Bull. Environ. Contam. Toxicol. (2003) 71:520–526

DOI: 10.1007/s00128-003-8346-7

Bulletin of Environmental Contamination and Toxicology

# DT-Diaphorase [NAD(P)H: (Quinone Acceptor) Oxidoreductase] in *Gammarus pulex*: Kinetics and Some Biochemical Properties

M. Kutlu,[1] S. Sümer,[2] A. Özata[1]

[1] Department of Biology, Faculty of Science, Anadolu University, 26470 Eskişehir, Turkey
[2] Department of Biology, Faculty of Science, Hacettepe University, 06532 Beytepe, Ankara, Turkey

Received: 23 November 2001/Accepted: 13 June 2003

It is very important to get some information on the acute toxicity of substances to aquatic invertebrates in the assessment of their hazard in aquatic environments. Amphipods are one of the most sensitive groups of organisms to toxic substances in acute tests (McCahon et al., 1988). *Gammarids* are common and abundant in many regions and also easily collected and maintained. Previous studies showed that freshwater *gammarids* are sensitive to a wide variety of chemicals (Arthur, 1980). *Gammarus* is also one of the most sensitive genus to some heavy metals such as Cd, Cu and Pb according to the US EPA sources (Diamond et al., 1994).

Lead is one of the most important heavy metal pollutants in aquatic environments. Transport, industrial and domestic waste products are main sources of this pollutant. It causes not only water pollution but also air pollution due to direct release into the atmosphere (Kutlu and Sümer, 1998). In the present study, we choose the freshwater amphipod *Gammarus pulex* as a sensitive indicator organism for environmental pollution.

The freshwater invertebrate has been used as test organism to aquatic toxicology for many years (Macek et al., 1976). The genus *Gammarus* has been particularly popular (Arthur, 1980). There are some published studies on the toxic effects of lead by assay in enzyme activities (Kutlu and Sümer, 1998). In this study, the toxic effect of lead on *Gammarus*, a sensitive indicator organism for environmental pollution, were studied for enzyme activities changes.

Some recent studies have employed DT-diaphorase [NAD(P)H: (quinone acceptor) oxidoreductase, DTD] as a potential biomarker for xenobiotic exposure in fish (Bagnosco et al., 1991).The flavine-containing enzyme, DTD has been studied extensively in mammalian species, but it has received relatively little attention in aquatic species. In this study, DTD activity (dicumarol-sensitive, dicholorophenolindophenol reduction) and its kinetics were characterized in *Gammarus pulex*.

To our knowledge, a characterization of DTD kinetics has not been reported previously for *Gammarids*. This study focuses on the effect of lead acetate on DTD activity and its characteristics in *Gammarus pulex*.

*Correspondence to:* M. Kutlu

## MATERIALS AND METHODS

*Gammarus pulex* were collected from the Porsuk River at Eskişehir (Turkey). They were taken to the laboratory and transferred into aquarium. Animals were acclimated to laboratory conditions in a recirculating aquarium containing tap water for at least 7 days prior to use in exposure studies. The temperature was controlled and maintained at 10-12 °C. The water was aerated continuously. At the end of acclimation the animals that appeared healthy were used in the experiments, that were sorted according to their length and only animals with 5-8 mm length were used. Pb solutions were prepared by dissolving lead acetate in distilled water. All experiments were carried out in triplicate.

For enzymatic studies animals were homogenized on ice with a teflon pestle and Potter-Elvehjen glass homogenizer after addition of 0,015 M KCl. The homogenate was centrifuged at 1000 g for 20 min at 0 °C. The pellet was discarded and the supernatant was centrifuged at 10000 g for 10 min at 0 °C and the supernatant was then centrifuged at 22 000 g for 60 min at 0 °C. The supernatant obtained was used for measuring DTD activity. DTD activity was determined by measuring the rate of formation of product, DCPIP (dicholorophenolindophenol ), using a modified version of method by Hasspieler and Di Giulio (1992). Protein concentrations were determined using the method described by Lowry et al (1951).

One unit of enzyme activity was defined as the amount of enzyme that produced one nanomole of DCPIP in per minute at 25 °C under the specified conditions. Specific activity was defined in terms of units per mg of protein.

In a series of studies, we observed that the treatment of lead acetate induced the activity of DTD in *Gammarus pulex.* Figure 1 shows the activity of DTD during the 64 hours of exposure at the toxicant concentration of $EC_{50}$.To assess the relationship between cytosolic DTD activity and the exposure time of Pb at $EC_{50}$, the activity was determined after 4, 8, 16, 32, and 64 hours of exposure.

The animals were exposed to a single toxicant concentration ($EC_{50}$) for various time periods (4,8,16,32 and 64 hours) (Kutlu and Sümer, 1998). Control groups of animals were subjected to the same procedures but exposed only to clean, conditioned tap water. At the end of the exposure times, animals were homogenized as mentioned above. After centrifugation enzyme activity was measured.

