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In vitro Toxicity Effects of Some Insecticides on Gilthead Sea Bream (*Sparus aurata*) Liver Glucose 6-Phosphate Dehydrogenase

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Abstract

In this study, effects of some insecticides (cypermethrin, deltamethrin, dichlorvos, imidacloprid and lambda-cyhalothrin) on glucose 6-phosphate dehydrogenase (D-glucose-6-phosphate: $NADP^+$ oksidoreductase, EC 1.1.1.49; G6PD) from gilthead sea bream (*Sparus aurata*) have been investigated *in vitro*. In this purpose, G6PD enzyme was purified 1864.3-fold with a yield of 54.6% from fish liver using 2',5'-ADP Sepharose 4B affinity chromatography. Activity of the enzyme was determined by Beutler method spectrophotometrically. The purify of the enzyme was checked by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and the purified enzyme showed a single band on the gel. Additionally, inhibitory effects of some insecticides (cypermethrin, deltamethrin, dichlorvos, imidacloprid and lambda-cyhalothrin) on G6PD were investigated. For this aim, IC_{50} values for cypermethrin, deltamethrin, dichlorvos, imidacloprid and lambda-cyhalothrin were determined by plotting activity % vs. [I]. IC_{50} values of cypermethrin, deltamethrin, dichlorvos, imidacloprid and lambda-cyhalothrin were 0.237, 0.016, 0.071, 0.021 and 0.187 mM, respectively. From these results, we showed that deltamethrin is the most potent inhibitor of fish liver G6PD enzyme.

Keywords: Glucose 6-phosphate dehydrogenase, Gilthead sea bream, Insecticides, Inhibition

INTRODUCTION

Glucose 6-phosphate dehydrogenase (D-glucose-6-phosphate: $NADP^+$ oxidoreductase EC 1.1.1.49: G6PD), which is the production of NADPH and ribose 5-phosphate, which are essential for reductive biosynthesis and nucleic acid synthesis, is the first enzyme in the pentose phosphate pathway [1, 2]. Pentose phosphate pathway has three important functions: (i) serving as the route of entry of pentoses to the glycolytic pathway production of reducing equivalents in the form of NADPH; (ii) production of pentose phosphates necessary for nucleotide biosynthesis; and (iii) production of reducing equivalents in the form of NADPH [3]. At the cellular level, a continuous supply of reducing equivalents in the form of NADPH is essential to growth and proliferation process, serving as they do as hydrogen and electronsources for a variety of reductive biosynthetic reactions [4, 5].

Pesticides are widely used in agriculture adversely affecting non-target organisms wherein fish are major components of the aquatic biota. Although there are some benefits to the use of pesticides, are also drawbacks, such as potential toxicity to humans and animals. Deltamethrin is extensively used as an ectoparasiticide in animals and as insecticides in crop production and public health programme. Some of the toxic actions of deltamethrin have been reported earlier, but reports on tissue residue level and effects after repeated daily oral administration on cytochrome P450, cytochrome b5, antioxidant status, blood biochemistry and histology of some tissue in rats are scarcely available [5]. Cypermethrin has been widely used in cotton, cereals, vegetables and fruit, for food storage, in public health and in animal husbandry. Its structure is based on pyrethrum, a natural insecticide which is contained in chrysanthemum flowers, but it has a higher biological activity and is more stable than its natural model [7]. Dichlorvos is a volatile organophosphorus insecticide that is used against a wide range of mite and insect pests of plants, farm animals and humans and as an anthelminthic. It has agricultural, public health and domestic uses. It is also used to control parasites in fish farming [8].

Imidacloprid is an insecticide that was made to mimic nicotine. Nicotine is naturally found in many plants, including tobacco, and is toxic to insects. Imidacloprid is used to control sucking insects, termites, some soil insects, and fleas on pets. Products containing imidacloprid come in many forms, including liquids, granules, dusts, and packages that dissolve in water. Imidacloprid disrupts the nerve's ability to send a normal signal, and the nervous system stops working the way it should. Imidacloprid is much more toxic to insects and other invertebrates than it is to mammals and birds because it binds better to the receptors of insect nerve cells [9]. Lambda-cyhalothrin is a pyrethroid insecticide. Pyrethroids are synthetic chemical analogues of pyrethrins, which are naturally occurring insecticidal compounds producedin the flowers of chrysanthemums (*Chrysanthemum cinerariaefolium*). Insecticidal products containing pyrethroids have been widely used to control insect pests in agriculture, public health, and homes and gardens. Lambda-cyhalothrin is highly toxic to a number of fish and shellfish [10, 11].

