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Cutinase Inhibition by Means of Insecticidal Organophosphates and Carbamates. 3. Oxidation of Phosphorothionates by Chloroperoxidase from *Caldariomyces fumago*

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Chloroperoxidase (CPO) from *Caldariomyces fumago* combined with hydrogen peroxide and chloride proved to be most efficient for the transformation of organophosphorothionate pesticides, i.e., chlorpyrifos, chlorpyrifos-methyl, parathion, and parathion-methyl, into their more potent serine esterase inhibiting oxon analogues. Following CPO pre-oxidation steps, the detection limit of a recently described spectrophotometric cutinase assay could be increased by about 2 orders of magnitude as a consequence of increased inhibition rates of the organophosphates. This type of enzymatic oxidation is easier to perform and more efficient, as compared to bromine or *N*-bromosuccinimide, used for acetylcholine esterase (AChE) assay in water analyses, but is insufficient for complex matrices such as plant sample extracts. The performance of a complete assay, including sample preparation, oxidation, and inhibition, takes about 3 h. Performing oxidations of organophosphorus compounds, two significant anomalies were observed. Upon CPO oxidation, chlorpyrifos-methyl showed a very strong cutinase inhibition as compared to the corresponding oxon standard, and oxidized malathion, contrarily to malaoxon, revealed cutinase inhibition, which however obeyed a reversible reaction mechanism in contrast to the usually irreversible reactions of organophosphates. Except for methomyl, no significant effects of CPO oxidation on the inhibition strength of insecticidal carbamates could be detected. The applicability of the assay was tested with fruit samples spiked with chlorpyrifos at 0.2–0.5 mg/kg, thereby regarding the role of the latter as the pesticide detected most often in fruits. Mean recoveries ranged between 30–50%. An enhanced recovery of 84% was obtained for an apple juice sample spiked with parathion-methyl (0.5 mg/L).

KEYWORDS: Chloroperoxidase from *Caldariomyces fumago*; oxidation; pesticides; cutinase inhibition; enzyme assay; organophosphorus insecticides; carbamates

INTRODUCTION

An enzyme assay enabling a rapid and sensitive screening for insecticidal organophosphates and carbamates based on inhibition of cutinase *Fusarium solani pisi* (EC 3.1.1.74) has been described previously (*1, 2*). Organophosphorus insecticides are frequently applied as less toxic phosphorothionates, characterized by a thiono moiety (P=S). However, the corresponding organophosphorus oxon analogues (P=O) are of stronger cutinase inhibiton (*2*), thus making a preceding oxidation step necessary to improve the sensitivity of the enzyme assay.

According to DIN 38415-1 (*3*), oxidations are performed by *N*-bromosuccinimide, whereafter the excess of reagent is destroyed by ascorbic acid. However, this DIN method is proposed only for the analysis of drinking water being characterized by a significantly less oxidizable matrix than plant sample extracts. Because *N*-bromosuccinimide exhibits restricted water solubility, increasing the oxidant concentration is rather limited. Bromine as a further less mild potential oxidant will also be rather consumed by plant matrices and, additionally, has considerable influence on the enzyme activity (*4*) in the following cutinase assay. Therefore, both techniques are insufficient for use in our assay.

In vivo, thionophosphates are transformed into their oxon analogues by cytochrome P-450 mono-oxygenases (*5, 6*). Application of a prokaryotic cytochrome P450 mutant for the oxidation of organophosphorothionates was shown to successfully increase the sensitivity of an acetylcholine esterase biosensor assay (*7*). However, this enzyme mutant is not commercially available.

Chloroperoxidase (CPO) produced by the imperfect marine fungus *Caldariomyces fumago* (EC 1.11.1.10), shows cytochrome P450-like mono-oxygenase activity, in addition to

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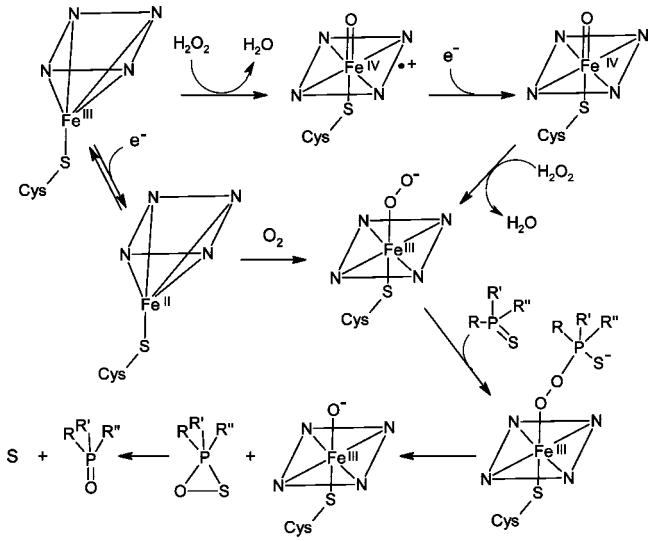


Figure 1. Proposed mechanism for phosphorothionate oxidation performed by means of chloroperoxidase from *Caldariomyces fumago* (modified, cf. Bello-Raminez et al. (*12*)).

peroxidase, halogenase, and catalase activity (*8*, *9*). CPO is a heme peroxidase containing iron(III) protoporphyrin as the prosthetic group and catalyses oxidations using hydrogen peroxide (H_2O_2) or other peroxide compounds as electron acceptors (*9*–*11*). The application of CPO to perform oxidations of organophosphorothionate pesticides, i.e., transformation of P=S into P=O, was first described by Hernandez et al. (*8*). Thereby, in the presence of H_2O_2 oxidation is achieved by the formation of a Fe^{III}-O-O^- complex at the active CPO site, which in turn interacts with the phosphorus of thionophosphates. Via a transiently formed oxidized intermediate containing P and S, the oxon analogue is formed (*12*) (**Figure 1**). Alternatively, the reactive Fe^{III}-O-O^- complex may be formed by interaction of the active CPO site with oxygen (*12*).

The aim of the present study was to evaluate the suitability of CPO and optimal reaction conditions for the thion-oxon tranformation of organophosphorous pesticides, both in standard solutions and spiked fruit samples. Additionally it had to be checked whether a CPO oxidation step has any effect on insecticidal carbamates, which are also determined by the cutinase inhibition assay.

