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Response of Superoxide Dismutase, Catalase, and ATPase Activity in Bacteria Exposed to Acetamiprid[1]

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Objective To investigate how acetamiprid, a new insecticide, affects the activity of superoxide dismutase (SOD), catalase (CAT), and ATPase and the SOD isozyme patterns in two G^- bacteria, *E. coli* K12 and *Pse*.FH2, and one G^+ bacterum, *B. subtilis*. **Methods** The SOD, CAT, and ATPase specific activities of cell lysates were determined spectrophotometrically at 550 nm, 240 nm, and 660 nm, respectively, with kits A001, A016, and A007. SOD isozyme patterns were detected by native PAGE analysis. **Results** SOD and CAT activities in the tested bacteria increased significantly in a concentration-dependent manner after different concentrations of acetamiprid were applied. The activity of SOD in *B. subtilis* and *Pse.*FH2 was stimulated and reached the highest level after treatment with 100 mg/L acetamiprid for 0.5 h. For *Pse*.FH2, there was another stimulation of SOD activity after acetamiprid application for about 8.0 h and the second stimulation was stronger than the first. The stimulation by acetamiprid showed a relative lag for *E. coli* K12. Acetamiprid seemed to exhibit a similar effect on CAT activity of the two G^- bacteria and had an evident influence on ATPase activity in the three bacteria within a relatively short period. Only one SOD isozyme was detectable in *Pse.*FH2 and *B. subtilis*, while different isozyme compositions in *E. coli* could be detected by native PAGE analysis. **Conclusion** Acetamiprid causes a certain oxidative stress on the three bacteria which may not only elevate SOD and CAT activities but also generate new SOD isozymes to antagonize oxidative stress. However, this oxidative stress lasts for a relatively short time and does not cause a long-term damage.

Key words: Acetamiprid; SOD; CAT; ATPase; Stress response; Native PAGE

INTRODUCTION

Organisms with aerobic metabolism face constant risk from reactive oxygen species (ROS), including superoxide radical (O_2^-), hydroxyl radical ($^{\cdot}OH$), and hydrogen peroxide (H_2O_2). The high toxic superoxide anion seriously disrupt normal metabolism through oxidative damage to cellular components. One of the most serious damaging effects on cells is the peroxidation of membrane lipids that may severely affect the functional and structural integrity of biological membranes, leading to leakage of potassium ion and other solutes, which may finally cause cells to die[1-2]. Oxygen free radicals can be also converted to reactive hydroxyl radicals, which attack and oxidize DNA, cell membrane, and others[3-4]. Therefore elimination of superoxide anion is definitely necessary for survival of cells.

Superoxide dismutase (SOD), as part of the defense systems against oxidative damage in aerobic organisms[5-6], catalyzes superoxide anion (O_2^-) to O_2 and H_2O_2, which then is reduced to H_2O by H_2O_2-scavenging enzyme-catalase[7]. Therefore, SOD and CAT are thought to limit the accumulation of reactive oxygen species.

Most environmental bacteria experience oxidative stress from a variety of sources. The reactive oxygen species (ROS) can be produced in cells not only during microbial aerobic growth as by-products of normal cellular metabolism but also under stress situations. Various pesticides may induce oxidative stress that causes generation of free radicals and alters antioxidants[8-9].

ATPase plays an important role in many intracellular physiological functions, such as nutrition transportation, energy transformation and information transfer. Given that ATPase activity is likely to undergo a series of changes under stress condition, Yadwad *et al*.[10] pointed out that it can be considered

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as a sensitive indicator of toxicity. The xenobiotics can alter ATPase activity by disrupting energy-producing metabolic pathways or interacting directly with the enzyme[11]. Since Daivis *et al.*[12] first reported that pesticide inhibits the activity of Na^+, K^+-ATPase in rainbow trout, more attention has been focused on the potential toxicological hazard induced by pesticide exposure. Sancho *et al.*[13] have investigated the acute toxicity of fenitrothion (an organophosphorus insecticide) to eel, *Anguilla anguilla*, and its toxic effect on ATPase activities in gill tissue. Lü and Min[9] observed that ATPase activity of *Burkholderia cepacia* WZ1 changes significantly after exposure to herbicide quinclorac.

