Pesticide Biochemistry and Physiology 101 (2011) 1–5

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

journal homepage: www.elsevier.com/locate/pest

Catalase activity as a potential vital biomarker of fish intoxication by the herbicide aminotriazole

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ARTICLE INFO

Article history:
Received 11 February 2011
Accepted 28 May 2011
Available online 7 June 2011

Keywords:
3-Amino-1,2,4-triazole
Catalase
Lactate dehydrogenase
Carassius auratus
Erythrocytes
Plasma
Hemoglobin

ABSTRACT

The objective of this study was to investigate the effects of the herbicide 3-amino-1,2,4-triazole (AMT) on the activities of catalase and lactate dehydrogenase (LDH) in blood (plasma and erythrocytes) and eight solid tissues of goldfish, *Carassius auratus*. Injection of goldfish with AMT (0.5 mg/gww AMT in 0.9% NaCl) resulted in a significant decrease in catalase activity 24 h post-injection in most tissues investigated. In white and red muscle, kidney, heart, liver, brain and erythrocytes the activity of catalase decreased by 61%, 69%, 64%, 48%, 40%, 27% and 26%, respectively, in comparison to the values seen in animals injected with physiological saline (0.9% NaCl). However, the activity of LDH decreased only in red muscle (by 19%) after AMT injection, whereas in plasma it increased by 137%. Protein carbonyl levels, a measure of oxidative damage to proteins, did not change in plasma in goldfish injected with AMT and total hemoglobin levels in AMT-injected fish, although lower compared with uninjected controls, did not differ from values in saline-injected controls. It is proposed that catalase activity in erythrocytes and white muscle might be usefully developed as a potential marker for fish intoxication by aminotriazole and other related herbicides.

1. Introduction

In the last five decades use of pesticides has increased sharply due to intensive agriculture practices [1]. As a result, a number of pesticides and their metabolites have been detected in aquatic environments, raising serious environmental concerns. Among aquatic organisms pesticides may have many negative effects, one in particular being the potential to cause oxidative stress through enhanced generation of reactive oxygen species (ROS) or corruption of antioxidant defense systems [2–4]. The uptake of pesticides by aquatic organisms may occur from the water, from sediments, from suspended particulate matter or from food. The final result of exposure to contaminants depends on the particular dietary and ecological lifestyles of the aquatic organisms [2]. It is known that aquatic organisms are more sensitive to pesticides than terrestrial organisms, including mammals, and in this respect they may provide experimental data for evaluation of oxidative stress induction, mutagenicity, and other adverse effects of pollutants [5]. Pesticides may alter metabolism, activating or inhibiting enzymes [6] and changes in enzymatic activity, in turn, cause modifications of metabolism and cellular damage to various organs [7].

Many recent laboratory and field studies have suggested that the measurement of enzymatic activities might be an effective indicator of exposure to chemical pollution [8,9]. In this regard, there is particular interest in antioxidant enzymes that oppose ROS-induced oxidative damage. Catalase, which protects tissues against damage by hydrogen peroxide, was one of the first enzymes proposed to be an effective marker of oxidative stress [10]. Other studies have focused on enzymes of intermediary metabolism such as lactate dehydrogenase (LDH) [11].

Aminotriazole (3-amino-1,2,4-triazole or AMT), also known commercially as amitrole or amitrol, is a nonselective systemic triazole herbicide used on nonfood croplands to control annual grasses, broadleaf and aquatic weeds [12]. The use of this compound as a herbicide on food crops in the USA was banned because of its carcinogenic properties [13,14]. However, it can still be found in aquatic ecosystems, where there is concern that it is toxic to various species of freshwater fish and invertebrates [15]. Hence, information is needed on the potential toxic effects of AMT on aquatic species and biomarkers need to be developed as indicators of AMT contamination. AMT is known to be an inhibitor of catalase in diverse systems [16,17] and that may be the reason for its toxicity and carcinogenicity. In previous studies we analyzed the effects of AMT on goldfish brain [18], liver and kidney [19] by monitoring the dynamics of catalase activity over 5–168 h after intraperitoneal injection of AMT at concentrations of 0.1, 0.5 or 1.0 mg/gram wet weight (gww). To extend our knowledge of the