MATERIALS AND METHODS

Reagents. Chloroperoxidase (EC 1.11.1.10) from *C. fumago* (suspension in 0.1 M sodium phosphate pH 4.0; >10,000 U/mL; applied charge 26,776 U/mL) was purchased from Sigma (Taufkirchen, Germany). Cutinase from *Fusarium solani pisi* (EC 3.1.1.74),lyophilized, was provided from Unilever Research Laboratory (Vlaardingen, The Netherlands), and the solution was prepared as described in ref *1*. Pesticide reference standards were obtained from Riedel-de-Haën (Taufkirchen, Germany) or Dr. Ehrenstorfer GmbH (Augsburg, Germany) (*2*). 3,5,6-Trichloro-2-pyridinol was from Riedel-de-Haën (Pestanal), and dimethyl phosphate was from Acros Organics (Geel, Belgium). Hydrogen peroxide 30% (i.e., 8.82 mol/L), 3-chloroperbenzoic acid (technical grade, ~70%), and monochlorodimedone (2-chloro-5,5-dimethyl-1,3-cyclohexanedione, ≥98.0%) were obtained from Fluka (Taufkirchen, Germany); *tert*-butyl hydroperoxide (70% aqueous solution, i.e., 7.77 mol/L) and magnesium sulphate (≥99.5%) were from Sigma-Aldrich (Taufkirchen, Germany); and peracetic acid (32%, in diluted acetic acid) and polyethylenimin (low mol wt, M_w 2,000, 50% solution in water) were from Aldrich (Taufkirchen, Germany). Sodium chloride (p.a.) was obtained from Merck (Darmstadt, Germany). Blank samples of kiwi, cherry, peach, and apple juice were purchased from a local organic produce store. All solvents used were of analytical grade (Merck). Water was prepared with a Milli-Q 185 Plus water purification system (Millipore, Eschborn, Germany).

Solutions. Chloroperoxidase solution was prepared by dissolving 192 mg in 5 mL of acetate buffer (0.05 mol/L, pH 4.8). Pesticide stock solutions of 50–1000 mg/L were prepared in methanol, and working standards of 0.01–100 mg/L (depending on inhibition strength and limits of detection (*2*)), were prepared by dilution with water or water/methanol mixtures, respectively, resulting in methanol concentrations of 10%. From a 3,5,6-trichloro-2-pyridinol stock solution (100 mg/L, in methanol) working standards of 0.1−10 mg/L were prepared by aqueous dilution. Dimethyl phosphate standards of 1–50 mg/L were obtained by aqueous dilution of a 500 mg/L methanolic stock solution. Peroxide solutions were prepared as follows: hydrogen peroxide (H_2O_2) was 10-fold diluted by water (i.e., 0.88 mol/L); *m*-chloroperbenzoic acid (*m*CPBA, 0.21 g, 0.85 mmol) was dissolved in 10 mL of methanol and diluted up to 25 mL with sodium acetate buffer (50 mM, pH 4.8) (i.e., 34 mmol/L); peracetic acid (AcOOH) solution was prepared by diluting 3 mL of 32% AcOOH up to 50 mL (i.e., 0.3 mol/L) with 1.2 M NaOH solution for adjustment to pH 7. Oxidations were performed in sodium acetate buffer (50 mM, pH 4.8) containing 0.5 M KCl (i.e., 37.28 g KCl/L) in the case of H_2O_2 oxidation, and 1.34 M KCl (i.e., 100 g KCl/L), respectively, for the oxidation applying *t*-BuOOH, *m*CPBA, and AcOOH. Polyethylenimin (PEI) solution (18.5 mM) was prepared by dissolving 1.5 mL of PEI in 20 mL of 50 mM acetate buffer under adjustment of the alkaline solution to pH 4.8 by 5 M hydrochloric acid. Monochlorodimedone (MCD) stock solution (20 mM) was prepared in sodium acetate buffer (50 mM, pH 4.8; containing 20 mM KCl) and diluted 1:250 with water, resulting in a 0.08 mM solution.

Analytical Procedures. *Principles and Conditions of the Cutinase Assay.* For determination of the inhibition effect of insecticides - after preceding oxidation - on cutinase, the enzyme solution was incubated with variously concentrated pesticide standards under defined reaction conditions (25°C; 30 min). Afterwards, the residual enzyme activity was measured by means of turnover of the substrate *p*-nitrophenyl butyrate at $\lambda = 405$ nm (*1*).

Oxidation by Means of Chloroperoxidase. Oxidations were performed in 0.5-mL flip-top polypropylene microcentrifuge tubes (Brand, Wertheim, Germany). Pipetting schemes for alternatively tested oxidation steps, i.e., applying chloroperoxidase combined with H_2O_2, *t*-BuOOH, *m*CPBA, and AcOOH, are given in **Table 1**. Oxidations combined with H_2O_2, *t*-BuOOH, and AcOOH were performed at 5 °C, and assays with *m*CPBA at 20 °C, respectively. After oxidation was complete, samples were sonicated (Sonorex super RK 106, 35 kHz, Bandelin electronic, Berlin, Germany) at 20 °C for about 15 min (until no more gas bubbles were formed) and either examined by means of TLC or subjected to the cutinase assay (*1*).

Determination of Chloroperoxidase Activity under Assay Conditions. Determination of chloroperoxidase activity was recorded with a dual-beam Cary 1 spectrometer (Varian, Darmstadt, Germany) in disposable semi-micro UV cuvettes of 1 cm path length (Brand, Wertheim, Germany). Monochlorodimedone (MCD) [molar extinction coefficient ($\lambda_{max} = 292$ nm) $\varepsilon = 20{,}683$ L mol^{-1} cm^{-1} (±5.0%, $n = 8$)] was applied as substrate, one unit being defined to convert 1 μmol of MCD into dichlorodimedon (DCD) per minute (*13*). Hereby, the pesticide solution was replaced by a 0.08 mM MCD solution, and all other solutions , i.e., chloride containing acetate buffer, CPO, and H_2O_2 (**Table 1**: H_2O_2), were diluted by a factor of 1:100 in order to avoid gas bubble formation. The initial batch absorbance was 0.6815 ± 0.9% ($n = 4$), i.e., <1.0. Absorbance decrease due to conversion of MCD into DCD was spectrophotometrically monitored at 292 nm as a function of time; DCD exhibited no absorbance at this wavelength, as proven by scanning the batch at the end of the transformation. The absorbance change ΔA/min was determined to be 0.1767 ± 2.0% ($n = 4$), the reaction time for complete transformation was 3.87 ± 3.3% min ($n = 4$), and the CPO activity was 184 U/mL (i.e., 0.178 U/mg).

