Oxidative metabolism of quinazoline insecticide fenazaquin by Aspergillus niger

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Abstract Fenazaquin (4-(2-(4-t-butylphenyl)ethoxy)quinazoline) is a quinazoline insecticide, which contains a rare pesticidal toxophore, quinazoline. Its metabolic fate in animals and plants was previously reported. However, the microbial metabolism of the compound has never been studied. Microbial transformation is an important research area for the investigation of environmental safety issues of pesticides. Aspergillus niger was selected as a model soil fungus since it is ubiquitous in agricultural soils, with extensive genetic studies undertaken. Fenazaquin was rapidly metabolized by A. niger (half-life, $t_{1/2} = 0.6$ day). 4-Hydroxyquinazoline and 4-t-butylphenethyl alcohol were identified as major metabolites from the cultures. Fenazaquin was also rapidly transformed into the same metabolites ($t_{1/2} = 0.1–0.5$ day) under chemical oxidation ($m$-chloroperoxybenzoic acid). Among the several metabolic inhibitors, flavin-dependent mono-oxygenase inhibitor, methimazole yielded no inhibitory activity ($t_{1/2} = 1.6$ day). Several cytochrome P450 inhibitors including piperonyl butoxide, ketoconazole, and myclobutanil were also tested. Piperonyl butoxide strongly reduced fenazaquin metabolism ($t_{1/2} = 58.7$ days). However, ketoconazole and myclobutanil showed no activity even at fungi-toxic concentrations ($t_{1/2} = 1.2–4.3$ days) with major metabolites similar to those of control experiments. The results suggest that oxidative metabolism of fenazaquin was catalyzed by specific cytochrome P450s, which are insensitive toazole fungicides. In addition, piperonyl butoxide was found to be one of the most promising synergists of pesticides, through cytochrome P450 inhibition.

Keywords Aspergillus niger · Cytochrome P450 · Fenazaquin · Metabolism · Quinazoline

Introduction

Quinazoline is a compound with a unique chemical configuration, which is found in a limited numbers of pesticides (e.g., fenazaquin, 4-(2-(4-t-butylphenyl)ethoxy)quinazoline). The insecticide can be used to control several mites [1, 2]. It inhibits mitochondrial electron transport chain at site I of the respiratory chain [2, 3]. Fenazaquin is a highly lipophilic compound ($\log P = 5.5$) and hence is not translocated into plants from the agricultural environment [4]. However, few studies indicated the widespread contamination with this pesticide compared with other lipophilic pesticides [5]. Its half-life ranges from 3 to 60 days in soils and aqueous systems [6, 7]. Metabolic transformation of fenazaquin has been studied in a few animals and plants [4–6]. However, its fate in microorganisms has never been reported.

Pesticides in soil and aqueous environment are subjected to various degradative reactions, among which biotransformation is often one of the most significant determinants of degradation rates [8]. Because of their large biomass, populations, and genetic adaptation, bacteria and fungi are usually considered as major contributors in pesticide biodegradation [9, 10]. For example, several recalcitrant pesticides (e.g., organochlorine insecticides) were mineralized by white rot fungi [11–13]. In addition, numerous
non-lignolytic fungi are also known to decompose structurally diverse pesticides [14–17]. Interestingly, several phytopathogenic fungi can rapidly metabolize pesticides [17–20]. For example, many Aspergillus and Fusarium species can efficiently metabolize organophosphate and carbamate insecticides [19, 20]. The Aspergillus species are well-known producers of extra-cellular hydrolase (e.g., lipases, proteinases, and other depolymerizing enzymes). Biodegradation of ester-type pesticides (e.g., organophosphates, carbamates, and pyrethroids) are usually catalyzed by hydrolases [21]. Aspergilli are also known to metabolize many other natural and synthetic compounds. For example, Aspergillus niger can metabolize numerous synthetic flavonoids via oxidative transformation [22]. The metabolites of these xenobiotics suggest that oxidative enzymes (e.g., cytochrome P450s, CYP) take a major metabolic role. Current genomic studies indicate that the Aspergillus genome contains more than 150 CYPs [23, 24]. Due to its metabolic versatility and genetic information, Aspergillus is a promising research tool for the study of microbial pesticide metabolism.