In some cases, pesticides contaminate to underground and spring waters. Thus these are hazard to vital function of organisms, including specific enzymes. The differences in enzyme activities are crucial for the continuity of physiological equilibriums. Although cypermethrin, deltamethrin, dichlorvos, imidacloprid and lambda-cyhalothrin are wellknown to interfere with a number processes such as general health, neurotoxic, hematotoxic, reproductive and developmental, genotoxic, respiratory, cardiovascular, hepatic and renal effects on vertebrate, there is little knowledge of about how they affect the specific enzymes in organisms, such as fish [5].

Therefore, the aim of this study is to purify G6PD from gilthead sea bream (*Sparus aurata*) liver and to investigatethe *in vitro* inhibitory effects of some commonly used insecticides on this enzyme.

MATERIALS and METHODS

Chemicals

2',5'-ADP Sepharose 4B was purchased from Pharmacia. $NADP^+$, glucose-6-phosphate, protein assay reagent, and chemicals for electrophoresis were purchased from Sigma. All other chemicals used were of analytical grade and were purchased from Merck.

Animal and Preparation of the Homogenate

S. aurata fish were obtained from a commercial fish farm in the Mediterranean region in Turkey. All procedures were conducted in strict compliance with the guidelines established by the Animal Care and Use Committee. The fish were decapitated and their livers were extracted and stored at -80°C. For analyses, the frozen liver was thawed and cut into small pieces by using a scalpel. Liver simples (8 g) were washed three times with 0.9% sodium chloride solution. The livers were homogenized by liquid nitrogen (-163°C) and transferred to the standard homogenizator buffer, containing 50 mM KH_2PO_4, 1 mM PMSF, 1 mM EDTA and 1 mM DTT. The homogenates was centrifuged for 2h at 13.000 rpm. The supernatant was collected and kept for analysis.

Activity Determination

In accordance with the Beutler [12] method, enzyme activity was spectrophotometrically measured at 37°C. This method is based on the fact that NADPH, which is formed as a result of reducing $NADP^+$, yields absorbance at 340 nm. One enzyme unit was described as the enzyme amount reducing 1µmol $NADP^+$ per minute.

Ammonium Sulphate Fractionation and Dialysis

G6PD enzyme homogenate was exposed to ammonium sulphate precipitation at 0–20, 20–30, 30–40, 40–50, 50–60, 60–70% ranges; and the precipitation range of the enzyme was determined. During each precipitation process, centrifugation was carried out at 13.000 rpm for 15 min. After ammonium sulphate, the precipitate was obtained and dissolved in 50 mM KH_2PO_4 (pH 7.2) buffer. Enzyme activity was measured in the precipitate and supernatant for each time. Then, the enzyme solution was dialysed at 4°C in 10 mM KH_2PO_4 including 1 mM EDTA (pH 7.2) for 2h with two changes of buffer [13].

2',5'-ADP Sepharose 4B Affinity Chromatography

For 10 ml of bed volume, 2 g of dry 2',5'-ADP Sepharose 4B was washed several times in 400 ml of distilled water. During several washings, the impurities were removed and the gel was conditioned. After the removal of the air in the gel, it was resuspended in the buffer (0.1 M K-acetate + 0.1 M K-phosphate, pH 6.0) at a ratio of 25% buffer to 75% gel and was packed in a column (1 x 10 cm). Precipitation of the gel, it was equilibrated with the same buffer by means of a peristaltic pump (flow rate: 50 ml/h). After the dialyzed enzyme solution was loaded on the column which was equilibrated with buffer (0.1 M K-acetate + 0.1 M K-phosphate, pH 6.0) and the flow rate was regulated to 20 ml/h. The column was respectively washed with 25 ml of 0.1 M K-acetate + 0.1 M K-phosphate (pH 6.0) and 25 ml of 0.1 M K-acetate + 0.1 M K-phosphate (pH 7.85). Eventually, the enzyme was eluted with a solution of 80 mM K-phosphate + 80 mM KC1 + 0.5 mM NADP+ + 10 mM EDTA (pH 7.8). The enzyme activity was measured and the activity-containing tubes were collected together [13].