Acetamiprid, a new insecticide, because of its broad spectrum propertiesand low toxicity, is regarded as a substitute to the organophosphate insecticides and has been used widely. To explore how defensive enzymes of bacteria changed when exposed to different concentrations of acetamiprid, the activities of CAT, SOD, and ATPase in three bacteria, typical G^- bacterium *Escherichia coli* K12, typical G^+ bacterium *Bacillus subtilis* B19, and acetamiprid-degrading bacterium *Pseudomonas sp.* FH2 were investigated. At the same time, the SOD isozyme profiles in the three bacteria were compared after acetamiprid was applied. The results demonstrate the stress response of bacteria to acetamiprid. It is reasonably believed that this information is valuable for appropriate usage of acetamiprid and prevention of its contamination.

MATERIAL AND METHODS

Bacterial Strains and Culture Conditions

E. coli K12 and *B. subtilis* B19 used in this experiment were kept in the laboratory of Environment Microbiology and Microbial Molecular Ecology, Zhejiang University, China. *Pseudomonas* FH2, an acetamiprid-degrading bacterium, was isolated from the soil of Dongfeng Pesticide Factory.

B. subtilis B19 and *Pseudomonas sp.* FH2 were grown aerobically in liquid LB medium at 30℃ at 150 r/min. *E. coli* K12 was grown in the same medium but incubated at 37℃ at 150 r/min.

Acetamiprid at the concentrations of 10, 100, and 1000 mg/L (final concentration) was added to cultures at the same time of inoculation which were then incubated for 24 h before harvesting for extraction of enzymes, or 100 mg/L (final concentration) acetamiprid was added to cultures that were incubated for 24 h in liquid LB medium.

Cell Harvest and Lysis Procedures

Cells were harvested from 6 mL liquid culture by centrifugation and washed twice using ice-cold 0.9% sodium chloride solution. Pellets were re-suspended in 3 mL 0.9% sodium chloride solution and then subjected to 99 cycling of sonication in an ice water bath for 3 s followed by cooling for another 3 s. Cellular debris was removed by centrifugation at 10 000 g at 4℃. The supernatants were collected for SOD, CAT, and ATPase activity assay and native PAGE and activity staining of SOD.

Measurement of Enzyme Activity

SOD, CAT, and ATPase specific activities of cell lysates were determined spectrophotometrically at 550 nm, 240 nm, and 660 nm, respectively, with kits A001, A016, and A007[9]. One unit of SOD activity was defined as the amount of lysate that inhibited the rate of xanthine/xanthine oxidase-dependent cytochrome C reduction at 25℃ by 50%. One unit of catalase activity was defined as the amount of lysate that catalyzed the decomposition of 1 μmol H_2O_2 per min at 25℃. The specific activity was expressed as units per milligram of cellular proteins. One unit of ATPase activity was defined as the amount of inorganic phosphorus produced by ATP decomposition per hour per mg protein.

Total protein concentration in cell lysates was determined by a modified Lowry procedure (kit A045) using bovine serum albumin as the standard.

All reagents and kits were purchased from Nanjing Jiancheng Bioengineering Institute, Jiangsu Province, China.