Rapid dissipation of pesticide is an important factor in food safety issues. However, it decreases the efficacy of pesticides, which results in reduced agricultural productivity. In addition, metabolic inactivation of pesticides is one of the most common mechanisms of pesticide resistance [25]. Inhibitors of pesticide metabolism are frequently included in commercial pesticide formulations (e.g., piperonyl butoxide).

In this study, fenazaquin was subjected to metabolic transformation by A. niger. Metabolic pathways were elucidated via chemical oxidation, specific enzyme inhibitor studies, and instrumental analyses.

Materials and methods

Chemicals

The following reagents were purchased from Sigma-Aldrich Korea Ltd (Seoul, Korea); including fenazaquin, 4-t-butylphenylacetic acid, piperonyl butoxide, methimazole, ketoconazole, myclobutanil, and m-chloroperoxybenzoic acid (MCPBA). 4-Hydroxyquinazoline and 2,4-quinazolinedione were obtained from Oakwood Products Inc (PA, USA). Other reagents for the syntheses were obtained from Alfa Aesar Korea (Seoul, Korea). Potato dextrose broth was obtained from BD Korea (Seoul, Korea). Solvent was HPLC grade or higher.

Synthesis of 4-t-butylphenethyl alcohol

4-t-Butylphenethyl alcohol was prepared by literature method [26].

Kinetic study of fenazaquin biotransformation by A. niger

Aspergillus niger KACC 45093 was kindly provided by the National Agrobiodiversity center, RDA-Genebank Information Center (Jeonju, Korea). The fungal seed culture was grown on a potato dextrose broth (PDB) for 3 days at 28 °C, 200 rpm. For the kinetics study, the mycelium from seed culture (0.2 g, fresh weight) was added to a freshly sterilized PDB (200 mL). Then, aliquots (0.5 mL) of fenazaquin solution (20 mg/10 mL in dimethyl sulfoxide, DMSO) were added. The cultures were further grown at 28 °C, 200 rpm for specific period (0, 1, 3, 5, 7, 14 days). For sterilized control experiments, 7-day cultures were sterilized at 110 °C, 30 min and fenazaquin solution (0.5 mL) was treated as described above. After further incubation at the same condition, the concentrations of fenazaquin were analyzed. Media control was also prepared according to the same procedure without fungal mycelia. All experiments were performed in triplicates.

Effects of metabolic inhibitors

The following metabolic inhibitors were selected, including piperonyl butoxide, ketoconazole, and myclobutanil as cytochrome P450 (CYP) inhibitors, and methimazole as a flavin-dependent mono-oxygenase (FMO) inhibitor. The seed culture of A. niger KACC 45093 (0.2 g, fresh weight) was added to a freshly sterilized PDB (200 mL), followed by stock solutions of metabolic inhibitors (500 mg/10 mL DMSO). The inhibitor concentrations were set to 1 mg/200 mL PDB, except myclobutanil (1, 5, and 20 mg/200 mL PDB). After 12 h of pre-incubation at 28 °C, 200 rpm, aliquot (0.5 mL) of fenazaquin solutions (20 mg/10 mL, DMSO) was added. After incubations, the amounts of parent and metabolites were measured. Triplicate cultures were prepared for each treatment.

Extraction of metabolites and instrumental analysis

The cultures, including mycelia and medium, were homogenized with Waring blender (2 min) and filtered. The filter cake was extracted with MeOH (100 mL × 2). The combined extracts were concentrated under reduced pressure. The residue was suspended in saturated NaCl (200 mL). Fenazaquin and its metabolites were extracted with ethyl acetate (EA, 100 mL × 3). The EA extracts were dried over anhydrous Na2SO4 and concentrated to
dryness. The residue was re-dissolved in EA (10 mL) and used for gas chromatography–mass spectrometry (GC–MS).

