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Inhibitory Impact of Pesticides on Glutathione Reductase Activity in Bonito Fish *(Sarda sarda)*

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ABSTRACT
Glutathione is a naturally occurring reducing agent that plays a critical role in protecting cells against oxidative stress. This protective function is facilitated by antioxidant enzymes with the inclusion of glutathione peroxidase and glutathione reductase (GR; EC 1.8.1.7). GR plays a key role in glutathione metabolism by continuously regenerating reduced glutathione (GSH), which is essential for combating oxidative stress within the cell. Therefore, GR activity is considered an important indicator of an organism's defence capacity against environmental stressors, including heavy metals and pesticides. In this study, the GR enzyme was purified from the gill tissue of bonito and its biochemical response to pesticides was evaluated. After purification process, the optimum conditions for enzyme activity were determined as 200 mM KH_2PO_4 buffer at pH 6.5, and 2 mM NADPH as the substrate. The IC_{50} values of the pesticides atrazine, oxamyl, and simazine were found to be 64.5 µM, 56.43 µM, and 53.45 µM, respectively. These findings suggest that GR enzyme could serve as a potential biomarker for assessing the toxicological impacts of environmental pollutants for aquatic organisms.

Keywords: Enzyme Purification, Glutathione Reductase, Pesticide, Bonito Fish

1. Introduction

The presence of free radicals in biological systems may cause disease and aging, due to their harmful effects; which are known to be controlled by some specific antioxidant systems (Harman 2002). Free radicals atoms or molecules with one or more unpaired electrons in their outermost orbit—can cause cellular damage, mutations, and biological aging. (Buyukguzel 2013). An increase in free radicals or a decline in intracellular antioxidant levels can lead to oxidative stress. The impact of free radicals on disease and the ageing process in biological systems, as well as the function of antioxidant defence systems, is highlighted by these sources (Liguori et al. 2018).

Aquatic ecosystems are polluted by toxic chemicals due to anthropogenic activities such as pesticides used in agricultural production, domestic wastes, and industrial activities. These pollutants can show cytotoxic effects in fish via reactive oxygen species (ROS) production (Li et al. 2010). Fish are key organisms in aquatic ecosystems, playing a crucial role in assessing the potential risks of pollutants. Due to their direct residence in aquatic environments and interaction with the food chain, fish serve as model organisms for monitoring pollution, as they can accumulate and metabolize xenobiotics. (Fazio et al. 2013; Ekinci & Beydemir 2010). Evidence suggest that unconscious use of pesticides in agricultural production cause cell and DNA damage in many organisms, trigger reactive ROS production, and affect enzyme activities (Morgan & Brunson 2002; Verma et al. 2007; Lee & Steinert 2003). Pesticides can be classified in two main ways, the first being based on the group of organisms they target. This group includes rodenticides (rodenticides), insecticides (insecticides), herbicides (herbicides), nematocides (roundwormicides), algicides (algaecides) and fungicides (fungicides). The second group comprises inorganic pesticides (mercury, arsenic, fluoride and elemental sulphur), natural organic pesticides (pyrethrum, rotenos, nicotine and allethrin) and synthetic organic pesticides (organophosphate, organochloride and carbamate) (Canyurt 1994). The active ingredients of most pesticides can be divided into organic or inorganic (Ekinci & Şentürk 2010). Pesticides interfere with various metabolic pathways in cells, leading to diverse cellular responses depending on their type and concentration. Pesticide exposure stimulates ROS production via cellular metabolism, resulting in oxidative stress in aquatic species (Trídico et al. 2010). Pesticide toxicity can cause induction of the oxidative system by causing a disruption in antioxidants or free oxygen radical scavenging enzyme systems (El-Sharkawy et al. 1994; Almeida et al. 1997). Pesticides induce oxidative stress by promoting the production of reactive oxygen species (ROS), such as hydroxyl radicals (OH), superoxide anion (O_2^-), singlet oxygen ($^1(O_2)$) and hydrogen peroxide (H_2O_2) which can damage vital biomolecules including DNA, protein, lipid and enzymes (dos Santos Carvalho et al. 2012). In healthy

organisms, formation and removal of ROS are in a balance. Enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione-s-transferase (GST) and glutathione reductase (GR) or non-enzymatic antioxidants such as glutathione (GSH) are involved in maintaining this balance (Martysiak- Żurowską & Wenta 2012).