Sample Preparation. According to the QuECHERS method (*14*), a 10-g aliquot of a previously homogenized and spiked sample was weighed into a 25-mL glass centrifuge tube (with screwable Teflon cap; Schott, Mainz, Germany), supplemented by 10 mL of acetonitrile

Table 1. Pipetting Schemes for Enzymatic Oxidations by Means of Chloroperoxidase and Different Peroxides, Given in the Sequence of Practicability

	H_2O_2	*t*-BuOOH	*m*CPBA	AcOOH
CPO solution	25 μL (0.17 U)	25 μL (0.17 U)	25 μL (0.17 U)	25 μL (0.17 U)
KCl containing acetate buffer (pH 4.8)	50 μL (KCl 0.5 mol/L, i.e., 25 μmol)	25 μL (KCl 1.34 mol/L, i.e., 33.5 μmol)	50 μL (KCl 1.34 mol/L, i.e., 33.5 μmol)	25 μL (KCl 1.34 mol/L, i.e., 33.5 μmol)
pesticide or sample extract solution	100 μL	100 μL	100 μL	100 μL
peroxide solution	25 μL (22 μmol)	25 μL (194 μmol)	50 μL (1.7 μmol)	50 μL (15 μmol)
		30 min Incubation		
CPO solution	25 μL	25 μL	25 μL	
peroxide solution	25 μL (22 μmol)		50 μL (1.7 μmol)	
		30 min Incubation		
CPO solution	25 μL			
peroxide solution	25 μL (22 μmol)			
		45 min Incubation		
ultrasonic bath	15 min	15 min	15 min	15 min
pesticide dilution	1:3	1:2	1:3	1:2

(MeCN), and vigorously shaken for 1 min. After 4 g of anhydrous $MgSO_4$ and 1 g of NaCl were added, the samples were shaken for another 1 min and subsequently centrifuged for 5 min at 3750 rpm (Biofuge primo R; Heraeus Instruments, Hanau, Germany). For juice samples, 10 mL was spiked and analogously extracted.

For solid-phase extraction (SPE) cleanup, a combination of (i) SAX/PSA Supelclean dual layer SPE cartridges (6 mL, 500 mg each) and (ii) Discovery DSC-18 SPE cartridges (6 mL, 1 g) (Supelco, Taufkirchen, Germany) was used by means of a Visiprep vacuum manifold (Supelco) operated at 600 mbar. To prevent the influence of extractable SPE materials on enzyme activity, cartridges must thoroughly be pretreated (SAX/PSA: 6 × 6 mL MeCN; DSC-18: 2 × 6 mL MeCN, 2 × 6 mL H_2O, 6 × 6 mL MeCN).

The supernating sample extract was given over the cartriges combination followed by rinsing with 6 mL of MeCN. The eluate was collected in a 25-mL pear-shaped flask, evaporated to about 2 mL by applying a rotary evaporator under vacuum, and afterwards evaporated to complete dryness under a stream of nitrogen. The residue was taken up in 1 mL of aqueous methanol (10%) and subjected to the oxidation step and subsequently to the cutinase assay. For pesticide standards, measurements were performed against blanks of aqueous methanol (10%), which also underwent the oxidation procedure. Spiked samples were evaluated against corresponding matrix blanks.

Thin-Layer Chromatography (TLC). A Linomat IV sample applicator (CAMAG, Muttenz, Switzerland) was used to apply samples as 12-mm zones onto 10 cm × 10 cm TLC plates (silica gel 60 F_{254} on aluminium foil, layer thickness 0.2 mm) (Merck, Darmstadt, Germany). The entire oxidation batch volume (**Table 1**) was applied. After development in a 10 cm × 10 cm glass twin-trough chamber (CAMAG), spots were evaluated by means of a CAMAG TLC Scanner 3 and winCats 4 software. Additionally, the following TLC spraying reagents were applied: palladium(II) acetate reagent prepared by dissolving 400 mg Pd(II) acetate (purum, 47% Pd, Fluka, Taufkirchen, Germany) in 50 mL of a mixture of ethanol/acetone/hydrochloric acid (37%) (5/4/1) (*15*); zinc chloride/diphenylamine reagent as solutions of 1.0 g $ZnCl_2$ (puriss. p.a.; Fluka) and 0.5 g diphenylamine (p.a., Merck) in 100 mL of acetone (*16*); according to ref *17*, solutions of Na_2CO_3 (20% (w/v) in water) and *p*-chloranil (tetrachloro-*p*-benzochinone, Merck, 0.5 % (w/v) in acetone) were sequentially sprayed for the detection of monocrotophos. Further details of the applied TLC conditions are given in **Table 2**.

High-Performance Liquid Chromatography/Mass Spectrometry (LC/MS). LC/MS analyses were performed on a HP1100 system consisting of an autosampler, a gradient pump, and a diode array detector (DAD) module (Hewlett-Packard, Waldbronn, Germany), coupled to a VG platform II quadrupole mass spectrometer (Micromass, Manchester, UK), equipped with an electrospray interface (ESI): MS parameters ESI+ or ESI–, source temperature 120 °C; capillary voltage 3.5 kV; HV lens 0.5 kV; cone voltage 30 V. For LC analyses, the MS system was operated either in the full scan mode (*m/z* 100–400) or in selected ion mode (SIM). MassLynx 3.2. software was used for data acquisition and processing. Separation was performed at 25 °C on a reversed phase column (5 μm, ReproSil-Pur C_{18}-AQ, 250 mm × 4 mm, Dr. A. Maisch, Ammerbuch, Germany). For gradient elution methanol (A) and ammonium formate buffer (10 mM, pH 4.0) (B) were used: %A (*t* [min]): 20 (0)–100 (20)–100 (25)–20 (30); flow rate 0.8 mL/min; injection volume 20 μL.

For LC/MS analyses the batches of oxidized 50 mg/L standards were 5-fold concentrated before measurements by means of a nitrogen stream at room temperature.

RESULTS AND DISCUSSION

Optimization of CPO Reaction Conditions. Oxidation of phosphorothionates is able to enhance the cutinase inhibiting strength, as expressed in terms of the coefficient k_i, by about two orders of magnitude (*2*). In order to achieve a biocatalytic oxidation, combinations of CPO and four different peroxides were tested with respect to their efficiency. The oxidation batches were controlled by thin-layer chromatography for complete conversion and the formation of oxidation byproducts. For the initial method development concerning optimum ratio conditions of CPO, peroxide compound, and chloride, parathion-methyl was exemplarily chosen.