**Chemical oxidation of fenazaquin**

Fenazaquin (30 mg) was dissolved in dichloromethane (80 mL). A solution of MCPBA (2, 6, and 20 mg/20 mL, dichloromethane) was added in one portion and stirred at 25 °C. Aliquots of reaction mixture was collected at several intervals and analyzed with GC–MS.

**Instrumental analyses of fenazaquin and metabolites**

Fenazaquin and its metabolites were analyzed with gas chromatograph–mass spectrometer (GC–MS). Analytical conditions for GC–MS were as follows: Shimadzu GC-2010 with GCMS-2010 SE equipped with Rtx-5MS column (30 m, 0.25 μm film thickness, 0.25 mm i.d.; Restek, USA). Helium was carrier gas at a flow rate of 1 mL/min. The column temperature was programmed as follows: 160 °C (10 min) and raised to 295 °C at a rate of 2.5 °C/min and held for 30 min. The mass spectra of metabolites were obtained in full scan mode.

**Results and discussion**

**Kinetics of fungal and chemical degradation of fenazaquin**

Fenazaquin was quite stable in control experiments (Fig. 1, Table 1). However, the concentration of remaining fenazaquin showed an exponential decay with reaction time. In addition, a concentration-dependent dissipation was observed in MCPBA-mediated oxidations. For example, the reaction rate with 200 mg/L MCPBA (approximately 10 molar excess to fenazaquin) was 5 times higher than that of 20 mg/L MCPBA (approximately 1 equivalent). Both results indicated that MCPBA-mediated fenazaquin oxidation followed first-order kinetics. MCPBA is frequently used as an oxidative enzyme mimic in pesticide metabolism [27–29]. Numerous functional groups can be oxidized with MCPBA (e.g., including alcohols, aldehydes, carbamates, and organophosphorus) [30]. MCPBA can catalyze oxidative transformation of ethers into ketones [31]. In addition to MCPBA, several peracids are also used in chemical synthesis and metabolic studies [27, 28]. Extensive studies showed that the reaction products of drugs and pesticides via peracid oxidation are frequently similar to those of CYP-catalyzed metabolism [27–30]. Based on these results, comparative analysis with the fungal metabolism yielded a detailed insight into fenazaquin metabolism.

Fenazaquin was rapidly dissipated under *A. niger* culture. The degradation pattern also showed exponential decay. Biological degradation of xenobiotics often follows first- or pseudo-first-order kinetics [32, 33]. Half-life and rate constants of fenazaquin were 0.6 and 1.13/day, respectively. No degradation was observed in sterilized mycelia and medium control (Fig. 1; Table 1).

**Metabolites from chemical and fungal degradation**

According to instrumental analyses, the major degradation products of MCPBA oxidation were 4-tert-butylphenethyl alcohol (II) and 4-hydroxyquinazoline (IV) (Table 2). All metabolites, except 2-(4-(2-hydroxyethyl)-phenyl)-2-methyl-1-propanol (VI), were identified with synthetic standards (Supplementary material). The concentrations of II and IV gradually increased during chemical oxidation and fungal cultures. However, the levels of these

![Fig. 1](image-url) Degradation of fenazaquin by MCPBA-catalyzed oxidation (A) and *A. niger* (B). Inserts in panel A indicate the concentrations of MCPBA (symbols for MCPBA concentration: filled circle, 20 mg/L; empty circle, 60 mg/L; filled triangle, 200 mg/L). Inserts in panel B are for non-sterile normal cultures (filled circle), sterilized cultures (empty circle), PDB culture medium without mycelium (filled triangle), respectively.