Glutathione is synthesised in the cell as a tripeptide (cysteine glycine, glutamic acid) and is present in the cell in reduced form (GSH). It provides reducing power to cells by using NADPH. Glutathione is an important antioxidant in the cell due to the high concentration (0.1-10 mM) of cysteine thiol group in its structure (Meister 1995; Meister 1983). The retention of glutathione in the reduced form depends on the pentose phosphate metabolic pathway. NADPH, generated through the pentose phosphote pathway, functions as a crucial coenzyme in the reaction catalysed by glutathione reductase (Thorburn & Kuchel 1985). GSH serves as a reducing agent in protecting cysteine residues, intracellular proteins, and thiol groups in molecules including coenzyme A and dihydrolipoate, as well as in maintaining antioxidant molecules like α-tocopherol and ascorbate, and in reducing ribonucleotides for the synthesis of DNA precursors. GSH is involved in the protection of cells against toxic compounds, radiation and oxidative damage, as well as in the metabolic processes of several endogenous compounds such as prostaglandins, leukotrienes and prostaglandins and in the inactivation of some drugs (Meister 1983; Meister 1991; Meister 1988).

The enzyme GR catalyses the reduction of oxidized glutathione (GSSG) to its reduced form (GSH), utilizing NADPH as a reducing cofactor. The GSH/GSSG ratio serves as a critical biomarker for asseing the cellular redox status (Rana et al. 2002). A primary target of the reaction catalyzed by GR is the regulation of the cellular GSH/GSSG ratio. Under oxidative stress conditions, the GR enzyme plays a critical role in maintaining this ratio. GR enzyme activity in fish has been shown to be influenced by both the concentration and exposure time of environmental pollutants, including pesticides. GR and other enzymatic antioxidants play a crucial role in maintaining the redox balance in fish cells and mitigating oxidative damage. GR activity is essential for detoxifying reactive oxygen species (ROS) produced by pesticide exposure (Meng et al. 2014).

Bonito *(Sarda sarda)* is a mackerel-like predatory fish species from the family *Scombridae* that inhabits the Atlantic Ocean and Mediterranean Sea (Ateş et al. 2008; Zaboukas et al. 2006), typically traveling in shoals. This fish has a blue-green colouration on the dorsal side, while the sides and abdomen are silver-white. It is also characterised by a pattern of nine stripes on the longitudinal dorsal part of its body (Zonn et al. 2021). With an average length of ∼50 cm and a weight of ∼2.0 kg, this migratory species is recognized as a valuable fishery resource (Baibat et al. 2020; Turan et al. 2016.) Its muscle tissue is characterized by a firm texture and dark coloratin and it is notably rich in crude protein. In addition, it contains significant amounts of polyunsaturated fatty acids, essential minerals (such as phosphorus, potassium, magnesium, iodine and iron), and vitamins including riboflavin, thiamine, niacin and vitamin B_{12} (Çatak et al. 2020; Özden 2010). Considering its high nutritional value, *Bonito (Sarda sarda)* is regarded as a commercially valuable species for the fish market.

The widespread use of pesticides is a major contributor to environmental contamination, particularly through their infiltration into water sources from application sites. Investigating the effects of various environmental factors on different animals and specific enzymes is of particular interest (Soydan & Kuran 2017; Alper Türkoğlu et al. 2017; Şentürk et al. 2009; Ekinci & Beydemir 2009). Pesticide pollution in fish in aquatic ecosystem not only disrupts the ecological balance of these organisms, but also causes great damage to fish, which is an important food source and economic value for humans (Oruc et al. 2004). Studying the effects of pesticides on fish can provide important data to better evaluate the negative effects of these substances on human health.