Native Gel Electrophoresis of SOD and Gel Activity Staining

Cell lysate was loaded onto an 8% native polyacrylamide gel bathed in 1×Tris-glycine buffer (pH8.3) and the proteins separated at constant current (20 mA) at 4℃. SOD activity staining of the gel was performed by the method of Rao *et al.*[14]. The gels were stained by incubation in a solution containing 2.5 mmol/L nitro-blue tetrazolium (NBT) in dark for 20 min, followed by incubation in 50 mmol/L potassium phosphate buffer (pH 7.8) containing 28 mmol/L riboflavin and 28 mmol/L tetramethylethylene-diamine in dark for 15 min. The gels were then placed in 50 mmol/L potassium phosphate buffer (pH 7.8) containing 100 μmol/L EDTA and exposed to light (4000 Lx) for 20 min at room temperature. The staining reaction for SOD was stopped by 7.5% glacial acetic acid. The gels in tubes

containing the 7.5% acetic acid solution were stored at 4℃ until photographed[15].

Statistical Analysis

Data were expressed as $\bar{x} \pm s$ and compared statistically by Duncan's new multiple range test at 5% level with SAS8.1 software.

RESULTS

Changes of Catalase, SOD, and ATPase Activity in E. coli, B. subtilis and Pse.FH2 after Treatment With Different Concentrations of Acetamiprid

The activities of SOD and CAT in the three bacteria treated with different concentrations of acetamiprid are presented in Table 1.

As shown in Table 1, all tested bacteria possessed SOD activity. There existed comparatively high specific SOD activity in *E. coli* and *Pse.* FH2 and was approximately 1.5-fold higher than that in *B. subtilis*. The SOD activities in the three bacteria increased after acetamiprid application, and more pronounced in bacteria treated with 100 mg/L of acetamiprid than in those treated with 10 mg/L of acetamiprid.

Table 1 also shows that all tested bacteria possessed CAT activity that was at the same range before acetamiprid addition. The CAT activity in all bacteria increased in a concentration-dependent manner after acetamiprid was added. The activity was 131.9 unit mg^{-1} protein in *B. subtilis*, as approximately 8-fold over that in *E. coli* (16.5 unit mg^{-1} protein) and 6-fold in *Pse.* FH2 (20.78 unit mg^{-1} protein), when these bacteria were exposed to 100 mg/L of acetamiprid. It is to be noted that CAT activity in *B. subtilis*, a G^+ bacterium, markedly declined from 131.9 units mg^{-1} protein to 71 units mg^{-1} protein, but that in the other two G^- bacteria still increased significantly from 16.5 to 22.87 and 20.78 to 26.7 units mg^{-1} protein, respectively, when the concentration of acetamiprid treating these bacteria was enhanced from 100 to 1000 mg/L.

TABLE 1

Changes of SOD and CAT Activities at Different Concentrations of Acetamiprid ($\bar{x} \pm s$)

Acetamiprid Concentration (mg/L)	SOD Activity (Units mg^{-1} Protein)			CAT Activity (Units mg^{-1} Protein)		
	E. coli	*B. subtilis*	*Pse.*FH2	*E. coli*	*B. subtilis*	*Pse.*FH2
0	51.08±7.78[a]	38.10±5.03[a]	54.87±2.72[a]	10.65±1.34[a]	12.88±0.70[a]	12.55±1.29[a]
10	74.13±3.27[b]	66.80±6.23[b]	74.78±0.85[b]	14.27±2.30[a]	24.53±2.02[b]	14.21±3.75[a]
100	86.24±2.23[c]	84.44±7.66[c]	81.41±1.72[c]	16.50±2.89[a]	131.9±2.04[c]	20.78±0.79[b]
1000	113.9±4.09[d]	82.17±2.87[c]	82.45±1.45[c]	22.87±1.06[b]	71.72±3.86[d]	26.7±1.83[c]

Note. Data are presented as mean plus the standard error of at least triplicate samples, and the different letters (a, b, c, and d) in each column mean there exists a significant difference between each other (P<0.05) according to Duncan's new multiple range test.