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doi:10.1016/j.pestbp.2011.05.005

tissue specificity of AMT-induced catalase inhibition in fish, the present study investigated the effects of AMT on the activities of catalase and LDH in six goldfish tissues (kidney, heart, red muscle, white muscle, brain and liver) as well as in blood plasma and erythrocyte hemolysate. The AMT dose and length of fish treatment were selected based on our previous work [18,19] where we demonstrated that an AMT dose of 0.5 mg/gww produced a well-recognized effect after 24 h. In the present study, we found that catalase activity in erythrocytes or white muscle samples might potentially be developed as a vital biomarker for fish contamination by AMT and other related herbicides. Although it is difficult to directly relate the AMT doses used in the present study to environmental concentrations of AMT which are very low [20], we suggest that measurement of catalase activity can provide some alternative or be complementary to physico-chemical methods of AMT detection and help to develop a biological detection method.

2. Material and methods

2.1. Reagents

Phenylmethylsulfonyl fluoride (PMSF), 2,4-dinitrophenylhydrazine (DNPH), potassium phosphate monobasic, pyruvate and ethylenediamine tetraacetic acid (EDTA) were purchased from Sigma–Aldrich Corporation (USA), NADH was obtained from Reanal (Hungary). All other reagents were of analytical grade.

2.2. Animals and AMT exposure

Goldfish (*Carassius auratus* L.) weighting 50–100 g were obtained commercially from a local fish farm (Kukilnyky, Ivano-Frankivsk district, Ukraine) in October 2009. They were held in a 1000 L open tank for 8 weeks before experimentation under natural photoperiod in aerated and dechlorinated tap water at 19.0–19.5 °C and pH 7.40–7.85; oxygen concentration was maintained in the range 7.5–8.0 mg L^{-1}. Fish were fed with commercial food during acclimation to laboratory conditions, but they were not fed during experimentation. For experiments, three groups of six fish were transferred into 120 L glass aquaria, in a static mode, under the same environmental conditions. Fish in the first group were injected intraperitoneally with sterile AMT dissolved in 0.9% (w/v) NaCl to reach a final concentration of 0.5 mg/gww (the injected volume was of 0.3% of body mass). The second group of fish was injected with 0.9% NaCl only (also 0.3% of body mass). The last group of goldfish was not treated and used as an initial control. After 24 h exposure, blood was quickly collected from caudal vessels using 50 mM EDTA as an anticoagulant. Fish were then sacrificed by transspinal transsection and tissues were dissected out, rinsed in ice-cold 0.9% NaCl solution and homogenized in pre-chilled homogenization buffer containing 50 mM phosphate buffer (KPi) and 0.5 mM EDTA (pH 7.0).

2.3. Hemoglobin determination and hemolysate preparation

Total hemoglobin concentration was measured as in [21] with some modifications according to the method of Bilyi and colleagues [22]. Aliquots (0.01 ml) of blood were added into 3 mM sodium–potassium phosphate buffer (pH 6.36) in a total sample volume of 2 ml; after 2–3 min incubation to allow erythrocyte hemolysis, sample absorbance was measured [22].

The rest of the blood was separated into plasma and erythrocyte fractions. After removal of plasma by centrifugation at 1500 g, 4 °C for 15 min in a CV 1500 centrifuge, erythrocytes were washed three times with 0.9% w/v NaCl at 4 °C and then disrupted osmotically by the addition of five volumes of ice-cold distilled water [23]. Hemolysates were centrifuged (8000 g, 15 min, 4 °C) to remove the ghosts. Plasma and hemolysates were used for the measurement of enzyme activities.

2.4. Assay of catalase and lactate dehydrogenase activities

Samples of solid tissues were homogenized (1:10, w/v) using a Potter–Elvejhem glass homogenizer in pre-chilled 50 mM KPi buffer (pH 7.0), containing 0.5 mM EDTA; a few crystals of phenylmethylsulfonyl fluoride (PMSF) were added prior to homogenization. Homogenates were centrifuged at 15,000 g, 4 °C for 15 min in an Eppendorf 5415 R centrifuge (Germany). Supernatants were removed, kept at 0–4 °C and used for enzyme assay.