2. Material and Methods

2.1. Chemicals

Analytical grade chemicals used in the experiments were purchased from Sigma-Aldrich, and other required reagents were obtained from Merck.

2.2. GR Enzyme activity

The Bonito fish (*Sarda sarda*) was supplied fresh from the local fish market and transferred to the laboratory via cold chain. The total weight of the fish is 703.686 g. Bonito gill tissue (5.784 g) was weighed and mechanically disrupted in liquid nitrogen using a mortar and pestle. The homogenized tissue was subsequently transferred into a 50 mL Falcon tube, followed by the addition of 40 mL of buffer containing 50 mM KH_2PO_4 (pH 7.2), 1 mM EDTA, 1 mM PMSF, 1 mM DDT, and 150 mM KCl. The sample was then centrifuged at 15,000 rpm at +4 °C for 60 minutes. Following centrifugation, the supernatant and precipitate were separated by filtration through filter paper, and enzyme activity was analysed. Enzyme activity was determined spectrophotometrically by monitoring the oxidation of NADPH and the corresponding decrease in absorbance at 340 nm. The measurements were performed kinetically at 340 nm for 3 minutes (Carlberg & Mannervik 1985).

2.3. Ammonium sulphate precipitation and dialysis

Ammonium sulphate ($(NH_4)_2SO_4$) precipitation is a commonly used method in protein purification. In this step, it was used to

selectively precipitate the target protein from other cellular proteins. Bonito gill tissue was subjected to ammonium sulphate precipitation between 0–100% saturation range to determine the optimal precipitation conditions. Enzymatic activity was observed within the 40–60% saturation interval. At each saturation step, the precipitate was dissolved in 200 mM KH_2PO_4 buffer (pH 6.5). Following the identification of the active range, the resulting protein solution was dialyzed to eliminate unwanted compounds and salts. Dialysis was performed in 20 mM KH_2PO_4 (pH:6.5) buffer for 3 hours.

2.4. Determination of kinetic parameters of the enzyme

After purification, different ionic strength, pH and substrate conditions were examined to determine the kinetic properties of the enzyme. To determine the optimum values, 50, 100, 150, 200, 300, 400, 400, 500, 600, 700 and 800 mM of potassium phosphate (KH_2PO_4) buffer was used, and to determine the optimum pH range, activity was determined at pH ranges of 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5 and 9. At the determined KH_2PO_4 and optimum pH, 70, 80, 90, 100, 120 and 150 µl of NADPH were used for activity determination.

As a result of the activity determination, the kinetic parameters of the enzyme were found as optimum ionic strength 200 mM KH_2PO_4 buffer, optimum pH 6.5 and optimum substrate 2 mM NADPH in 100 µl, respectively.

2.5. In vitro effects of pesticides

To assess the effects of pesticides on (GR) activity in bonito gill tissue, various concentrations of pesticides were introduced in to the reaction medium. Enzyme activity was measured, and a pesticide-free assay was used as the control, representing 100% relative activity.

The effects of varying concentrations of atrazine, oxamyl and simazine pesticides on GR activity in gill tissue were measured spectrophotometrically. The graph of inhibitory concentration versus percentage of activity for each pesticide was determined using typical polynomial software. The pesticide concentrations (IC_{50}) required to inhibit the enzyme activity by 50% were determined and shown in Table 1.

