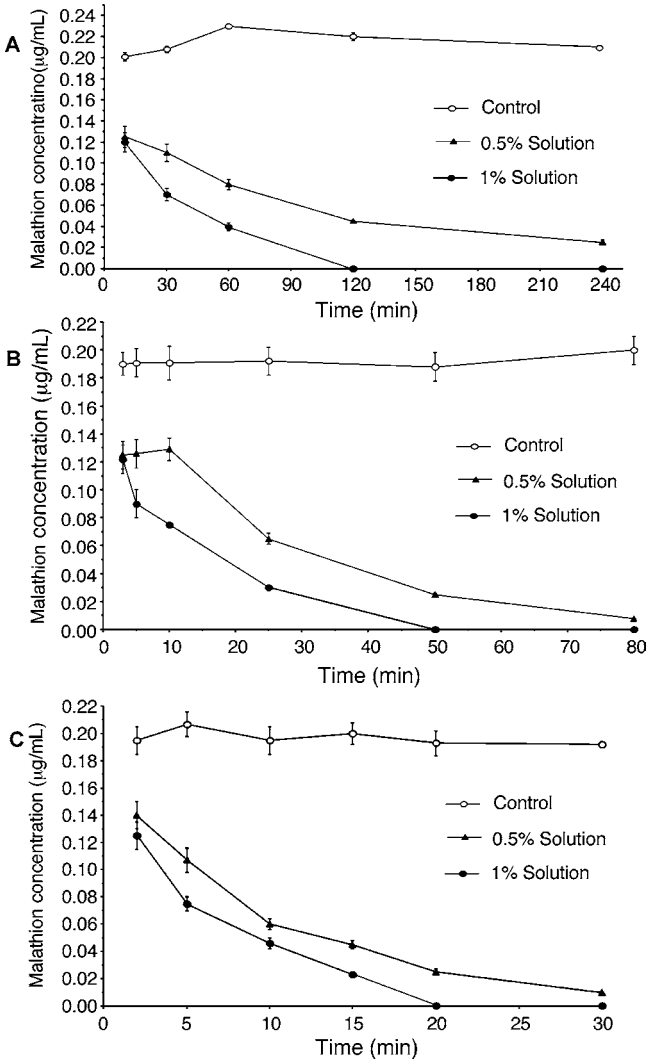

Figure 3. (**A**) Degradations of malathion in 0.5 and 1% asparagus solutions at pH 7.4 and 5 °C for various times (bars indicate standard deviation). (**B**) Degradations of malathion in 0.5 and 1% asparagus solutions at pH 7.4 and 25 °C for various times (bars indicate standard deviation). (**C**) Degradations of malathion in 0.5 and 1% asparagus solutions at pH 7.4 and 37 °C for various times (bars indicate standard deviation).

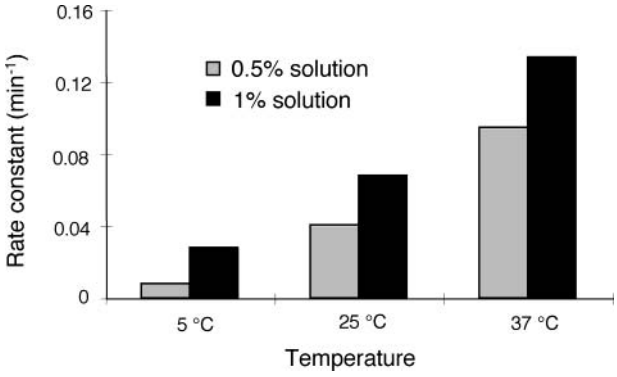

Figure 4. Rate constants of malathion degradation in asparagus solutions treated in the present study (bars indicate standard deviation).

observed at 37 °C (in vivo condition) among the three different temperatures tested at pH 7.4−0.134 min^{-1} from a 1% solution and 0.095 min^{-1} from a 0.5% solution. The degradation rate was the slowest (0.008 min^{-1}) in a 0.5% solution at 5 °C (refrigeration condition). The results of this study indicate that malathion degradation occurs faster in proportion to the

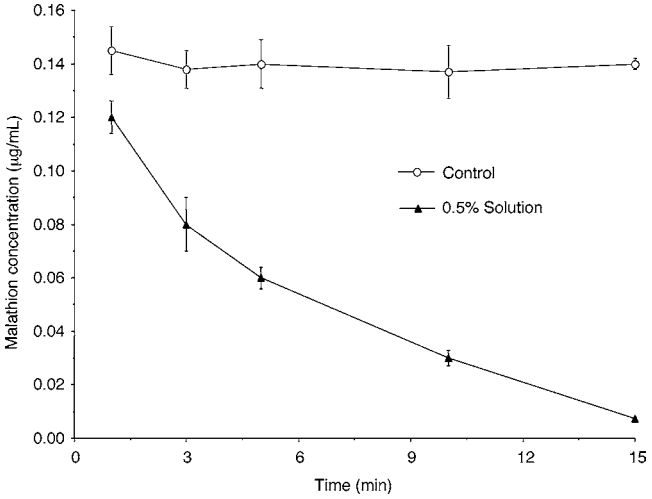

Figure 5. Degradation of malathion in a 0.5% asparagus solution at pH 9 and 37 °C for various times (bars indicate standard deviation).