Protein Determination

Quantitative protein determination was spectrophotometrically measured at 595 nm according to Bradford's method, with bovine serum albumin used as a standard [14].

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The control of enzyme purity, using Laemmli's procedure [15], was carried out in 3% and 8% acrylamide concentrations for running and stacking gel, respectively. To the gel solution was added 10% SDS. The gel was stabilized in the solution containing 50% propanol + 10% TCA + 40% distilled water for 30 min. The staining was performed for about 2 h in a solution of 0.1% Coomassie Brilliant Blue R-250 + 50% methanol + 10% acetic acid. Finally, the washing was carried out in a solution of 50% methanol + 10% acetic acid + 40% distilled water until the protein bands were cleared.

In vitro Studies for Insecticides

Cypermethrin (0.02, 0.03, 0.09, 0.2 and 0.57 mM), deltamethrin (0.005, 0.01, 0.015, 0.02 and 0.025 mM), dichlorvos (0.01, 0.02, 0.03, 0.035 and 0.2 mM), imidacloprid (0.005, 0.01, 0.02, 0.025 and 0.03 mM) and lambda-cyhalothrin (0.02, 0.05, 0.1, 0.15 and 0.57 mM) were used as inhibitors. Assays were carried out under standard conditions with varying concentrations of cypermethrin, deltamethrin, dichlorvos, imidacloprid and lambda-cyhalothrin. The inhibition of enzyme by cypermethrin, deltamethrin, dichlorvos, imidacloprid and lambda-cyhalothrin was further examined by varying G6-P concentrations at a fixed NADP concentration and at six different constant concentrations of each insectisides. The activity of control cuvette in the absence of an inhibitor was taken as 100%. All compounds were tested in triplicates for each concentration. For each inhibitor, an activity %-[Inhibitor] graph was drawn. Insectisides concentrations that produced 50% inhibition (IC_{50}) were calculated from the regression graphs.

RESULTS and DISCUSSION

Pesticides are chemical substances used as biological agent, antimicrobial, disinfectant or device against any pest. Some pesticides are persistent organic pollutants and contribute to soil contamination. They are also one of the considerable causes for fresh water pollution [16, 17].

Pesticides can interfere into freshwater such as river and lake by rain water or irrigation water. This situation may be hazardous for living systems, including specific enzymes. It is well-known that enzymes catalyze almost all chemical reactions in the metabolism of the living systems. These chemical substances including pesticides, drugs and metal ions influence metabolism at low concentrations by decreasing or increasing enzyme activities [17-19]. Inhibition of some important enzymes, that play a key role in a metabolic pathway, may lead to pathologic conditions or disorders. Also G6PD has a vital function

in many kinds of tissues and plays an important role in metabolism. It was shown that metabolic disease such as diabetes mellitus affected the some enzyme activity [20]. Cypermethrin, deltamethrin, dichlorvos, imidacloprid and lambda-cyhalothrin are wellknown to interfere with a number processes since they have neurotoxic, hematotoxic, genotoxic, hepatic and renal effects on vertebrate. However, little is known about how their affects on specific enzymes in organisms, such as fish. For this reason, we aimed in this study at evaluating the influences of pesticides on G6PD enzyme from *S. aurata* liver.

In this study, we investigated the effects of cypermethrin, deltamethrin, dichlorvos, imidacloprid and lambda-cyhalothrin on liver G6PD enzyme activity of *S. aurata*, which is an important source of food for all humanity. For this purpose, G6PD, with a specific activity of 68.98 U/mg protein, was purified 1864.3-fold with a yield of 54.6 % from fish liver using hemolysate preparation, ammonium sulfate precipitation, and 2', 5'-ADP Sepharose 4B affinity gel chromatography (Table 1). The purity of the enzyme was determined by SDS-PAGE and showed single bands on the gel (Figure 1).

Table 1. Purification scheme of G6PD from fish liver.