Because enzyme activity is known to be significantly influenced by solvents as a result of protein denaturation (*1*), the effect of methanol used for the preparation of standards on CPO activity was tested. Methanol concentrations above 20% in the oxidation batch exhibited great influence on the completeness of oxidation by means of CPO. The restricted oxidation became obvious in a drastically reduced formation of gas bubbles as compared to aqueous pesticide solutions. However, methanol concentrations below 20% did not affect CPO activity, so that standards and sample extracts prepared in 10% methanol (*2*) proved to be well appropriate.

First, inspired by Hernandez et al. (*8*), H_2O_2 was tested as oxidant in combination with CPO. Oxidation was performed at pH 4.8 because, for this value, CPO-catalyzed oxygen release from H_2O_2 was described to be at optimum and halide anion independent (*18*, *19*). The aim was to perform oxidations in the absence of chloride, thus excluding halogenations catalyzed by CPO, thereby keeping pH < 5 to avoid CPO deactivation at pH > 6 (*5*). However, in contrast to the preceding experiences (*18*, *19*), chloride made a most important contribution to successful oxidation, whereas in its absence oxidation was incomplete under the same reaction conditions.

Optimization of H_2O_2 concentration proved to be crucial for successful oxidation. Whereas low H_2O_2 concentrations caused incomplete oxidation, high excess was responsible for enzyme inactivation. For instance, application of 44 μmol H_2O_2 instead

Table 2. Experimental Conditions of Thin-Layer Chromatography

mobile phase[a]	pesticide (hR_F)	detection wavelength (nm)	LOD ng/zone	LOD mg/L[b]
	Organophosphorus Insecticides			
n-hexane/diethyl ether/ethanol/ethyl acetate/formic acid, 90/4/2.6/2.5/0.1 (*13*)	chlorpyrifos (68)	290	230	0.8
	chlorpyrifos oxon (15)	290	135	0.5
	parathion (37)	286	50	0.2
	paraoxon (9)	286	50	0.2
	parathion-methyl (27)	286	10	0.03
	paraoxon-methyl (4)	278	25	0.08
n-hexane/diethyl ether/ethanol/ethyl acetate/formic acid, 90/8/5.2/5/0.2	chlorpyrifos-methyl (82)	290	320	1.1
	chlorpyrifos-methyl oxon (23)	290	45	0.2
	3,5,6-trichloro-2-pyridinol (39)	318	490	1.6
n-hexane/diethyl ether/ethanol/ethyl acetate/formic acid, 54/20/13/13/0.1	dichlorvos (65)	200	2500	8.3
	monocrotophos (25)	220	580	1.9
		500[d]	4000	10
	chlorfenvinphos (87)	290	345	1.2
	demeton-*S*-methyl (n.d.[c])			
	acephate (n.d.[c])			
n-hexane/tetrahydrofuran, 72/20 (*13*)	malathion (52)	390[e]	150	0.5
	malaoxon (14)	390[e]	440	1.5
	Carbamate Insecticides			
n-hexane/diethyl ether/ethanol/ethyl acetate/formic acid, 65/20/13/13/0.1	methomyl (46)[e]	230	95	0.3
	carbaryl (81)	210	110	0.4
	propoxur (61)[f]	200	250	0.8
	carbofuran (59)[e,f]	200	215	0.7
	ethiofencarb (64)[e]	200	250	0.8
	oxidation byproducts (41, 21)[e]	200		
	pirimicarb (58)	240	850	2.8
	oxidation byproducts (63, 21, 15)	300		

[a] Mixing by volume. [b] If the batch volume is 300 μL as in the case of oxidation with chloroperoxidase and hydrogen peroxide. [c] n.d.: not detectable by either UV absorption or by means of spraying reagents. [d] Na_2CO_3/chloranil reagent: red zone. [e] Pd(II) reagent: light to dark brown zone. [f] $ZnCl_2$-DPA reagent: blue zone.

of 22 μmol drastically reduced the conversion of parathion-methyl. This observation is in accordance with previous results (*9*, *20*, *21*) reporting CPO inactivation by H_2O_2 due to oxidation of the porphyrin ring (*22*). However, inactivation was successfully avoided by stepwise addition of a low concentration H_2O_2 solution, combined with further CPO dosages (**Table 1**). Whilst one dosage of reagents was sufficient for the transformation of a 10 mg/L parathion-methyl standard, for a 50 mg/L standard transformation was still incomplete after the second addition of oxidation reagents but successful after a third reagents dosage. However, pipetting only H_2O_2 after 30 and 60 min, without CPO, failed to complete oxidation. To slow down oxygen formation and to keep it in the reaction solution, batches were kept at 5 °C (*10*) during the whole oxidation procedure.

The presence of polyethylenimin (PEI), a cationic polymer, is reported to diminish peroxide dependent CPO inactivation (*9*) and, therefore, was tested to increase the CPO tolerance against H_2O_2 and, consequently, to avoid stepwise oxidation reagent dosages. However, the addition of PEI to the oxidation batch proved to go along with a significant reduction of CPO activity. Thus, addition of 25 μL of PEI solution (0.5 μmol PEI, i.e., 0.8%) to the CPO/H_2O_2 oxidation batch did not succeed in complete oxidation of a 50 mg/L parathion-methyl standard. In the presence of 25 μL of 1:10 diluted PEI solution (0.05 μmol PEI, i.e., 0.08%), oxidation was also incomplete. Therefore, the stabilizing effect of PEI was not usable, due to the decrease of CPO activity, which was also described by Andersson et al., suggesting the stabilizing effect of the polymer to be of shielding type (*9*). Thus, the peroxide concentration nearby CPO is reduced, leading to a slowed down oxidation activity.

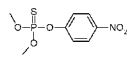
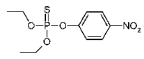
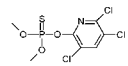
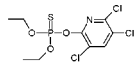
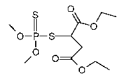
Under the optimized conditions, the oxidation assay by CPO and H_2O_2 was able to completely convert parathion-methyl in standard concentrations of up to 50 mg/L into their corresponding oxons. The obtained inhibition constants agreed well with those determined for the corresponding oxon standards (**Table 3**), and the measured calibration curves of parathion-methyl oxon and CPO/H_2O_2 oxidized parathion-methyl, exemplarily shown in **Figure 2**, were quite identical.

Application of Further Peroxides. Chloroperoxidase is described as being able to use a variety of hydroperoxides and peroxy acids (*10*, *18*) for the performance of its reactions. With the aim to minimize oxidation time and batch volume alternative peroxide compounds, i.e., *tert*-butyl hydroperoxide (*t*-BuOOH, known for enhanced CPO tolerance (*9*)), *m*CPBA, and AcOOH, were tested. Pipetting schemes of these alternative co-oxidants were also optimized by means of TLC screening (**Table 1**).

