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Effect of Cyprodinil and Fludioxonil Pesticides on Human Superoxide Dismutase

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Copper-zinc superoxide dismutase (CuZnSOD), (E.C:1.15.1.1) catalyzes the dismutation of the superoxide radical to hydrogen peroxide and oxygen. In this study, CuZnSOD was purified from human erythrocytes by DEAE-cellulose chromatography and copper-iminodiacetic acid agarose chromatography by 196.3 fold and 33.8 % efficiency. Specific activity of CuZnSOD was measured in purified enzyme extract and human erythrocytes treated with and without varying concentrations of cyprodinil and fludioxonil. CuZnSOD activity in 500 ppm cyprodinil treated erythrocytes was about 68.7 % of the initial CuZnSOD activity of control. Whereas CuZnSOD activity of erythrocytes treated with 500 ppm fludioxonil was about 33.9 % of the initial activity CuZnSOD activity of control. In the case of purified CuZnSOD activity treated with 500 ppm cyprodinil was about 76.2 % of the initial purified CuZnSOD activity of control. In same way purified CuZnSOD activity treated with 500 ppm fludioxonil was about 40.7 % of the initial purified CuZnSOD activity of control. In short, fludioxonil according to cyprodinil more inhibited CuZnSOD. Cyprodinil inhibited CuZnSOD competitively and fludioxonil inhibited CuZnSOD non-competitively.

Key Words: Superoxide dismutase, Erythrocyte, Pesticide, Cyprodinil, Fludioxonil.

INTRODUCTION

In 1938, Mann and Keilin described a blue-green protein containing copper (Haemocuprein) that they had isolated from bovine blood. In 1953, a similar protein was isolated from horse liver and named hepatocuprein. Other proteins of this type were later isolated, such as cerbrocuprein from brain. In 1970, it was discovered that the erythrocyte protein contains zinc as well as copper. No enzymic function was detected in any of these proteins, so it was often suggested that they served as metal stores[1]. However, in 1969 McCord and Fridovich reported that the erythrocyte protein is able to remove the superoxide radical catalytically, *i.e.*, it functions as a superoxide dismutase enzyme (SOD)[2,3]. SOD, catalyzes the dismutation of the superoxide anion to hydrogen peroxide[4]. Thus, SOD has antiinflammatory effects[5].

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Cyprodinil and fludioxonil fungicides are active ingredients of Switch 62.5WG. Switch 62.5 WG has been evaluated around the world for biological efficacy since the early 1990's. It has been shown to protect plants against many economically important pathogens and is particularly effective against *Botrytis* species. Created with four hurdles to infection, switch provides peace of mind and consistently better control of *Botrytis*[6].

Biochemical studies indicate cyprodinil is anilinopyrimidine compound and interferes with the biosynthesis of methionine and so disrupts protein structure and function. Studies indicate that cyprodinil also impedes the secretion of fungal hydrolytic enzymes and so fungal pathogenesis is impaired. Fludioxonil is phenylpyrroles compound and blocks the protein kinase which catalyzes phosphorylation of a regulatory enzyme involved in glycerol synthesis. Ultimately, the phenylpyrrole mode of action associated with fludioxonil disrupts fungal membranes[6].

Pesticides remain on surfaces of vegetables and fruits or penetrate into vegetables and fruits. Because of that we take the pesticides to our body. Pesticides affect our organism consequently affect our enzyme activity.

In our work, human erythrocytes CuZnSOD was isolated by using DEAE-cellulose chromatography and copper chelate affinity chromatography[7] and incubated with pesticides. At the same time, human erythrocytes were incubated with pesticides. Then changings of specific activity of CuZnSOD were determined.

EXPERIMENTAL

Fludioxonil (4-(2,2-difluoro-1,3-benzodioxol-4-yl) pyrrole-3-carbonitrile), Pestanal 46102 and cyprodinil (4-cyclopropyl-6-methyl-N-phenylpyrimidin-2-amine), Pestanal 34389 were obtained from Riedel-de Haen. Xanthine (X-7375), xanthine oxidase (X-4500), nitrotetrazolium blue chloride (N-6876) were obtained from Sigma. All other chemicals used were analytical grade.