Purification Step	Activity (U/ml)	Protein (mg/ml)	Total Volume (ml)	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg protein)	Purification Factor	Yield (%)
Hemolysate	0.284	7.67	8.31	2.36	63.7	0.037	1	100
Ammonium Sulphate Precipitation and Dialysis	0.687	6.62	2	1.356	13.24	0.1024	2.77	57.4
2',5'-ADP Sepharose 4B Affinity Chromatography	0.861	0.012	1.5	1.29	0.018	68.98	1864.3	54.6

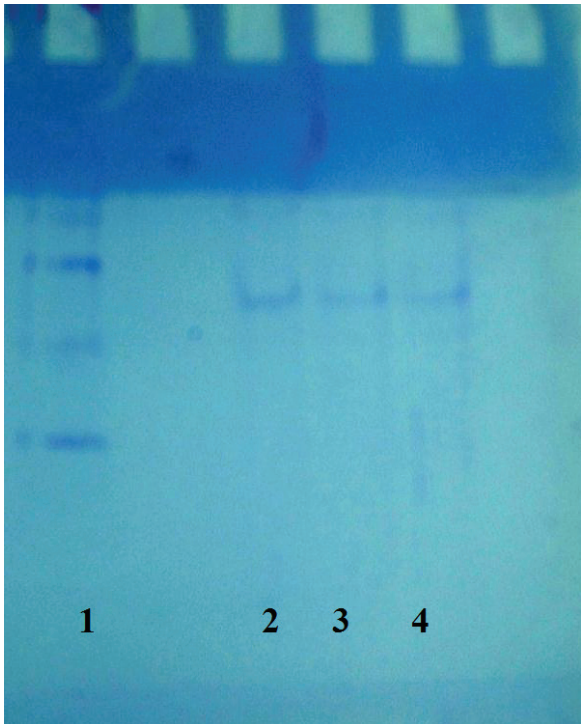


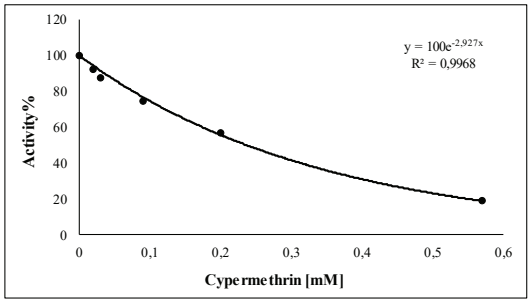
Figure 1. SDS–PAGE of purified G6PD. Lane 1: Standard proteins and Lane 2, 3, 4: fish liver G6PD

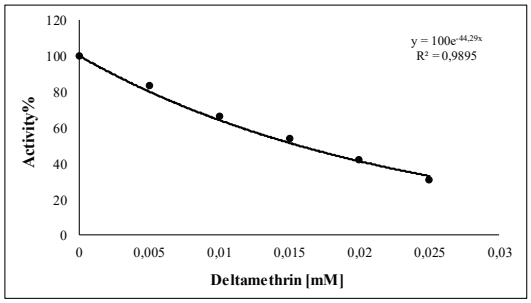
G6PD was first isolated from human erythrocytes by Yoshida [21]. In the following years, the enzyme was purified from ion-exchange materials by using the natural substrates, G6-P and $NADP^+$. The affinity chromatography, 2',5'-ADP Sepharose 4B, was used first by Ninfali and co-workers [13]. Up to now, the enzyme has been purified from many sources using, including fish, various methods and chemicals on the enzyme, such as, sheep brain cortex [22], dog liver [23], *Taenia crassiceps* [24], pea leaves[25], mouse liver [26], *Chalcalburnus tarischii* [27], rainbow trout erythrocytes [5], rat kidney [28], rainbow trout liver [29], human erythrocyte [30], *Capoeta umbla* kidney [2. 31]. These studies are very valuable in terms of G6PD's physiological importance in living organisms.