Catalase activity was measured at 240 nm in tissue supernatants, plasma and erythrocyte hemolysates in a medium containing (final concentrations are given): 50 mM KPi-buffer (pH 7.0), 0.5 mM EDTA, 10 mM H_2O_2, and 5–50 μL supernatant [24]. LDH activity was assayed spectrophotometrically using a Specol 211 spectrophotometer (Germany) by monitoring the change in NADH absorbance at 340 nm. The reaction mixture contained (final concentrations): 50 mM KPi-buffer (pH 7.0), 1 mM EDTA, 0.2 mM NADH, 1 mM pyruvate, and 2–40 μL of supernatant [25]. One unit of enzyme activity is defined as the amount of enzyme consuming 1 μmol of substrate per minute. Activities were expressed as international units per milligram of protein.

2.5. Protein carbonyl determination

A 50 μl aliquot of plasma was mixed 1:1 v/v with 40% w:v trichloroacetic acid and then centrifuged for 5 min at 5000 g. Protein carbonyl (CP) content was measured in the resulting pellets, by the reaction with 2,4-dinitrophenylhydrazine (DNPH), as described previously [24]. The content of carbonyl protein groups was evaluated spectrophotometrically at 370 nm. Data are expressed as nanomoles CP per milligram protein.

2.6. Protein measurements and statistics

Protein concentration was measured by the Bradford method with Coomassie Brilliant Blue G-250 [26] using bovine serum albumin as a standard. Data are presented as means ± SEM. Statistical analysis was performed using the Student's *t*-test.

3. Results and discussion

3.1. Total hemoglobin concentration in goldfish blood and protein carbonyls in plasma

Several studies have investigated effects of some pesticides on hematological parameters of different fish species. For example, the effect of dichlorvos was studied in *Cyprinus carpio* [27] and *Clarias batrachus* [28], and the organophosphorus preparation ekalux was studied in *Oreochromis mossambicus* [29]. Many publications have analyzed different biochemical and physiological changes induced by pesticides [30,31]. Blood parameters can be a useful tool for evaluation of the effects of pesticides on fish [32], particularly because tests can be performed non-lethally.

We tested whether fish injection with AMT affected total hemoglobin content. Fig. 1 shows total hemoglobin concentration in blood of fish injected with either 0.9% NaCl or with AMT dissolved in 0.9% NaCl; hemoglobin levels decreased by 28% and 19%, respectively, in comparison with uninjected fish. Similar results were reported by Regz and colleagues in pesticide-exposed rats [33]. On the other hand, our results disagree with data obtained by Heim and colleagues [34] who found that AMT did not affect hemoglobin concentration in rats. The differences between these studies may

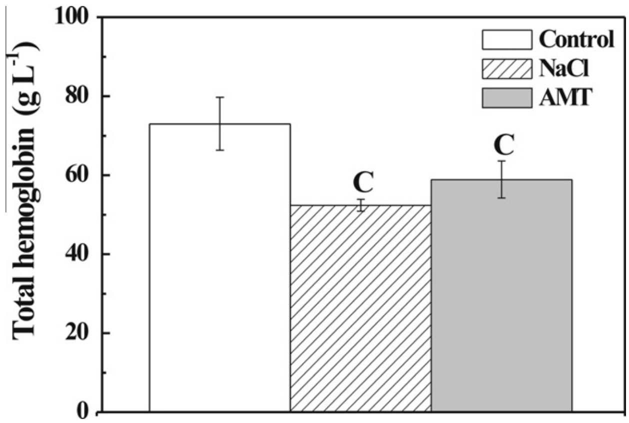

Fig. 1. The concentration of total hemoglobin ($g\ L^{-1}$) in blood after goldfish injection with either 0.9% NaCl or AMT (0.5 mg/gww in 0.9% NaCl). Data are expressed as means ± SEM, $n = 4–6$. [C]Significantly different from the control (uninjected) group ($P < 0.05$).