Protein measurements: Protein concentration was determined by the method described by Lowry *et al.*[8], using bovine serum albumin as standard.

CuZnSOD activity assay: This method based on generation of superoxide radicals by xanthine and xanthine oxidase which react with nitrotetrazolium blue to form blue formazan dye. The superoxide dismutase activity is then measured by the degree of inhibition of this reaction. One unit of CuZnSOD is the amount of CuZnSOD, 50 % inhibition of the rate of reduction of nitrotetrazolium blue under the conditions of the assay[9].

Effect of pesticide on enzyme activity: Cyprodinil and fludioxonil which dissolved in ethyl alcohol were incubated at 37 °C with erythrocyte and purified CuZnSOD an hour. Then activity of CuZnSOD was measured.

RESULTS AND DISCUSSION

Effect of cyprodinil on erythrocyte: Erythrocytes were incubated with cyprodinil from 0-500 ppm and their enzyme activities were measured. These conclusions were shown in Fig. 1.

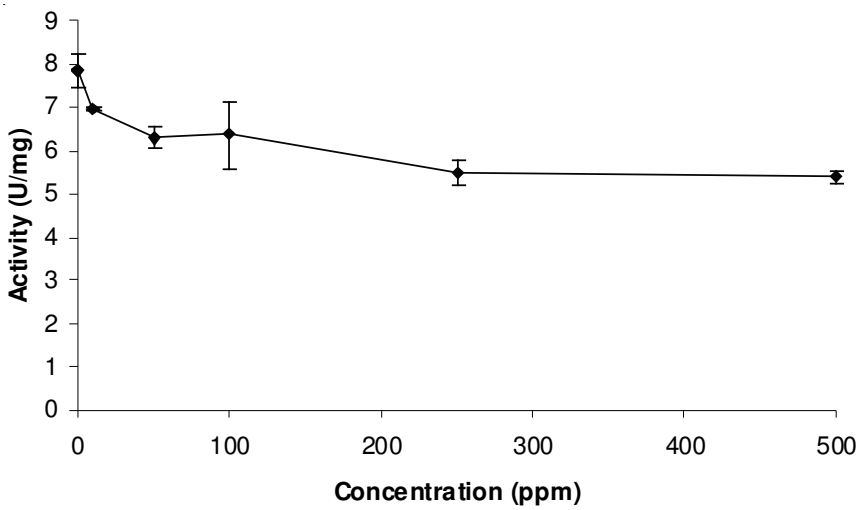

Fig. 1. Effect of cyprodinil concentrations on erythrocyte CuZnSOD activity

As shown from Fig. 1. CuZnSOD activity decreased from 7.85 U/mg initial activity to 5.39 U/mg last activity with increasing cyprodinil concentrations.

Effect of cyprodinil on purified CuZnSOD: Purified CuZnSOD was incubated with cyprodinil from 0-500 ppm and their enzyme activities were measured. These conclusions were shown in Fig. 2.