Table 1- GR enzyme inhibition data of bonito fish *(Sarda sarda)* with pesticide

Metal ion	***IC_{50} (μM)***
Atrazine	64.5
Oxamyl	56.43
Simazine	53.45

3. Results and Discussion

ROS, generated as by-products of redox reactions during cellular metabolism, can lead to oxidative stress when produced in excess or not efficiently eliminated, thereby disrupting essential metabolic functions (Assi 2017). As a result, high amounts of ROS can react with biomolecules such as lipids, nucleic acids and proteins, damaging cellular structure and leading to the emergence of chronic diseases (Jones 2008; Federico et al. 2007; Perše 2013). Glutathione (GSH), a ubiquitous antioxidant found in all eukaryotic cells, plays a significant role in cellular defence due to its high intracellular concentration and strong reactivity with electrophiles through the sulfhydryl group of its cysteine moiety. Therefore, it is involved in several oxidative stress–related diseases (Giustarini el al. 2023). An imbalance or deficiency in the GSH/GSSG ratio contributes to cellular oxidative damage, which has been associated with the development of neurodegenerative disorders such as Alzheimer's and Parkinson's diseases, as well as liver and lung pathologies (Towsend et al. 2003; Wu et al. 2004).

Aquatic ecosystems constitute the terminal component of the food chain and fish serve as a vital nutritional resource for humans (Uysal et al. 2008). Although aquatic organisms provide significant benefits, the destruction of their ecosystems has become a significant concern (Verbeke et al. 2005). Pesticides constitute a primary source of pollution in aquatic ecosystems, which are frequently preferred in agricultural regions due to their easy application and short-term effect (Ekinci & Şentürk 2010). They are directly transmitted to aquatic environments through wastewater, rivers or atmospheric transport. Pesticides contaminating aquatic environments tend to accumulate in aquatic organisms, and finally transferred to humans through the food chain (Korkmaz et al. 2020).

In this study, certain characteristic properties of GR enzyme extracted from the gill tissue of bonito fish *(Sarda sarda)* were investigated. The purification procedure was carried out through successive steps of homogenate preparation, ammonium sulphate precipitation and dialysis. The purification process began with the preparation of a homogenate from gill tissue. Subsequently, ammonium sulphate precipitation was performed over a saturation range of %0-100 to fractionate the proteins present in the homogenate. Precipitation of the GR enzyme was achieved within the 40–60% saturation range, resulting in the removal of impurities and effective concentration of proteins at this stage. Ekinci and Şentürk (2013) reported a precipitation interval of 30–70% for GR enzyme derived from the liver of rainbow trout. In another study, a 0–70% saturation range for GR enzyme was determined in sheep liver (Ulusu et al. 2005).

Ammonium sulphate treatment was followed by dialysis to remove undesired ions and salts. Atrazine, oxamyl and simazine pesticides were applied on the enzyme. IC_{50} values of the applied pesticides were calculated as 64.5 µM, 56.43 µM, 53.45 µM, respectively. The IC_{50} graphs are presented in Figures 1-3 and Table 1.