Enhanced stability of CPO towards *t*-BuOOH was confirmed by the non-necessity of further *t*-BuOOH dosages and, consequently, reduced batch volume and pesticide dilution. However, comparing the k_i values of oxidized parathion-methyl and the corresponding oxon standard the deviation was significantly higher than for the CPO/H_2O_2 oxidation procedure (**Table 3**).

When applying *m*-chloroperbenzoic acid as peroxide compound, the reduced solubility of *m*CPBA in aqueous buffers, especially at lower temperatures, was a handicap. Therefore, the oxidation was performed at room temperature, in contrast to the other peroxides. Comparing the k_i values of oxidized parathion-methyl and the corresponding oxon standard, a deviation of about 30% was obtained (**Table 3**). The combination of CPO and peracetic acid (AcOOH) offered the best performance as compared with the former co-oxidants, resulting in a smaller batch volume going along with the lowest pesticide dilution in addition to reduction of assay time (**Table 1**). Deviations of determined k_i values of oxidized parathion-methyl and the oxon standard were tolerable (**Table 3**). However, in the case of AcOOH the strong acid pH was a disadvantage. Thus, CPO and un-neutralized 2% AcOOH solution successfully performed oxidation as proven by TLC. The cutinase assay,

Table 3. Oxidation of Organophosphorus Thion Pesticides by Chloroperoxidase from *Caldariomyces fumago*[a]

				k_i [L/(mol·min)]			
				after oxidation			
pesticide	structure formula	completeness of oxidation[1)]		calculated (RSD, n)[4)]	slope[4)]	oxon standard *(2)*	deviation[2)]
parathion-methyl		1	+	$2.5 \cdot 10^3$ (±12.0%, 20)	$2.2 \cdot 10^3$	$2.1 \cdot 10^3$	+4.8%
		2	+	$4.0 \cdot 10^3$ (±11.9%, 17)	$3.6 \cdot 10^3$		+71.4%
		3	+	$1.5 \cdot 10^3$ (±13.2%, 19)	$1.5 \cdot 10^3$		-28.6%
		4	+	$2.4 \cdot 10^3$ (±25.5%, 34)	$1.8 \cdot 10^3$		-14.3%
parathion		1	+	$1.4 \cdot 10^4$ (±4.8%, 16)	$1.4 \cdot 10^4$	$1.6 \cdot 10^4$	-12.5%
		2	+	$1.9 \cdot 10^4$ (±12.1%, 19)	$1.9 \cdot 10^4$		+18.8%
		3	n.t.	$1.1 \cdot 10^4$ (±3.4%, 21)	$1.1 \cdot 10^4$		-31.3%
		4	+	$1.4 \cdot 10^4$ (±11.7%, 34)	$1.2 \cdot 10^4$		-25.0%
chlorpyrifos-methyl		1	+	$7.4 \cdot 10^5$ (±4.0%, 13)	$7.4 \cdot 10^5$	$1.3 \cdot 10^4$	+5592%
chlorpyrifos		1	+	$6.4 \cdot 10^5$ (±6.8%, 13)	$6.0 \cdot 10^5$	$9.4 \cdot 10^5$	-36.2%
malathion		1	+	$2.6 \cdot 10^3$ (±14.0%, 9)[3)]	$4.7 \cdot 10^2$	0	(∞)
		2	+	$3.0 \cdot 10^3$ (±31.3%, 31)[3)]			
		3	n.t.	$4.8 \cdot 10^2$ (±21%, 15)			
		4	+	$1.5 \cdot 10^3$ (±24.7%, 15)[3)]			

[a] Legend for notes: (1) Controlled by TLC; standard concentrations 50 mg/L, except 25 mg/L for chlorpyrifos; peroxide used: H_2O_2 (**1**), *t*-BuOOH (**2**), mCPBA (**3**), AcOOH (**4**). (2) Relative difference between the k_i value after oxidation and that of the corresponding oxon standard, calculated with k_i values obtained from slopes. (3) Reversible inhibition, k_i calculated for standard concentration ≤25 mg/L. (4) Compare ref *1*.

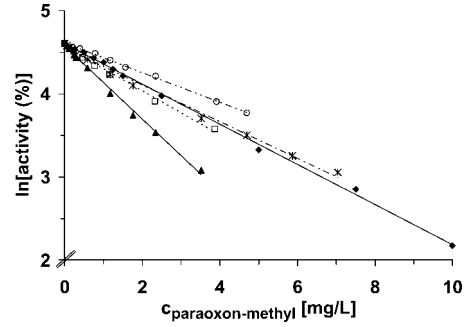


Figure 2. Inhibition of cutinase as a function of paraoxon-methyl concentration in the inhibition batch, i.e., oxidized parathion-methyl: paraoxon-methyl standard (♦); parathion-methyl oxidized by means of chloroperoxidase in the presence of *m*-chloroperbenzoic acid (○), peracetic acid (*), hydrogen peroxide (□), *tert*-butyl hydroperoxide (▲); semi-logarithmic plot, i.e., natural logarithm of the residual cutinase activity after incubation (%), ln[activity (%)], vs inhibitor concentration. For clarity error bars were omitted; relative standard deviations (RSD) were in the range of 1–10%.

however, did not work on batches exhibiting pH 5, as indicated by releasing protonated and, therefore, colourless *p*-nitrophenol and also by reduced cutinase activity in acidic medium. On the other hand, AcOOH solution was not stable at neutral pH, as indicated by gas bubble formation, so that, necessarily, solutions had to be freshly prepared, a significant drawback for the application of this peroxide compound.

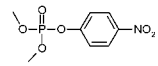
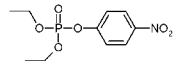
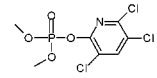
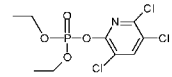
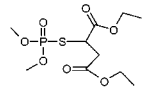
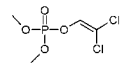
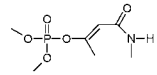
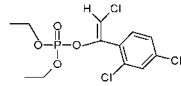
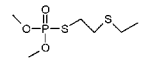
Summarizing the advantages and disadvantages of the various peroxides, application of H_2O_2 proved to be the oxidant of choice, yielding the least deviations of k_i values for oxidized parathion-methyl and parathion as compared to the corresponding oxon standards (**Table 3**).

Influence of Chloride on Oxidation. Chloride proved to be the key component in performing complete oxidations for combinations of CPO with all tested peroxides, in agreement with the report of previous studies (*8*, *23*). Furthermore, oxidations without Cl^- by means of CPO and different peroxide compounds have been found to take several days, yielding rates of only ≤60% (*10*).