Fig. 1 Degradation of fenazaquin by MCPBA-catalyzed oxidation (A) and *A. niger* (B). Inserts in panel A indicate the concentrations of MCPBA (symbols for MCPBA concentration: filled circle, 20 mg/L; empty circle, 60 mg/L; filled triangle, 200 mg/L). Inserts in panel B are for non-sterile normal cultures (filled circle), sterilized cultures (empty circle), PDB culture medium without mycelium (filled triangle), respectively.
metabolites decreased at the end of the experiments (Fig. 2). One of the most notable differences between chemical and biological oxidations was detected with the metabolites 4-t-butylacetic acid (III) and 2,4-quinazolinedione (V) in MCPBA oxidation. Because of its strong oxidative activity, MCPBA is commonly used for the

**Table 1** First- or pseudo-first-order kinetic parameters of fenazaquin degradation

| Classification | Inhibitors | Oxidants (mg/L) | Rate (/day) | Half-lives (day) | $R^2$ |
|----------------|------------|-----------------|-------------|------------------|-------|
| Chemical oxidation by MCPBA | – | 20 | 0.06 | 0.5 | 0.9591 |
| | | 60 | 0.11 | 0.3 | 0.9693 |
| | | 200 | 0.26 | 0.1 | 0.9622 |
| Biodegradation | Non-sterile | – | 1.13 | 0.6 | 0.9912 |
| | Sterile | – | ND$^b$ | – | – |
| | Medium | – | ND | – | – |
| Biodegradation inhibitors$^c$ | MTZ$^d$ | – | 0.44 | 1.6 | 0.9962 |
| | PBO | – | 0.01 | 58.7 | 0.9407 |
| | KCZ | – | 0.58 | 1.2 | 0.9107 |
| | MCN | – | 0.42 | 1.7 | 0.9415 |
| | MCN$^e$ | – | 0.38 | 1.8 | 0.9696 |
| | MCNe | – | 0.16 | 4.3 | 0.9815 |

$^a$Regression coefficient
$^b$No degradation (ND)
$^c$Inhibitor concentration (5 mg/L)
$^d$MTZ methimazole, PBO piperonyl butoxide, KCZ ketoconazole, MCN myclobutanil
$^e$Concentration of myclobutanil: 25 and 100 mg/L

**Table 2** GC–MS retention times and mass spectral data of fenazaquin and metabolites derived from MCPBA oxidation and cultures of *A. niger*

| ID | Name | Retention time (min) | Molecular and fragment ions (% relative abundance) |
|----|------|----------------------|--------------------------------------------------|
| I  | Fenazaquin | 45.02 | 306 (M$^+$, 1), 207 (1), 160 (47), 145 (100), 131 (10), 117 (29) |
| II | 4-t-Butylphenethyl alcohol | 23.75 | 178 (M$^+$, 23), 163 (100), 147 (21), 132 (19), 117 (33), 105 (25), 91 (25) |
| III| 4-t-Butylphenylacetic acid | 27.24 | 192 (M$^+$, 22), 177 (100), 149 (19), 131 (50), 117 (22), 91 (25), 77 (9) |
| IV | 4-Hydroxyquinazoline | 30.33 | 146 (M$^+$, 100), 118 (32), 91 (24), 64 (23) |
| V  | 2,4-Quinazolinedione | 34.18 | 162 (M$^+$, 82), 133 (7), 119 (100), 92 (75), 64 (25) |
| VI | 2-(4-(2-hydroxyethyl)-phenyl)-2-methyl-1-propanol | 30.75 | 194 (M$^+$, 1), 176 (2), 163 (100), 145 (21), 133 (18), 117 (32), 105 (25), 91 (21), 77 (8) |

metabolites 4-t-butylacetic acid (III) and 2,4-quinazolinedione (V) in MCPBA oxidation. Because of its strong oxidative activity, MCPBA is commonly used for the