TABLE 2

Changes of ATPase Activity at Different Concentrations of Acetamiprid ($\bar{x} \pm s$)

Acetamiprid Concentration (mg/L)	ATPase Activity (μmol Pi mg^{-1} Protein h^{-1})		
	E. coli	*B. subtilis*	*Pse.*FH2
0	4.95±0.26[a]	2.25±0.14[a]	2.85±0.18[a]
10	4.94±0.29[a]	1.01±0.09[b]	0.88±0.05[b]
100	4.44±0.21[a]	1.09±0.05[b]	1.02±0.03[b]
1000	3.58±0.10[b]	0.63±0.09[c]	1.24±0.11[c]

Note. Data are presented as mean plus the standard error of at least triplicate samples, and the different letters (a, b, c, and d) in each column mean there exists a significant difference between each other (P<0.05) according to Duncan's new multiple range test.

The effects of acetamiprid on ATPase activity in bacteria are summarized in Table 2. ATPase activity decreased significantly (P<0.05) in *Pse.*FH2 and *B. subtilis* when they were grown in medium with 100 mg/L of acetamiprid compared with that in medium without acetamiprid, and ATPase in *E. coli* decreased significantly (P<0.05) when acetamiprid concentration was 1000 mg/L. The result implied that acetamiprid inhibited ATPase activity in bacteria.

Effect of 100 mg/L Acetamiprid on SOD, CAT, and ATPase in Bacteria Grown for 24 h

To determine whether the three enzyme activities and SOD isozyme patterns varied at different growth intervals, 100 mg/L (final concentration) of acetamiprid was added into cultures of the three bacteria incubated for 24 h in LB medium. Then the samples were taken from the culture for enzyme activity assays after acetamiprid was applied for 0.0, 0.5, 1.5, 3.0, 5.0, 8.0, 14, and 24 h.

The regulation of SOD activity in the three bacteria differed (Fig. 1). SOD activity in *B. subtilis* was stimulated after acetamiprid application and reached the highest level (about 120 units mg^{-1}

protein) after incubation for 0.5 h, and then rapidly dropped down and kept the original level before addition of acetamiprid. For *Pse*.FH2, SOD activity was also stimulated immediately and reached the maximum at 0.5 h after acetamiprid was applied. However, there was another stimulation of SOD activity after acetamiprid application for about 8.0 h. Obviously, the second stimulation, during which the SOD activity was nearly 80 units mg^{-1} protein, was stronger than the first, during which the SOD activity was about 65.9 units mg^{-1}. The stimulation caused by acetamiprid comparatively lagged for *E. coli* K12. The SOD activity slightly decreased at first after acetamiprid was applied but began to increase half an hour later, and achieved the highest level (nearly 120 units mg^{-1} protein) after incubation for 1.5 h.

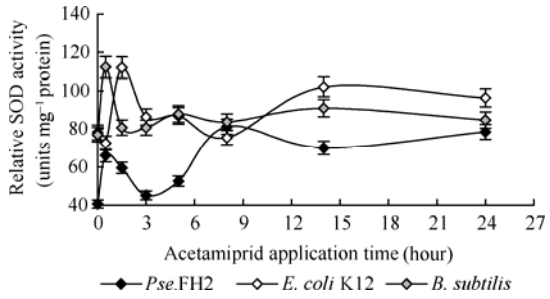

FIG. 1. Effect of 100 mg/L acetamiprid on SOD activity in *E. coli* K12, *B. subtilis* and *Pse*.FH2 after being grown for 24 h.

The effect of acetamiprid on CAT activity in *E. coli* K12 seemed to have the tendency similar to that in *Pse*.FH2 (Fig. 2). After acetamiprid treatment for 1.5 h, CAT activities in the two G^- bacteria were enhanced and reached 28.1 and 31.3 units mg^{-1} protein, respectively. Distinct oxidative stress was also observed on *B. subtilis* after acetamiprid application and CAT activity was enhanced significantly ($P<0.05$) to 85.1 units mg^{-1} protein.