Acceleration of oxidation in the presence of chloride may be explained by the formation of a certain content of chlorine, catalyzing the oxygen release. In terms of the oxidation scheme discussed for organophosphorus pesticide (**Figure 1**) (*12*), a reactive Fe^{III}–O–O^- intermediate may be formed by reaction of the active site with O_2, alternatively to a reaction with two molecules of peroxide, which as a two-step reaction is expected to occur with slower speed than the former one-step reaction.

However, for all four oxidizing steps, the chloride concentration was strictly minimized. Surprisingly, CPO in the presence of Cl^- was able to completely oxidize even a 10 mg/L parathion-

Table 4. Effect of Chloroperoxidase/H_2O_2 Oxidation of Organophosphorus Oxons on Cutinase Inhibition Constants (k_i)

		k_i [L/(mol·min)]		
pesticide	structure formula	after exposure to oxidation reagents (RSD, n)	without exposure to oxidation reagents (RSD) *(2)*	deviation
paraoxon-methyl		$2.0 \cdot 10^3$ (±8.7%, 6)	$2.1 \cdot 10^3$ ($2.0 \cdot 10^3$ ± 12.2%)	-4.8%
paraoxon		$1.5 \cdot 10^4$ (±6.8%, 7)	$1.6 \cdot 10^4$ ($1.6 \cdot 10^4$ ± 6.1%)	-6.3%
chlorpyrifos-methyl oxon		$2.9 \cdot 10^4$ (±26.8%, 4)	$1.3 \cdot 10^4$ ($1.3 \cdot 10^4$ ± 24.8%)	+123%
chlorpyrifos oxon		$6.4 \cdot 10^5$ (±6.0%, 4)	$9.4 \cdot 10^5$ ($9.6 \cdot 10^5$ ± 9.0%)	-31.3%
malaoxon		$1.1 \cdot 10^3$ (±9.0%, 4)[1)]	0	(∞)
dichlorvos		$6.2 \cdot 10^3$ (±19.6%, 4)	$6.0 \cdot 10^3$ ($6.9 \cdot 10^3$ ± 4.4%)	+3.3%
monocrotophos		$4.8 \cdot 10^1$ (±32.4%, 3)	$5.8 \cdot 10^1$ (49 ± 48.6%)	-17.2%
chlorfenvinphos		0	0	0
demeton-S-methyl		$4.8 \cdot 10^1$ (±39.6%, 3)	0	(∞)
acephat		0	0	0

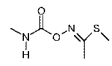
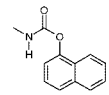
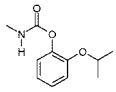
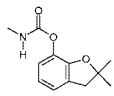
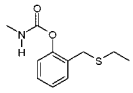
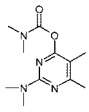
methyl standard in the absence of any peroxide compound, probably by using oxygen from the air, in agreement with the proposal of O_2 supported oxidation (*12*).

Subjection of Organophosphorus Thions, Oxons, and Carbamates to the Oxidation Step. For most oxidized thions under study, deviations in k_i values from those of the oxon standards were negligible (**Table 3**), and no further oxidation products other than the corresponding oxons were detectable by TLC. However, large deviations occurred in the cases of chlorpyrifos-methyl and malathion, which therefore will be separately discussed.

The influence of oxidation reagents on organophosphates and carbamates, both featuring no necessity of oxidation, was also tested by TLC and the cutinase assay. Although biocatalytic oxidation is a mild reaction, oxidative reagents were not without any influence on all organophosphates and carbamates (**Tables 4** and **5**). Whereas the phosphorothiolate demeton-*S*-methyl had no effect on cutinase (*2*), a weak inhibition was found after CPO oxidation (**Table 4**). A possible explanation can be seen in sulfoxidation of the mercaptane rest, since CPO is known to perform sulfoxidations of thio ethers (*24*). To confirm this theory, concentrated oxidation batches of demeton-*S*-methyl were subjected to LC/MS analyses. Surprisingly, the parent compound had completely disappeared, and one more polar peak was detected offering quasimolecular ions at *m*/*z* 247 and 263 of nearly the same intensities (**Table 6**). Thus, demeton-*S*-methyl was quantitatively oxidized to both the sulfoxide and sulfone under CPO conditions. Concerning the slight cutinase inhibition, it must be concluded that, due to the sulfoxidized side chain, a nucleophilic attack of cutinase's active site is facilitated as compared to the parent insecticide. This is in agreement with toxicological data: I_{50} values of the corresponding sulfoxide and sulfone are slightly reduced as compared to demeton-*S*-methyl (*25*), the same as described for the ethyl analogues (*26*).

Therefore, the stronger inhibition effect of the carbamate insecticides ethiofencarb and methomyl on cutinase after the CPO assay (**Table 5**) might also be the result of sulfoxidation. As in the case of demeton-*S*-methyl, ethiofencarb was not present in the oxidation batch anymore, but two zones with lower R_f values were visible on TLC (**Table 2**). Whilst the stronger retention is already in agreement with sulfoxidation, the results could be confirmed by means of LC/MS measurements. Instead of ethiofencarb, two peaks were obtained providing quasi-molecular ions at *m*/*z* 242 and 258, respectively, thus proving the formation of ethiofencarb sulfoxide and sulfone (**Table 6**). Additionally, a third compound co-eluting with the sulfoxide was detected and, in terms of molecular mass and fragmentation, tentatively identified as the benzaldehyde derivative resulting from oxidation of the ethiofencarb benzyl group. This compound was already described as a photoproduct of CIBA's C-8353 (2-(1,3-dioxolane-2-yl)-phenyl-*N*-methyl carbamate) offering identical MS data (*27*). Thus ethiofencarb was nearly evenly transformed into the sulfoxide, the sulfone, and 2-(methylcarbamoyl)-benzaldehyde, which in combination must

Table 5. Effect of Chloroperoxidase/H_2O_2 Oxidation of Carbamate Insecticides on Cutinase Inhibition Constants (k_i)[a]

		k_i [L/(mol·min)]		
pesticide	structure formula	after exposure to oxidation reagents (RSD, n)	without exposure to oxidation reagents *(2)*	deviation
methomyl		$2.2 \cdot 10^3$ (±6.4%, 21)	$7.5 \cdot 10^2$	+193%
carbaryl		$2.9 \cdot 10^2$ (±13.3%, 4)	$2.6 \cdot 10^2$	+12%
propoxur		$1.3 \cdot 10^2$ (±30.6%, 4)	$7.3 \cdot 10^1$	+78%
carbofuran		$1.7 \cdot 10^2$ (31.7%, 4)	0	$(\infty)^{1)}$
ethiofencarb		$1.1 \cdot 10^2$ (±46.0%, 3)	$5.5 \cdot 10^1$	+100%
pirimicarb		$1.1 \cdot 10^2$ (±53.0%, 4)	$9.4 \cdot 10^1$	+17%[2)]