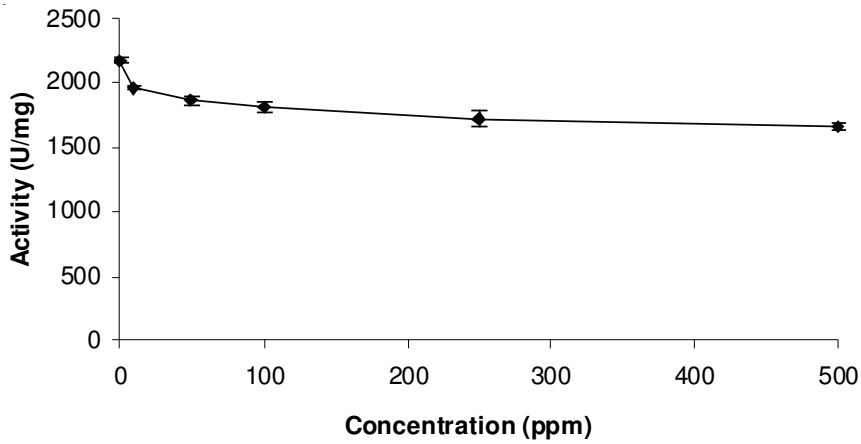

Fig. 2. Effect of cyprodinil concentrations on purified CuZnSOD activity

Specific activity of CuZnSOD was 2178 U/mg in the absence of cyprodinil, whereas in the presence of 500 ppm of cyprodinil, specific activity of CuZnSOD decreased to 1660 U/mg (Fig. 2).

Inhibition of cyprodinil: At different substrate concentrations of xanthine from 4.08×10^{-4} to 2.04×10^{-2} mM and at different concentrations of cyprodinil 50 and 100 ppm, activities of purified CuZnSOD were determined. Lineweaver-Burk graph of CuZnSOD was drawn by using the obtained results (Fig. 3).

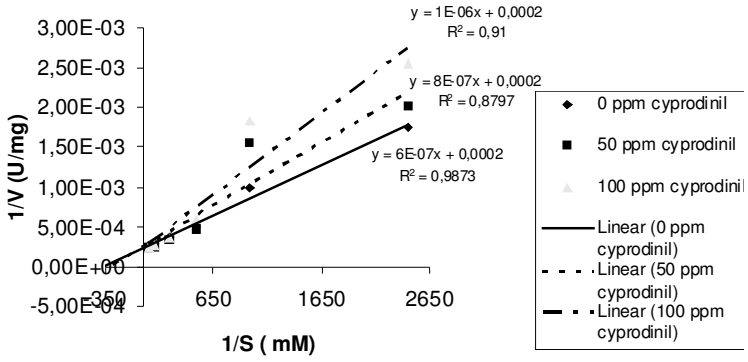

Fig. 3. Lineweaver-Burk graph of CuZnSOD incubated with cyprodinil

According to Fig. 3, V_{max}, K_m and K_i (inhibition constant) of CuZnSOD at different concentrations of cyprodinil were calculated and were shown in Table-1.

TABLE-1
EFFECT OF CYPRODINIL AND FLUDIOXONIL ON KINETIC PARAMETERS OF CuZnSOD

	V_{max} (U/mg)	K_m (mM xanthine)	K_i (mM)
0 ppm cyprodinil	5000	3×10^{-3}	0.000
50 ppm cyprodinil	5000	4×10^{-3}	0.666
100 ppm cyprodinil	5000	5×10^{-3}	0.666
0 ppm fludioxonil	2000	8×10^{-4}	0.000
100 ppm fludioxonil	833	8×10^{-4}	0.269
250 ppm fludioxonil	625	$6 \times .10^{-4}$	0.671

Effect of fludioxonil on erythrocyte: Erythrocytes were incubated with fludioxonil from 0 to 500 ppm and specific activities of CuZnSOD were measured. While CuZnSOD activity was initially 8.27 U/mg, after incubation with 500 ppm of fludioxonil CuZnSOD activity decreased to 2.80 U/mg (Fig. 4).

Effect of fludioxonil on purified CuZnSOD: Purified CuZnSOD was incubated with fludioxonil from 0 to 500 ppm and CuZnSOD activities were measured. Specific activity of CuZnSOD was 523 U/mg in the absence of fludioxonil, whereas in the presence of 500 ppm of fludioxonil, specific activity of CuZnSOD decreased to 213 U/mg (Fig. 5).