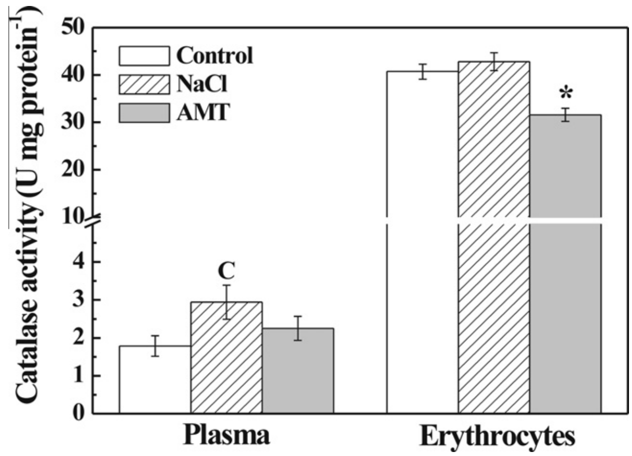

Fig. 2. The activity of catalase in erythrocytes and plasma after goldfish injection with either 0.9% NaCl or AMT (0.5 mg/gww in 0.9% NaCl). Data are expressed as means ± SEM, $n = 4–6$. [C]Significantly different from the control (uninjected) group or *from the NaCl-injected group ($P < 0.05$).

be connected with animal species, concentrations or methods used for animal treatment. Overall, however, it is clear that hemoglobin levels would not be a useful indicator of fish intoxication by AMT or related pesticides.

We next determined whether AMT injection induced oxidative stress in goldfish blood by measuring the level of protein carbonyl (CP) groups, a common indicator of oxidative stress. In the control group CP content in plasma was 2.28 ± 0.11 nmol mg protein^{-1}. Neither injection of 0.9% NaCl, nor AMT dissolved in 0.9% NaCl affected CP levels, which were 2.08 ± 0.38 and 1.63 ± 0.21 nmol mg protein^{-1}, respectively. These data demonstrate that AMT treatment did not influence the process of ROS-induced protein oxidation in fish plasma.

3.2. Blood catalase and lactate dehydrogenase activities

Fish exposed to environmental pollutants exhibit a variety of physiological responses, including disturbances of blood homeostasis [35]. Biochemical profiles of blood can provide important information about the internal environment of the organism [36]. The erythrocytes represent a substantial portion of the antioxidant capacity of the blood and catalase is one of the critical enzyme components of their antioxidant defense system [37].

Fig. 2 shows the catalase activity in erythrocytes and plasma of the three groups studied. Injection with physiological saline did not affect catalase activity in erythrocytes, but injection with AMT depressed activity in erythrocytes by 26% as compared with NaCl-injected fish. This shows that AMT inhibited catalase in goldfish erythrocytes. Our results do not correspond to data obtained by Johnson and colleagues [38], who investigated AMT effects on catalase *in vitro* in mice erythrocytes and found no inactivation of the enzyme by AMT. Aminotriazole did not influence catalase activity in blood *in vivo* [39] and activity in hemolysates *in vitro* decreased slowly and only after the addition of hydrogen peroxide [40]. This probably indicates that mammalian blood contained substances that could act as catalase–hydrogen peroxide complex I donors in concentrations high enough to prevent or retard enzyme inhibition by aminotriazole.

Catalase activity in plasma was 39% higher in NaCl-injected fish than in fish of the control (uninjected) group (Fig. 2). However, fish injected with AMT showed no difference in catalase activity relative to either the control or NaCl-injected groups.

Fish injection with AMT also resulted in a substantial increase (by 137%) in LDH activity in plasma as compared to injection with NaCl, whereas no difference was found between control and NaCl-injected groups (Fig. 3). These data suggest that monitoring of plasma LDH activity might be of potential use for estimating fish intoxication with AMT. However, in erythrocytes, the AMT-injected group did not show a significant difference in LDH activity compared with either of the other two groups, whereas the NaCl-injected fish had 20% lower LDH activity than the control group.