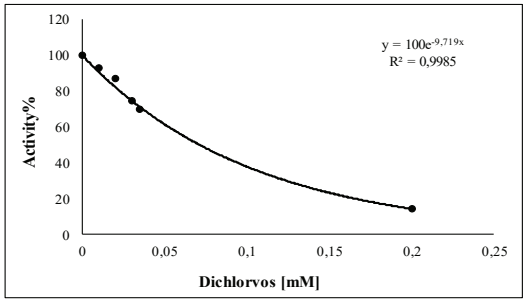
Besides, In this study, cypermethrin, deltamethrin, dichlorvos, imidacloprid and lambda-cyhalothrinwere chosen to investigate their inhibitory effects on fish liver G6PD. These insecticide showed inhibitory action against the enzyme. For each insecticides, IC_{50} parameters were determined by activity%-[Insecticide] graphs (Figure 2). IC_{50} values of cypermethrin, deltamethrin, dichlorvos, imidacloprid and lambda-cyhalothrin were 0.237, 0.016, 0.071, 0.021 and 0.187 mM, respectively (Table 2). Our results showed that deltamethrin is the most potent inhibitor of fish liver G6PD enzyme. Our results are in good agreement with others reported literature. For example, Şentürk et al. [5] investigated *in vitro* and *in vivo* effects of deltamethrin, cypermethrin, and propoxuron G6PD enzyme, which was purified from rainbow trout erythrocyte. They found IC50 values of deltamethrin, cypermethrin, and propoxur were 0.63, 1.02, and 12 mM, respectively. *In vivo* experiments showed that deltamethrin significantly inhibited the G6PD enzyme activity after the 48th h ($p < 0.05$). *In vivo* and *in vitro* experiments showed that deltamethrin is the most potent inhibitor of rainbow trout erythrocyte G6PD. In another study, Ceyhun et al. [17] investigated effects of the pesticides, cypermethrin, deltamethrin, diazinon and propoxur on the activity of rainbow trout gill carbonic anhydrase. IC_{50} values for deltamethrin, diazinon, propoxur and cypermethrin were 0.137, 0.267, 0.420 and 0.460 µM, respectively. *In vitro* results showed that pesticides inhibit rainbow trout gill carbonic anhydrase activity with rank order of deltamethrin > diazinon > propoxur > cypermethrin. Their results showed that deltamethrin is the most potent inhibitor of rainbow trout erythrocyte carbonic anhydrase enzyme.

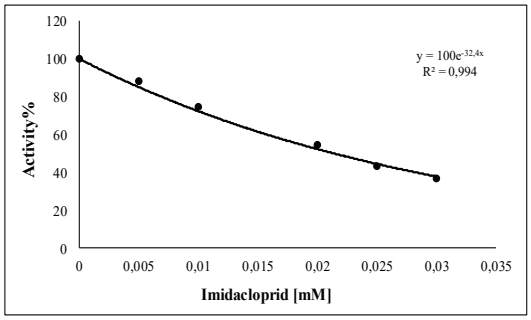
Table 2. IC_{50} values obtained from regression graphs for fish liver G6PD in the presence of different insecticides concentrations.

Insecticides	IC_{50} (mM)
Cypermethrin	0.237
Deltamethrin	0.016
Dichlorvos	0.071
Imidacloprid	0.021
Lambda-cyhalothrin	0.187









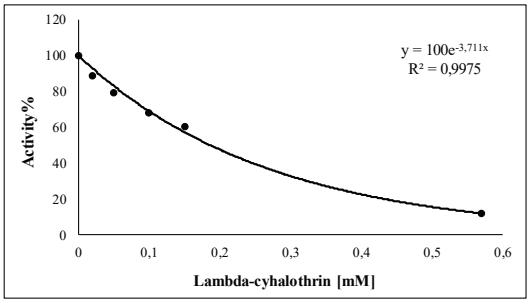


Figure 2. Activity %–[Insecticide] regression analysis graphs for fish liver G6PD in the presence of five different insecticide concentrations.

Consequently, *S. aurata* has a very high economic value because of its high protein/low-fat ratio in terms of nutritional value and its suitability for aquaculture. This fish is consumed as an important source of protein. Many different formations of pesticides may enter human cells along with water, air, and various foods, and humansare negatively affected by these conditions, either directly or indirectly. These effects may cause irreversible damage to human metabolism. Because of this, it is necessary to investigate the effects of pesticides pollution onthis fish to protect the ecological balance and human health. Therefore, in this study, the inhibitory effects of some insecticides (cypermethrin, deltamethrin, dichlorvos, imidacloprid and lambda-cyhalothrin) on the enzymatic activity of *S. aurata* liver G6PD were determined. All insecticides were inhibit the enzyme. But the most effective insecticide is deltamethrin which is widely used at homes and in agricultural fields. All insecticides, used in this study, were inhibit the enzyme at very low doses. This four insecticides can be cause high mortality in fish population. This would lead to the increase in food insufficiency for increasing populations and cause disruption of ecological balance. For this reason, the usage of insecticides must be well controlled and in their use with agricultural aims, dose adjustment should be paid attention

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