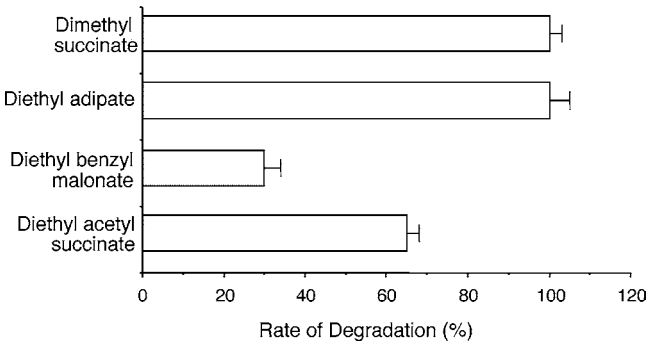

Figure 6. Degradation (%) of various carboxylic acid esters in a 0.5% asparagus solution at pH 9 and 25 °C after 20 min (bars indicate standard deviation).

concentration of asparagus extract and at higher temperatures.

Figure 5 shows malathion degradation in a 0.5% asparagus solution incubated at pH 9 and 37 °C. Malathion degraded 95% after 15 min. The rate constant in this solution was 0.222 min^{-1}, which is 2.34 times higher than that obtained from a 0.5% solution incubated at pH 7.4 and 37 °C. It took 30 min to degrade 95% of the malathion in a 0.5% solution at pH 7.4 (**Figure 3C**). On the other hand, malathion did not degrade after 4 h in a 0.5% solution at pH 4 and 37 °C. The results suggest that the enzyme that contributes to malathion degradation in an asparagus solution is not active under acidic conditions.

Among the organophosphate pesticides tested, only malathion contains a moiety of dicarboxylic acid ester. Therefore, simple chemicals containing a dicarboxylic acid ester (dimethyl succinate, diethyl adipate, diethyl benzyl malonate, and diethyl acetyl succinate) were used to examine the nature of enzyme activity in the asparagus extract.

Figure 6 shows the degradation (%) of various carboxylic acid esters in a 0.5% asparagus solution at pH 9 and 25 °C after 20 min. Dimethyl succinate and diethyl adipate degraded to the nondetectable level after 20 min, while diethyl benzyl malonate and diethyl acetyl succinate degraded by 30 and 64%, respectively, after 20 min. The results suggest that asparagus extract contains a carboxylesterase. Carboxylesterases are a common group of hydrolytic enzymes found in both prokaryotes and eukaryotes; they play an important role in the detoxification of organophosphorus pesticides, as well as carbamates and pyrethroids, by cleaving the carboxylic acid esters off by hydrolysis (*10*). The detoxification of malathion by carboxylesterase in mammals and insects was reported in the 1960s (*11*, *12*). Malathion α-monocarboxylic acid (MCA) and malathion dicarboxylic acid (DCA) were found in both rat and human urine as major metabolites (*13*). MCA and DCA were also reported in urine samples collected from adult ewes that had been administered malathion (*14*). Recently, a study on a toxicokinetic model of malathion and its metabolites (mono- and dicarboxylic acids) in the blood and urine of human volunteers was reported. These mono- and dicarboxylic acids were produced from malathion by a carboxylesterase (*15*). There are, however, only a few reports on malathion degradation in a vegetable extract. For example, some malathion metabolites, including deesterification products (e.g., MCA), were found in radish (*16*). As **Figure 2B** shows, these deesterification products were not recovered in the present study. MCA and DCA may remain in the water layer after isolation of residual malathion because they are highly water soluble. It is necessary to methylate carboxylic acid moiety(s) of MCA and DCA to recover from aqueous solution for analysis as previously reported (*14*). However, analysis of malathion metabolites was not within the scope of the present study.

It has been reported that the hydrolysis catalyzed by carboxylesterases involves charge relay among a catalytic triad comprised of an acidic amino acid residue (glutamic acid) a basic residue (histidine), and a nucleophilic residue (serine) (*17*, *18*). Therefore, the pH of the solution plays an important role in the activity of carboxylesterases. The high malathion degradation activities at pH 7.4 and 9 may be because both histidine and glutamic acid residues are in the activated form for catalytic hydrolysis. On the other hand, an active form of the enzyme for catalytic hydrolysis is not present at pH 4 because the charge relay in the enzyme does not occur under acidic conditions (*19*). These reports, in addition to the present study, support the argument that the enzyme degraded malathion in asparagus extract is a carboxylesterase.