3.3. Catalase and lactate dehydrogenase activities in solid tissues

Catalase is a major antioxidant enzyme found in virtually all aerobic organisms. The activity of the enzyme varies in different tissues, being higher in organs with high oxidative potential [41]. This was also confirmed in the present study with goldfish. Catalase activity decreased in the following order: liver > kidney ≈ heart > red muscle > brain > white muscle (Table 1). We observed similar trends in an earlier investigation of AMT influence on catalase activity in frog tissues [17]. The differences in catalase activity can be explained by the functions of these tissues. Thus, liver actively performs biosynthetic and detoxifying activities, which needs extensive energy supply provided by oxidative metabolism [42]. By contrast, white muscle possesses low catalase activity, correlated with the low intensity of oxidative metabolism in this tissue [43].

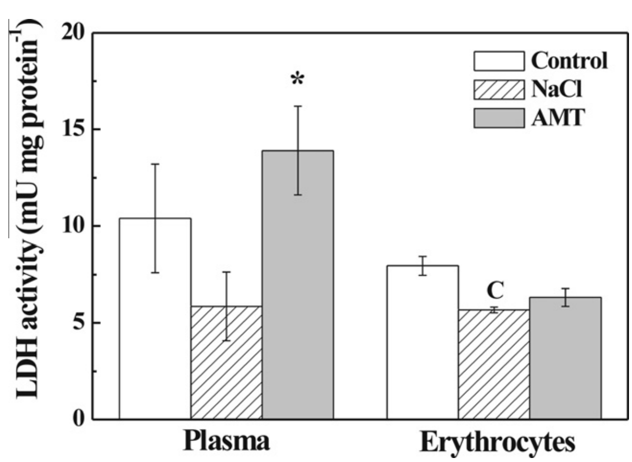

Fig. 3. The activity of lactate dehydrogenase in plasma and erythrocytes after goldfish injection with either 0.9% NaCl or AMT (0.5 mg/gww in 0.9% NaCl). Data are expressed as means ± SEM, $n = 4–6$. [C]Significantly different from the control (uninjected) group or *from the NaCl-injected group ($P < 0.05$).

Table 1
The effect of injection with 0.9% NaCl and AMT (0.5 mg/gww in 0.9% NaCl) on the activity of catalase (U mg protein-1) in different goldfish tissues.

Tissue	Fish group		
	Control	NaCl	AMT
White muscle	3.74 ± 0.32	3.79 ± 0.45	1.49 ± 0.23*
Red muscle	8.64 ± 0.12	10.9 ± 0.7[a]	3.34 ± 0.58*
Liver	32.6 ± 1.8	30.4 ± 1.4	18.3 ± 1.1*
Heart	13.6 ± 1.3	18.1 ± 1.4[a]	9.41 ± 1.90*
Kidney	14.0 ± 0.9	32.0 ± 1.8[a]	11.6 ± 1.3*
Brain	5.45 ± 0.49	5.75 ± 0.38	4.22 ± 0.68*

NaCl and AMT groups were injected with sterile physiological saline (0.9% NaCl) or AMT (0.5 mg/gww in 0.9% NaCl), respectively, and sampled after 24 exposure. Control fish were not injected. Data are expressed as means ± SEM n = 3–5.
[a] Significantly different from the control (uninjected) group.
* Significantly different from the NaCl-injected group ($P < 0.05$).

Table 2
The effect of injection with 0.9% NaCl and AMT (0.5 mg/gww in 0.9% NaCl) on the activity of LDH (U mg protein-1) in different goldfish tissues.

Tissue	Fish group		
	Control	NaCl	AMT
White muscle	0.63 ± 0.05	0.75 ± 0.05	0.77 ± 0.21
Red muscle	2.06 ± 0.40	1.87 ± 0.07	1.51 ± 0.09*
Liver	1.95 ± 0.22	2.01 ± 0.07	1.90 ± 0.04
Heart	2.08 ± 0.13	1.40 ± 0.13[a]	1.70 ± 0.21
Kidney	0.51 ± 0.08	0.45 ± 0.02	0.42 ± 0.02
Brain	1.33 ± 0.05	1.08 ± 0.08[a]	1.01 ± 0.12

NaCl and AMT groups were injected with sterile physiological saline (0.9% NaCl) or AMT (0.5 mg/gww in 0.9% NaCl), respectively, and sampled after 24 exposure. Control fish were not injected. Data are expressed as means ± SEM n = 4–6.
[a] Significantly different from the control (uninjected) group.
* Significantly different from the NaCl-injected group ($P < 0.05$).