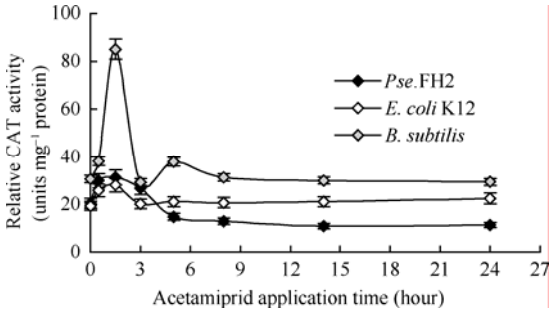

FIG. 2. Effect of 100 $mg \cdot L^{-1}$ acetamiprid on CAT activity in *E. coli* K12, *B. subtilis* and *Pse*.FH2 after being grown for 24 h.

The results shown in Fig. 3 indicate that acetamiprid application could evidently influence ATPase activity in these three bacteria within a relatively short period. ATPase activities in *E. coli* and *Pse*.FH2 were stimulated and increased to the highest recorded level (10.5 at 1.5 h and 4.46 μmol Pi mg^{-1} protein h^{-1} at 0.5 h, respectively) after acetamiprid application. The ATPase activity in *B. subtilis* increased to the peak of 6.64 μmol Pi mg^{-1} protein h^{-1} at 0.5 h after actamiprid application. The ATPase activities in the three bacteria kept stable after incubation for 5 h.

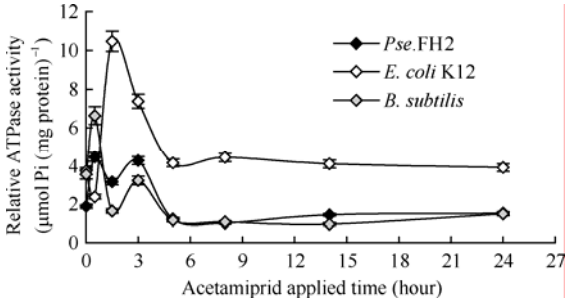

FIG. 3. Effect of 100 mg/L acetamiprid on ATPase in *E. coli* K12, *B. subtilis* and *Pse*.FH2 after being grown for 24 h.

Effect of Acetamiprid on SOD Isozyme Patterns

Samples incubated for 0.0, 0.5, 1.5, 3.0, and 5.0 h were used for native polyacrylamide gel electrophoresis and gel activity staining of SOD.

Native PAGE analysis (Fig. 4) indicated that SOD isozyme profile in *Pse*.FH2 and *B. subtilis* was different from that in *E. coli* K12. There appeared two SOD isozyme bands, namely bands a and b (Fig. 4 C) at the original time in *E. coli* K12. The samples in the bacterium, however, retained only one SOD isozyme band (band a) but produced a new isozyme band (band c) at 0.5 h after acetamiprid treatment. Interestingly, band c almost completely disappeared and an additional slight staining intensity band d appeared when *E. coli* K12 was incubated for 1.5 h after acetamiprid application. Finally, band c reappeared and band d disappeared drastically at 3 and 5 h after incubation. In contrast, only a single SOD isozyme band appeared and no detectable changes occurred in SOD isozyme pattern of *Pse*.FH2 and *B. subtilis* at the selected observation phase. It was clear that the SOD isozyme profile in degradation bacteria and *B. subtilis* was almost unaffected by treatment of acetamiprid.