LITERATURE CITED

(1) Efremenko, E. N.; Sergeeva, V. S. Organophosphate hydrolase—an enzyme catalyzing degradation of phosphorus-containing toxins and pesticides. *Russian Chem. Bull.* **2001**, *50*, 1826−1832.

(2) Bull, D. L. Metabolism of organophosphorus insecticides in animals and plants. *Residue Rev.* **1972**, *43*, 1−22.

(3) Miyata, T.; Matsumura, F. Organophosphate degrading enzymes in the crude supernatant fraction from the rat liver. *J. Agric. Food Chem.* **1972**, *20*, 30−32.

(4) Yang, H.; Carr, P. D.; McLoughlin, S. Y.; Kiu, J. W.; Home, I.; Qiu, X.; Jeffries, C. M. J.; Russell, R. J.; Oakeshott, J. G.; Ollis, D. L. Evolution of an organophosphate-degrading enzyme: a comparison of natural and directed evolution. *Protein Eng.* **2003**, *16*, 135−145.

(5) Durham, J. J.; Ogata, J.; Nakajima, S.; Hagiwara, Y.; Hagiwara, H.; Shibamoto, T. Degradation of organophosporus pesticides in aqueous extracts of young green barley leaves (*Hordeum vulgare L*). *J. Sci. Food Agric.* **1999**, *79*, 1311−1314.

(6) Ettre, L. S. Interpretation of analytical results. In *The Practice of Gas Chromatography*; Ettre, L. S., Zlatkis, A., Eds.; Interscience Publishers: New York, 1967; pp 402−440.

(7) Ganthavorn, C.; Nagel, C. W.; Powers, J. R. Thermal inactivation of asparagus lipoxygenase and peroxidase. *J. Food Sci.* **1991**, *56*, 47−49.

(8) Rodrigo, C.; Rodrigo, M.; Alvarruiz, A.; Frigola, A. Thermal inactivation at high temperatures and regeneration of green asparagus peroxidase. *J. Food Protec.* **1996**, *59*, 1065−1071.

(9) Morales-Blancas, E. F.; Chandia, V. E.; Cisneros-Zevallos, L. Thermal inactivation kinetics of peroxidase and lipoxygenase from broccoli, green asparagus, and carrots. *J. Food Sci.* **2002**, *67*, 146−154.
(10) Sogorb, M. A.; Vilanova, E. Enzymes involves in the detoxification of organophosphorus, carbamate, and pyrethroid insecticides through hydrolysis. *Toxicol. Lett.* **2002**, *128*, 215−228.
(11) Seume, F. W.; O'Brien, R. D. Metabolism of malathion by rat tissue preparations and its modification by EPN. *J. Agric. Food Chem.* **1960**, *8*, 36−41.
(12) Main, A. R.; Braid, P. E. Hydrolysis of malathion by aliesterases in vitro and in vivo. *J. Biochem.* **1962**, *84*, 255−263.
(13) Bradway, D. E.; Shafik, T. M. Malathion exposure studies. Determination of mono- and dicarboxylic acids and alkyl phosphates in urine. *J. Agric. Food Chem.* **1977**, *25*, 1342−1344.
(14) Muan, B.; Skare, J. U. A method for the determination of the main metabolites of malathion in biological samples. *J. Agric. Food Chem.* **1989**, *37*, 1081−1085.
(15) Bouchard, M.; Gosselin, N. H.; Brunet, R. C.; Samuel, O. A toxicokinetic model of malathion and its metabolites as a tool to assess human exposure and risk through measurements of urinary biomarkers. *Toxicol. Sci.* **2003**, *73*, 182−194.
(16) Kaur, I.; Mathur, R. P.; Tandon, S. N.; Dureja, P. Identification of metabolies of malathion in plant, water, and soil by GC-MS. *Biomed. Chromatogr.* **1997**, *11*, 352−355.
(17) Yan, B.; Yang, D.; Brady, M.; Parkinson, A. Rat kidney carboxylesterase: cloning sequencing, cellular localization, and relationship to rat liver hydrolase. *J. Biol. Chem.* **1994**, *269*, 29688−29696.
(18) Satoh, T.; Hosokawa, M. The mammalian carboxylesterases: From molecules to functions. *Annu. Rev. Pharmacol. Toxicol.* **1998**, *38*, 257−288.
(19) Parkinson, A. Biotransformation of xenobiotics. In *Casarett and Doull's Toxicology*, 6th ed.; Klaassen, C. D., Ed.; McGraw-Hill Medical Publication Division: New York, 2001; pp 133−224.

Received for review May 25, 2004. Revised manuscript received June 29, 2004. Accepted July 14, 2004.

JF049158J