Aminotriazole is a known catalase inhibitor that is widely used to analyze the role of catalase *in vivo* and *in vitro* [17–19,45]. AMT irreversibly inactivates human catalase by covalent interaction with His75 in the active center of the enzyme to prevent binding of hydrogen peroxide [44]. In previous experiments, we [17,18,41] and others [46] demonstrated that inhibition of catalase by AMT caused oxidative stress and induced protective compensatory mechanisms. Inhibition of catalase also resulted in oxidative stress in tissues of rainbow trout [47].

In the present experiments, fish injection with 0.9% NaCl increased catalase activity in red muscle, heart and kidney by 26%, 33% and 129%, respectively. However, catalase activity was reduced significantly in all tissues of fish injected with AMT in comparison with NaCl-injected fish. The reduction in catalase activity due to AMT injection was highest in red muscle, where catalase activity was reduced to ~31% of value of NaCl-injected fish. In kidney, catalase activity was 64% lower after AMT injection whereas levels in other tissues were 61%, 48%, 40% and 27% lower in white muscle, heart, liver and brain, respectively, compared with the respective values in saline-injected fish (Table 1). The lowest sensitivity of catalase to AMT was found in goldfish brain which might be due to an inability of AMT to cross the hematoencephalic barrier, or, if crossed by a fast restoration of catalase due *de novo* enzyme synthesis. It is impossible to choose between these possible explanations.

The tissue-specific effects of AMT injection on catalase activity can be explained in several ways. For example, effects might depend on the distribution of the inhibitor in different organs *in vivo* or a different amount of hydrogen peroxide production in the various tissues. Similar tendencies were observed in our previous study [19], when we registered a significant decrease in catalase activity by 50% and 80% in goldfish kidney and liver, respectively, after injection with 0.5 mg/gww of AMT and 24 h exposure as compared with control uninjected fish. Catalase activities in rat liver and kidney also decreased profoundly within the first 3 h after intraperitoneal or intravenous injection of AMT [39].

Perturbation of the prooxidant–antioxidant balance through a decrease in antioxidant capacity may lead to changes in the activity of other enzymes. Although LDH is generally rather stable to oxidation [48], in our previous study it was shown that LDH could be inactivated in the presence of hydrogen peroxide *in vitro* [49]. Because of this, we suggested that this enzyme might also be inactivated by ROS *in vivo*. Some authors suggest that LDH activity can be a marker of increased ROS levels [50]. Taking this into consideration, we measured LDH activity after injection of goldfish with NaCl or AMT. Table 2 shows that LDH activity decreased in heart and brain of NaCl-injected fish by 33% and 19%, respectively. However, AMT treatment had no significant effect on LDH activity in any tissue, compared with uninjected controls, although LDH activity in red muscle of AMT treated fish was 19% lower than the corresponding value in NaCl-injected fish. These data clearly show that LDH activity was virtually unaffected by AMT injection. This work has demonstrated that goldfish injection with aminotriazole inhibited catalase in all six solid tissues and blood. At the same time, LDH activity in these tissues was virtually unaffected by AMT, as well as protein carbonyls in plasma. AMT effects on catalase activity are probably due to a specific interaction of this herbicide with the heme part of catalase [45], whereas LDH, which does not possess this group, was unaffected. The present experiments identifying fish tissues that display high and reliable sensitivity to AMT suggest the possibility that the effects of environmental concentrations of AMT in waters exposed to agricultural run-off could be assessed by monitoring catalase activity in fish. Furthermore, the high sensitivity to AMT by catalase in both erythrocytes and muscle suggests that non-lethal techniques could be used (vital blood sampling or muscle biopsy) to acquire samples and that these could provide a relatively specific approach to identify fish intoxication by aminotriazole and related pesticides.

Acknowledgments

We are thankful for the excellent technical assistance of Viktor V. Husak and J.M. Storey is acknowledged for critical reading of the manuscript. The research received partial support from the Ministry of Education and Science of Ukraine to VIL (#0106U002245) and from a discovery grant from the Natural Sciences and Engineering Research Council of Canada to KBS.

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