[a] Legend for notes: (1) Only weak inhibition after exposure to oxidative reagents: 50 mg/L (std concn) ≙ 90.9% residual activity; no further zones on TLC. (2) Despite occurrence of further TLC zones under oxidative conditions, there is no influence on cutinase inhibition.

be responsible for the increased cutinase inhibition as compared to the parent compound. Although oxidized methomyl exhibited significantly stronger inhibitory power, no LC/MS peaks at all were found, either in positive or negative ESI mode, after exposure of methomyl to the CPO oxidation. The insecticide will apparently be completely degraded under the oxidative conditions, resulting in products of molecular masses below 100, which were not accessible by the LC/MS settings and also not detectable by TLC. After oxidation of pirimicarb, three further but rather small zones were detectable by TLC; however, there was no change in cutinase inhibition (**Table 5**).

Malathion. Both malathion and malaoxon proved not to be cutinase inhibitors (*2*). In contrast, oxidized malathion unambiguously caused cutinase inhibition (**Table 3**), and even oxidized malaoxon resulted in the same inhibition quality (**Table 4**), which probably originates from the same reaction product. According to the TLC results, oxidative conversion of malathion into malaoxon arose completely applying all tested peroxides, but additional zones of byproducts were not detectable. By means of LC/MS, however, a small additional peak was detected in the oxidation batch, offering quasi-molecular ions at *m/z* 331 and 329 (**Table 6**). The first one is well in agreement with monoxygenase-type oxygen insertion into the activated thiomalate methylene group as the only position coming into question. The resulting "hydroxymalathion" was partly oxidized to the corresponding oxon derivative.

Concerning the cutinase assay, the results deviated from the expectations regarding the experiences with other organophosphorous pesticides. There was no exponential correlation between residual cutinase activities and inhibitor concentrations, i.e., a linear correlation for semilogarithmic plotting as exemplarily shown in **Figure 2**, but the plots rather approached a constant, concentration-independent level (**Figure 3**), indicating a reversible enzyme inhibition (*28*). However, in respect to the cutinase inactivity of malaoxon and the low amount of oxidation byproducts formed during the CPO assay, the oxidized malaoxon compounds must have a high inhibition power (estimated to $k_i \approx 3 \times 10^4$ L mol^{-1} min^{-1}), although reversible in type.

Chlorpyrifos-methyl. Oxidized chlorpyrifos-methyl exhibited an inhibitory effect on cutinase significantly stronger than that of the corresponding oxon standard (**Table 3**). Whilst the parent compound completely disappeared and the corresponding oxon was formed, a further compound was detected by TLC. This oxidation byproduct was also found after exposure of chlorpyrifos-methyl oxon to the CPO oxidation. "Oxidized" chlorpyrifos-methyl oxon also proved to be a stronger cutinase inhibitor than the oxon standard (**Table 4**). LC/MS measurement provided a most significant contribution to the identification of the oxidation byproduct of chlorpyrifos-methyl. Only in the negative ESI mode was a significant signal obtained, which was assigned to the quasi-molecular ion of 3,5,6-trichloro-2-pyridinol at *m/z* 196 (**Table 6**). This could be confirmed by comparison with a purchased standard exhibiting the same retention time and mass spectrum. A rather small peak was additionally detectable offering a $[M - H]^-$ at *m/z* 290, quite in agreement with singly demethylated chlorpyrifos-methyl oxon.

To explain the increased k_i value of oxidized chlorpyrifos-methyl as compared to the oxon standard, both 3,5,6-trichloro-2-pyridinol and dimethylphosphate as hydrolysis products formed during CPO oxidation were checked with respect to their inhibition effect on cutinase. Both proved to be cutinase inhibitors, however, of inferior strength to chlorpyrifos-methyl oxon. Thus, inhibition constants of 1.4×10^3 and 1.3×10^2 L mol^{-1} min^{-1} were determined for 3,5,6-trichloro-2-pyridinol and dimethyl-phosphate, respectively. Whilst in literature dimethyl phosphate is described to be about as inhibitory to human plasma choline esterase as parathion (*29*), trichloro-pyridinol is described as not inhibiting the AChE of the electric eel (*30*). Regarding these references and our results, the increased inhibition effect on cutinase after oxidation is unfortunately not explained.

Interestingly, no byproducts were detectable by TLC for chlorpyrifos and chlorpyrifos oxon under identical conditions of CPO oxidation. However, analysing the concentrated oxidation batch of chlorpyrifos by LC/MS established that 3,5,6-trichloro-2-pyridinol was also formed, however, in rather low amounts as compared to chlorpyrifos-methyl. The enhanced stability under oxidative conditions is reflected in the deviation of k_i values of about 30% reduction, which corresponds to the general trend.

Analysis of Spiked Fruit Samples. Because of its special significance as most frequently detected pesticide (*31*, *32*), fruit samples were exemplarily spiked with chlorpyrifos, extracted following the QuChERS method (*14*), and subjected to the oxidation step and subsequent cutinase assay. Kiwi was chosen in view of its chlorophyll- and carotene-containing matrix, cherry because of its anthocyan plant pigment, and peach as carotene- and anthocyan-containing representative, respectively. In addition, apple juice was spiked with parathion-methyl, the pesticide applied for the oxidation steps optimization.