**Fig. 2** Concentrations of fenazaquin metabolites during MCPBA-mediated oxidation (A) and cultures of *Aspergillus niger* (B). Concentration of MCPBA, 60 mg/L. Metabolites II–V are listed in Table 2
oxidation of alcohols to aldehyde or carboxylic acids. Heterocycles are also susceptible to oxidation with the same reagent [27, 29, 30]. Recent studies showed that the same oxidant can oxidize cycloalkyl methyl ether to cyclic ketones [31]. Based on these findings, II and IV may be further oxidized to III and V by MCPBA. Instrumental analysis of A. niger cultures yielded II and IV as the major metabolites, with concentrations accounting for 30–50% of fenazaquin transformation. The levels of III and V in fungal culture were negligible. The metabolite VI was tentatively identified as 2-(4-(2-hydroxyethyl)-phenyl)-2-methyl-1-propanol, derived from the oxidation of 4-t-butyl group of II or via cleavage of the ether bond cleavage in undetected metabolite(s). Notably, fenazaquin metabolites containing oxidized 4-t-butyl group (alcohol and acid) were reported in rats and a few plants [6]. In a photochemical degradation experiment, preferential oxidation of t-butyl group over alcohols was observed [34]. Previous study with synthetic flavonoids showed that A. niger can rapidly oxidize t-butyl group to alcohols and acids by cytochrome P450 [22]. These results indicated that metabolite VI may be originated from metabolite II or fenazaquin alcohol. However, previous studies showed that II and IV were the major metabolites occurring in most biological systems [6, 34, 35]. Accordingly, it is plausible that metabolic pathways of fenazaquin in A. niger resemble those of animal and plants.

Effects of inhibitors on fungal metabolism of fenazaquin

Xenobiotics in biological system are subjected to metabolic transformation. Such structural modification of pesticides can result in potentiation or reduction of bioactivity [36, 37]. The binary or ternary mixture of pesticides frequently showed a synergistic effect of acetylcholine esterase inhibition [37]. Similar results were also found in drugs. For example, several antifungal agents (e.g., ketoconazole) are strong inhibitors of drug metabolizing CYP isforms [38]. Co-application of these fungicides and other drugs reduces the metabolic clearance of drugs, which results in enhanced efficacy. Piperonyl butoxide (PBO) is an important synergistic agent included in many pesticide formulations. It is an established and irreversible inhibitor of CYPs [39]. In this study, three CYP inhibitors were tested for their effects on fenazaquin metabolism. PBO strongly inhibited fenazaquin metabolism, while azole antifungal reagents (ketoconazole and myclobutanil) showed a limited inhibition (Figs. 3, 4; Table 1). The degradation of fenazaquin showed a concentration-dependent reduction by myclobutanil (Table 1). For example, the rate constant at the highest concentration of myclobutanil was one-third of the value observed at the lowest treatment level (0.16 and 0.42/day, respectively). However, these effects may result from the fungi-toxic effects of myclobutanil (Fig. 4). Accordingly, it can be concluded that certain CYPs that are susceptible to PBO-mediated inhibition catalyze fenazaquin metabolism. Flavin-dependent mono-oxygenase (FMO) is another common mediator of xenobiotic metabolism [40]. Until now, no FMO inhibitors have been registered as pesticide synergists. However, methimazole is frequently used as FMO inhibitor in
metabolic studies of drugs and pesticides [40, 41]. Methimazole slightly reduced the transformation rate of fenazaquin (Fig. 3; Table 1). The finding indicates that the contribution of FMO is limited in fenazaquin metabolism. In summary, the fungal metabolic pathway of fenazaquin resembles that of animal and plants (Fig. 5). PBO-selective inhibition indicates that CYPs may be the major enzymes in fenazaquin metabolism. These findings showed that PBO or related synergists enhance the efficacy of pesticides, which in turn reduces the pesticide application via PBO combination. Environmental safety can also be achieved via efficient use of synergists.

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