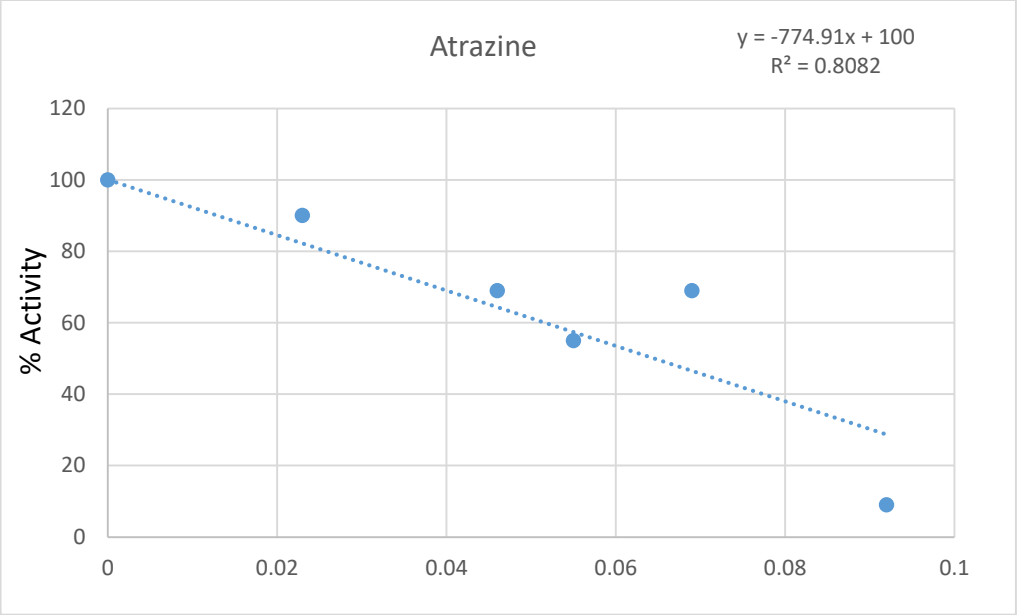


Figure 1- Regression analysis plot of % activity-[Atrazine] for bonito GR enzyme at different pesticide concentrations

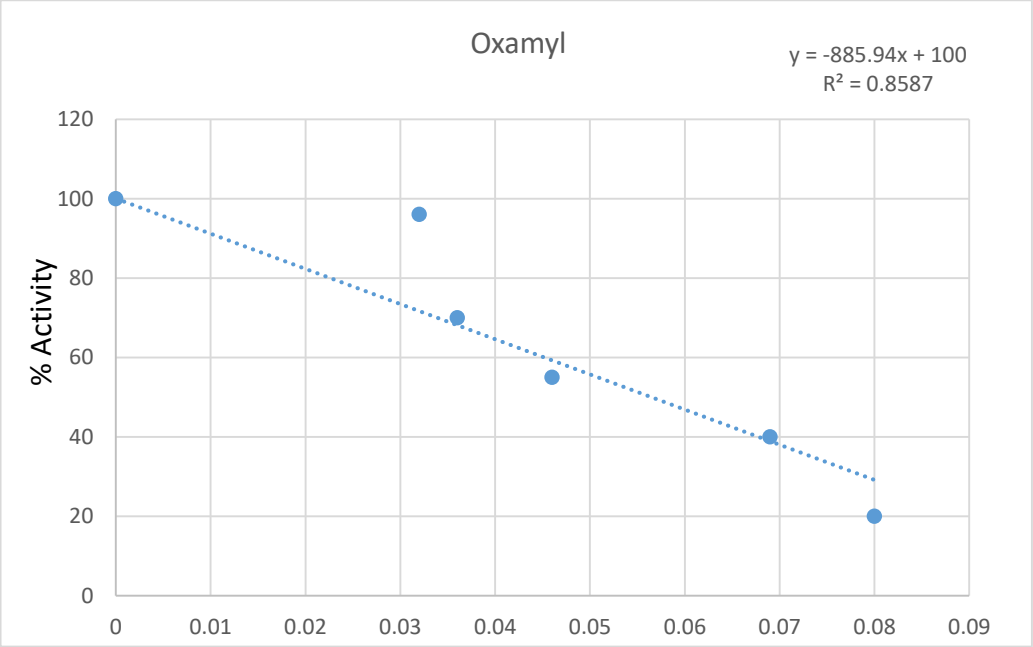


Figure 2- Regression analysis plot of % activity-[Oxamly] for bonito GR enzyme at different pesticide concentrations