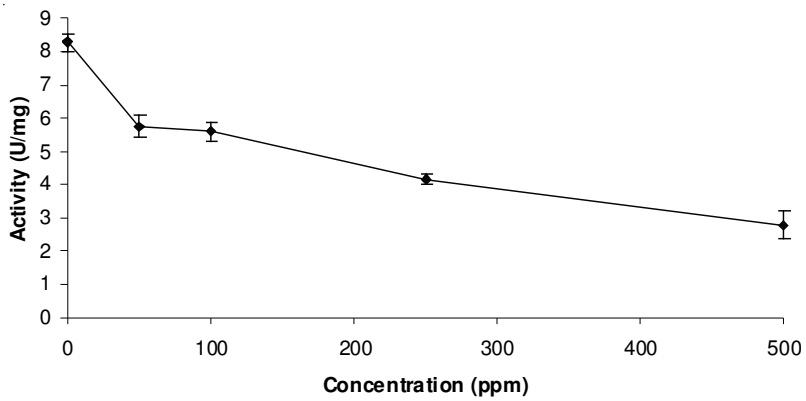

Fig. 4. Effect of fludioxonil concentrations on erythrocyte CuZnSOD activity

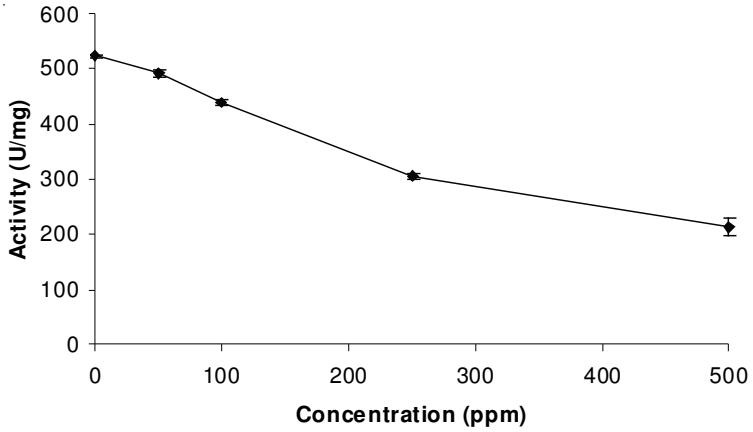

Fig. 5. Effect of fludioxonil concentrations on purified CuZnSOD activity

Inhibition of fludioxonil: At different substrate concentrations of xanthine from 4.08×10^{-4} to 2.04×10^{-2} mM and at different concentrations of fludioxonil 100 and 250 ppm, activities of purified CuZnSOD were determined (Fig. 6).

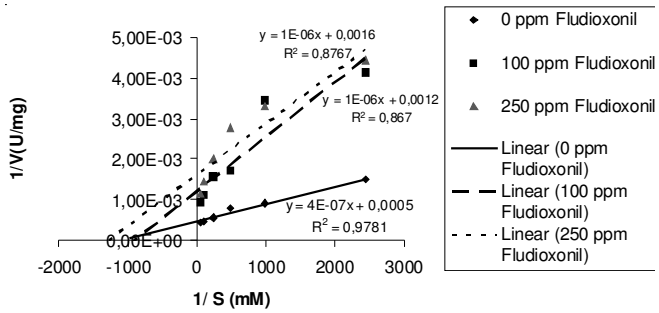

Fig. 6. Lineweaver-Burk graph of CuZnSOD incubated with fludioxonil

According to Fig. 6, V_{max}, K_m and K_i (inhibition constant) of CuZn SOD at different concentrations of fludioxonil were calculated and shown in Table-1.

Although there are some studies to investigate effects of various pesticides on enzymatic antioxidants. But, we could not find any study related to effects of cyprodinil and fludioxonil on human erythrocyte CuZnSOD.

Suwalsky *et al.*[10] revealed that because of lipophilic character of lindane, lipid-rich membranes are a plausible target of it. They interacted lindane with human erythrocytes and molecular models of the red cell membrane to evaluate its toxic effect on cell membranes. These models were bilayers of dimyristoyl phosphatidyl choline (DMPC) and of dimyristoylphosphatidylethanolamine (DMPE), representative of phospholipid classes located in the outer and inner monolayers of the erythrocyte membrane, respectively. They showed by fluorescence spectroscopy that lindane interacted with DMPC large unilamellar vesicles fluidizing both its polar head and its acyl chain regions. They claimed accordance with the bilayer couple hypothesis and preferential interaction of lindane with DMPE that lindane inserted in the inner leaflet of the erythrocyte membrane. Therefore they concluded that the toxic effects of the pesticide can be related to its capacity to interact with the lipid moiety of cell membranes.