DISCUSSION

Now there are considerable studies on free radical-

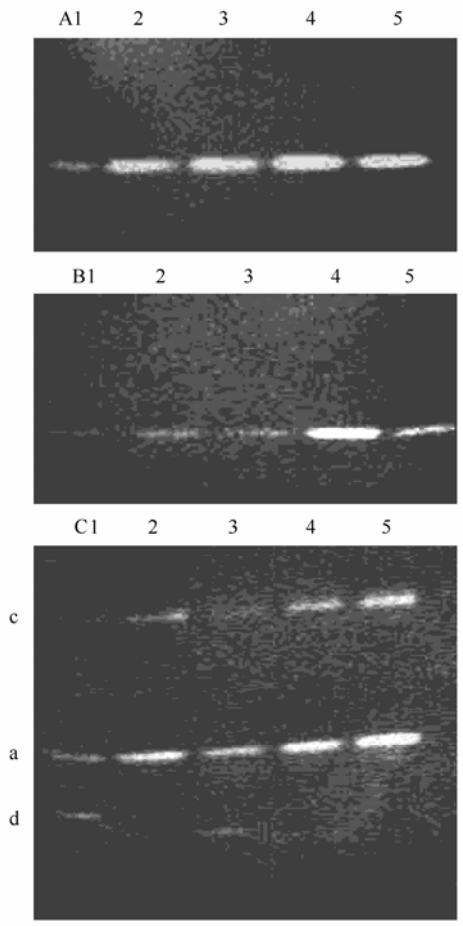

FIG. 4. Gel activity stained for SOD isozyme profiles. Lanes 1-5 : SOD isozyme profiles in *Pse*.FH2 (A), *B. subtilis* (B) and *E. coli* K12 (C) harvested after acetamiprid treatment for 0.0, 0.5, 1.5, 3, and 5.0 h, respectively.

mediated damages to biological systems following insecticide exposure. But these studies mainly focused on damages to animals and plants. In fact, the activity of catalase and SOD in a number of *Pseudomonas spp*. and other Gram-negative bacteria is well known and the regulatory and environmental role of these enzymes have been reported[16-18]. Although investigations of the stress response in microorganisms caused by insecticide application are still scarce, microorganisms frequently undergo this stress factor, and have evolved a series of protection mechanisms[19].

In the present work, the activity of SOD and CAT increased significantly (P<0.05) in a concentration-dependent manner (Table 1) after application of acetamiprid. One possible reason is that acetamiprid causes a certain oxidative stress and much ROS is produced. In order to antagonize ROS, defensive enzyme systems, such as CAT and SOD activities, are involved. At the same time, ATPase activity decreases after the pesticide application, suggesting that acetamiprid may affect the metabolism of ATP to a certain degree.

However, the effect of acetamiprid on these enzymes lasts for a relatively short time, and does not cause a long-term damage. For these strains, changes in the activities of SOD, CAT, and ATPase occur within 9 h after acetamiprid application and these enzyme activities maintain at a corresponding level as that at the original time.

In addition, as in Gram negative bacteria, SOD activity in *Pse*.FH2 is different from that in *E. coli* K12. SOD activity in *Pse*.FH2 is lower than that in the non-degrading bacterium *E. coli* K12 after acetamiprid application (Fig. 1). The behavior of *Pse*.FH2 capable of using acetamiprid implies that the bacterium may reduce some oxidative stress. It is suggested that oxidative stress caused by acetamiprid for degrading bacteria is lower than that for non-degrading bacteria.

Compared with the fluctuation of enzyme activity, PAGE results indicate that the pattern of SOD inozyme band in *Pse*.FH2 and *B. subtilis* is stable. No detectable variation of the SOD isozyme band was observed in our study, but a new SOD isozyme band was found in *E. coli* K12, suggesting that acetamiprid may influence SOD isozyme compositions. It can be seen from the above results that bacteria may not only elevate SOD activity but also generate new SOD isozymes against oxidative stress caused by acetamiprid. Therefore, SOD, CAT, and ATPase can be used as indicators for acetamiprid contamination.

At present, no consensus is available on the optimal quantitative criteria of insecticide-induced oxidative stress and the effect of antioxidant interventions. The precise action of insecticides on microorganisms still needs to be further demonstrated. In addition, it is still unknown whether ROS are generated following the treatment of acetamiprid. To elucidate the role of antioxidant enzymes in the defense mechanisms of bacteria against acetamiprid, more researches are needed.

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