Mean recovery rates for chlorpyrifos-spiked samples were between 30% and 50% (**Table 7**), well comparable to those obtained by means of an AChE biosensor for paraoxon-spiked apple (44%) and peach puree (52%) (*35*). For parathion-methyl spiked apple juice, a recovery rate of 84% was obtained. An

Table 6. LC/MS Data (ESI Positive or Negative) of Oxidation Byproducts As Compared to the Corresponding Oxon Standards or the Parent Carbamates, Respectively

	t_R (min)		*m/z*	intensity
chlorpyrifos-methyl oxon	19.6	306	$[M + H]^+$	100%
oxidized chlorpyrifos-methyl	19.6	306	$[M + H]^+$	100%
	18.9 (90%)[a]	196	$[M - H]^-$	100%
	14.1 (10%)[a]	290	$[M - H]^-$	100%
		196	$[M - H]^- - CH_3OPO_2$	44%
malaoxon	15.7	315	$[M + H]^+$	100%
		269	$[M + H]^+ - C_2H_5OH$	17%
		127	$[M + H]^+ - C_2H_5OH$, $- (CH_3O)_2P(O)SH$	14%
oxidized malathion	15.7 (90%)[a]	315	$[M + H]^+$	100%
		269	$[M + H]^+ - C_2H_5OH$	11%
		127	$[M + H]^+ - C_2H_5OH$, $- (CH_3O)_2P(O)SH$	8%
	17.2 (10%)[a]	331	$[M + H]^+$ (hydroxymalathion)	100%
		285	$[M + H]^+ - C_2H_5OH$	18%
		329	$[M + H]^+$ (oxomalathion)	45%
		283	$[M + H]^+ - C_2H_5OH$	21%
demeton-*S*-methyl	7.5	231	$[M + H]^+$	100%
oxidized demeton-*S*-methyl	5.4	263	$[M + H]^+$ (sulfone)	100%
		247	$[M + H]^+$ (sulfoxide)	92%
		169	$[M + H]^+ - C_2H_5SO_2H/C_2H_5SOH$	10%
ethiofencarb	16.8	226	$[M + H]^+$	35%
		164	$[M + H]^+ - C_2H_5SH$	78%
		169	$[M + H]^+ - CH_3NCO]$	30%
		107	$[M + H]^+ - CH_3NCO$, $- C_2H_5SH$	100%
oxidized ethiofencarb	9.6 (72%)[a]	242	$[M + H]^+$ (sulfoxide)	54%
		185	$[M + H]^+ - CH_3NCO$	95%
		164	$[M + H]^+ - C_2H_5SOH$	49%
		107	$[(CH_3O)_2P(O)SCH{=}CH_2{+}H]^+$	84%
		180	$[M + H]^+$ (2-(methylcarbamoyl)-benzaldehyde)	77%
		162	$[M + H]^+ - H_2$	100%
		123	$[M + H]^+ - CH_3NCO$	97%
	9.2 (28%)[a]	258	$[M + H]^+$ (sulfone)	37%
		201	$[M + H]^+ - CH_3NCO$	100%
		107	$[M + H]^+ - CH_3NCO$, $- C_2H_5SOH$	100%
methomyl	7.7	163	$[M + H]^+$	30%
		106	$[M + H]^+ - CH_3NCO$	100%
oxidized methomyl	n.d.[b]			

[a] Related to the peak areas of the total ion current chromatogram. [b] No peaks detectable.

Table 7. Cutinase Assay Results of Spiked Fruit Samples after Preceding Oxidation by Chloroperoxidase and H_2O_2

sample	apple juice	kiwi	cherry	peach
spiked pesticide	parathion-methyl	chlorpyrifos	chlorpyrifos	chlorpyrifos
maximum residue limit (*33*)	0.02 mg/kg	2.0 mg/kg	0.3 mg/kg	0.2 mg/kg
spiking level	0.50 mg/L	0.50 mg/kg	0.30 mg/kg	0.20 mg/kg
determined concentration[a]	0.42 mg/L(±36%; $n = 4$)	0.25 mg/kg (±43%; $n = 5$)	0.09 mg/kg (±92%; $n = 4$)	0.08 mg/kg (±13%; n=3)
recovery	84%	50%	30%	40%
cutinase activity in blank samples	100% (±2%; $n = 4$) (undiluted)	104% (±3%; n=3) (1:100 diluted)	97% (±3%; $n = 4$); (1:10 diluted)	99% (±7%; $n = 3$) (1:10 diluted)

[a] Results were tested for outliers after Grubbs on the level of 95% (*34*). For cherry and kiwi samples no outliers could be detected; for peach samples one outlier was identified (0.37 mg/kg).

enhanced recovery of 84% was also obtained for paraoxon from spiked orange juice by means of the mentioned AChE-biosensor assay (*35*).

The sample matrix exhibited no influence on cutinase activity under the given assay conditions, since cutinase activity in matrix blank samples was about 100%, as measured against a 10% methanolic water blank (**Table 7**).

These exemplarily performed sample measurements clearly demonstrated that the developed CPO oxidation step is well suitable for practical application. In contrast, oxidation of parathion in surface extracts of apples by means of *N*-bromosuccinimide according to ref *3* did not result in any paraoxon formation (*36*). Thus, even methanolic extracts of fruit surfaces only contain such a lot of matrix consuming the added oxidant completely.

The advantage of the enzymatic oxidation consists in the high catalytic activity of biocatalysts combined with substrate specificity, whereby *in situ* performed oxidation is milder than exposure to oxidative reagents in excess. Due to the high reliability of the following cutinase assay without disturbance of chloroperoxidase, the presented enzymatic method is a most elegant means to perform oxidations. This phenomenon is advantaged by different optimum pH levels of the two enzymes. Whilst cutinase is mostly active at alkaline pH, chloroperoxidase possesses highest stability at pH 5.5-6, whereas alkaline media are causing total loss of enzyme activity (*37*, *40*).

Regarding number and diversity of tested pesticides, the obtained results are acceptable, especially with respect to the objective of this enzymatic method, i.e., fast screening-out of positive from negative samples. As demonstrated for spiked

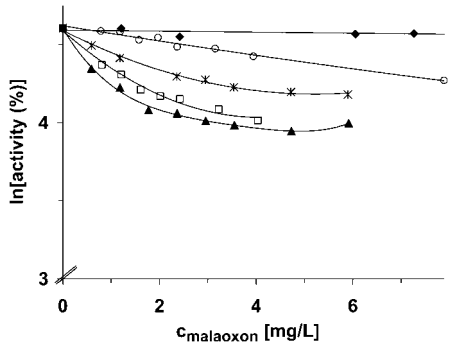


Figure 3. Inhibition of cutinase as a function of malaoxon concentration in the inhibition batch, i.e., oxidized malathion: malaoxon standard (♦); malathion oxidized by means of chloroperoxidase in the presence of *m*-chloroperbenzoic acid (○), peracetic acid (*), hydrogen peroxide (□), and *tert*-butyl hydroperoxide (▲); semi-logarithmic plot, i.e., natural logarithm of the residual cutinase activity after incubation (%), ln[activity (%)], vs inhibitor concentration. For clarity error bars were omitted; relative standard deviations (RSD) were in the range of 1–20%.

samples, the presented assay is able to detect pesticides in plant samples according to maximum residue limit requirements and thus fulfils the demands to a screening method. The here described spectrophotometric enzyme inhibition assay, including CPO oxidation, is the first one for detection of organophosphorus and carbamate insecticides in food.

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