Çömelekoglu *et al.*[11] took blood samples from farm workers (n = 40) who had been long-term exposed with pesticides (16.52 ± 6.92 years) in Içel and surrounding agricultural areas and measured erythrocyte superoxide dismutase (SOD) and catalase (CAT) activities. They also measured antioxidant enzymes in people who had not been exposed to pesticides (n = 30). They observed that erythrocyte SOD levels were significantly higher ($p < 0.001$) but erythrocyte catalase activity was significantly lower ($p < 0.001$) in farm workers than in control groups.

Ürek *et al.*[12] purified superoxide dismutase (SOD) from chicken liver and characterized partially. They found that DTT and β-mercaptoethanol didn't inhibit the SOD enzyme but CN^- and H_2O_2 inhibited the SOD enzyme. They observed that in the presence of 2 mM iodoacetamide, the enzyme showed an approximately 40 % activity loss.

Altuntas *et al.*[13] aimed to investigate how an organophosphate insecticide, phosalone, affects lipid peroxidation (LPO) *i.e.*, the levels of malondialdehyde (MDA) and the antioxidant defence system *i.e.*, activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) in erythrocytes *in vitro*. They determined that phosalone caused an increase in MDA formation and a decrease in the activities of SOD, GSH-Px and CAT. However, they saw these effects only at extremely high concentrations of phosalone and these concentrations were in the lethal range.

Bukowska *et al.*[14] studied effects of different concentrations of 2,4-dichloro phenoxyacetic acid (2,4-D) and its metabolite 2,4-dichlorophenol (2,4-DCP) on human erythrocytes *in vitro*. They measured the activity of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and the level of reduced glutathione (GSH).

They determined that the activity of erythrocyte superoxide dismutase SOD decreased with increasing dose of 2,4-D and 2,4-DCP, while glutathione peroxidase activity increased and 2,4-D (500 ppm) decreased the level of reduced glutathione in erythrocytes by 18 % and 2,4-DCP (250 ppm) by 32 %, respectively, in comparison with the controls. They revealed that comparison of the toxicity of 2,4-D and 2,4-DCP, the most prominent changes occurred in human erythrocytes incubated with 2,4-DCP.

Mavi *et al*.[15], purified superoxide dismutase enzyme (SOD) from human erythrocytes by copper chelate affinity chromatography techniques. Then they investigated the inhibition and activation effects of some drugs on the activities of CuZnSOD in human erythrocyte and leukocyte cells. They investigated inhibition or activation effects of 14 drugs on CuZnSOD. They determined that 5-fluorouracil showed activation effects on CuZnSOD at 3.33 and 4.00 mg/mL concentrations with 33 and 32 % activation, respectively. They isolated leukocytes from healthy human blood, lysed in liquid nitrogen and investigated the effect of 5-fluorouracil on the lysate SOD activity. They found that 5-fluorouracil showed inhibition effects on total SOD activity of human leukocytes at 2 and 4 mg/mL concentrations with 42 and 62 % inhibition, respectively.

In present study, it is found that when cyprodinil and fludioxonil fungicides interacts with erythrocytes and purified enzyme, they inhibit CuZnSOD.

Conclusion

Because of lipophilic properties of cyprodinil and disruption of cell membranes by fludioxonil, we consider these pesticides penetrate into erythrocytes and affect enzyme activity. Present results suit this idea. It is found that fludioxonil inhibited CuZnSOD non-competitively and cyprodinil inhibited CuZnSOD competitively. Although cyprodinil molecules don't resemble superoxide molecules, we consider that cyprodinil molecules constitute radicals and these radicals compete with superoxide molecules to tie active region of enzyme.

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