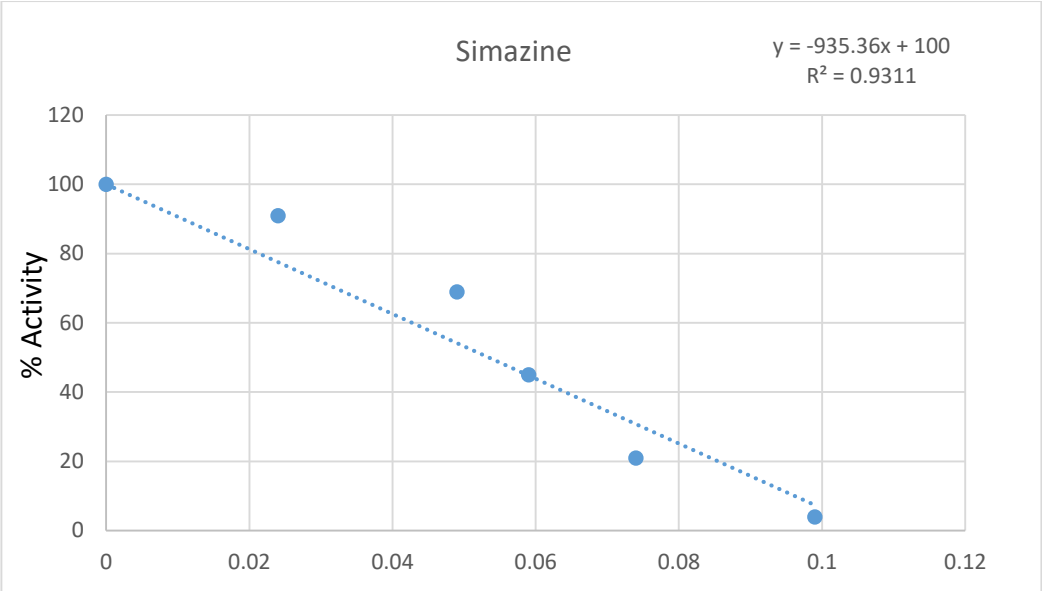


Figure 3- Regression analysis graph of % activity-[Simazine] for bonito GR enzyme at different pesticide concentrations

In this study, the GR enzyme was characterized, with optimal conditions identified as pH 6.5, 2 mM NADPH as the substrate concentration, and 200 mM KH_2PO_4 as the ionic strength. Çoban et al. (2024) reported that the GR enzyme isolated from sheep spleen exhibited optimal activity at 100 mM KH_2PO_4 (ionic strength) and pH 8.0. Similarly, Taşer & Çiftçi (2012) found that GR from turkey liver showed optimal activity at 100 mM KH_2PO_4 and pH 7.5.

The adverse effects of pesticides emphasise the relevance of this study. Notably, these chemicals pose a significant environmental threat by contaminating aquatic habitats such as rivers, lakes, and seas. Pesticide residues accumulated in aquatic organisms may be transferred to humans through diet, which may lead to serious health risks. Taş & Ekinci (2017) investigated the inhibition interaction of GR enzyme isolated from turbot gill tissue with atrazine, carbaryl, carbofuran, oxamyl, propoxur, simazine and tebuconazole pesticides. IC_{50} values of the pesticides were found to be 18.728 µM, 14.395 µM, 19.716 µM, 17.854 µM, 16.709 µM, 14.281 µM and 9.516 µM, respectively. Güller et al. (2018) investigated the inhibition kinetics of cypermethrin, chlorpyrifos, dichlorvos and glyphosate isoproplyamine pesticides on GR enzyme purified from turkey liver. IC_{50} values of the pesticides were determined as 1.79 µM, 40.59 µM, 1.79 µM and 32.86 µM, respectively.

Compared with the literature data, our findings are compatible with previous studies involving GR enzymes from different organisms.

4. Conclusions

GR is a key antioxidant enzyme that plays a vital role in living cells. Pesticides that accumulate in fish can be transmitted to humans through consumption, leading to oxidative stress and the development of various pathological conditions. The GR enzyme, partially purified from bonito gill tissue, plays a crucial role in maintaining the GSH/GSSG balance. Given their potential to disrupt this balance by inhibiting GR activity, the use of these pesticides should be approached with caution, and their dosages strictly regulated. This study is the first report in the literature on characterisation and kinetic properties of GR enzyme from bonito gill tissue, and the findings are expected to contribute to future research on antioxidant enzyme studies.

Author Declarations

Authors' contributions: Concept: E.S., Design: K.I., Data collection: B.Ç., Analysis: S.B.D., Writing: All Authors.
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