The effect of pH on DTD activity was also investigated. For this purpose, various buffer solutions (pH: 4.8, 5.2, 6.8, 7.0, 8.8-9.2) were used as working buffer solutions.

In kinetic studies, various substrate concentrations were tested to determine the $K_m$ value of DTD in *Gammarus pulex.* $K_m$ value was obtained by analyses of 1/V vs. 1/[S].

We have also determined the molecular weight of DTD from partially purified enzyme by using SDS-PAGE electrophoresis (Sambrook et al. 1989).

The method of Laemmli (1970) was used to prepare % 10 acrylamide slab gels with %5 stacking gel. The solution applied to the gel contained 20 μg protein, 0.0625 M Tris-HCl (pH: 6.8), %2 SDS, %50 glycerol, % 5 β-mercaptoethanol and % 0.001 bromophenol blue (preheated to 100 °C for 5-10 min.). The molecular weight standards (Sigma SDS-70) used were: bovine albumin (66.000), egg albumin (45.000), pepsin (34.700), trypsinogen (24.000), β-lactoglobulin (18.400) and lysozyme (14.300). The electrophoresis was performed 8 mA / gel at 15 °C for 6-7 hours. The proteins were stained with Coomassie blue-R.

## RESULTS AND DISCUSSION

Lead is a heavy metal commonly found in our environment. It can be an acute and chronic toxin. Lead is also found in soil near abandoned industrial sites at which lead may have been used. Water transported through lead or lead-soldered pipe contains some lead, with higher concentrations found in water that is weakly acidic. Some foods and some traditional home medicines also contain lead. Exposure to lead from any of these sources by ingestion, inhalation or dermal contact can cause significant toxicity (Chadzynski, L;1986).

Lead expresses its toxicity by several mechanisms. It avidly inhibits amino levulinic acid dehydratase (ALAD), one of the enzymes that catalyse synthesis of heme from porphyrin. Inhibition of ALAD causes accumulation of protoporphyrin in erythrocytes which is a significant marker for lead exposure.

Lead also is an electrophile that avidly forms covalent bonds with the sulfhydryl group of cysteine in proteins. Thus, proteins in all tissues exposed to lead will have lead bound to them. Some proteins become labile as lead binds with them because lead causes the tertiary structure of the protein to change; cells of the nervous system are particularly susceptible to this effect. Some lead-bound proteins change their tertiary configuration sufficiently so that they become antigenic; renal tubular cells are particularly susceptible to this effect because they are exposed to relatively high lead concentrations during clearance ( Burtis and Ashwood; 1994).

DT-diaphorase is a cytosolic enzyme, which is localized mainly in liver, kidney and gastrointestinal tract (Ernster et al. 1962 and Lind et al. 1982). DTD has a role as a cellular control devise against radical formation. Moreover it is considered that DT-diaphorase has an important role in the cellular defence mechanism against cytotoxic and mutagenic compound (Riley and Workman, 1992; Munday et all, 1999). This enzyme is induced by aromatic hydrocarbons such as 3-methyl cholanthrene (Williams et al., 1986) and antioxidants (Benson et al., 1980), and is an interesting enzyme from drug metabolism and toxicological aspects. Some recent studies have employed DTD as a potential biomarker for xenobiotic exposure in fish (Bagnosco et al., 1991).

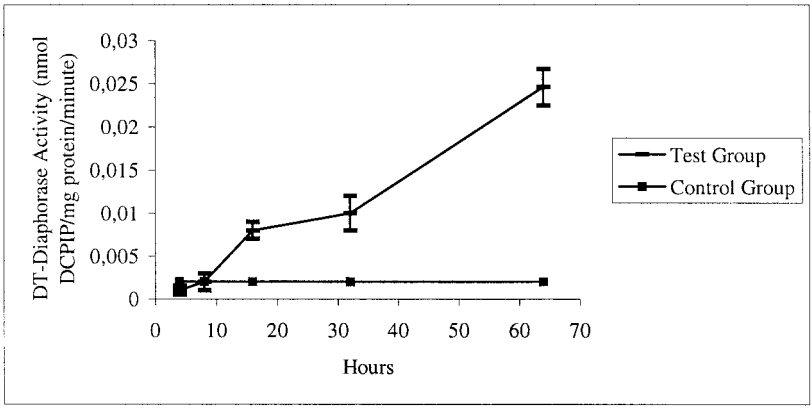


**Figure 1.** Cytosolic DT-diaphorase activity for *Gammarus pulex* at different hours ($EC_{50}$ concentrations). Points represent the mean of two assay trials using the same tissue preparation.

In a series of studies, we first observed that the treatment of lead acetate induces the activity of DT-diaphorase in *Gammarus pulex*. Figure 1 shows the activity of DTD during the 64 hours of exposure at the toxicant concentration of $EC_{50}$. To assess the relationship between cytosolic DTD activity and the exposure time of Pb at $EC_{50}$, the activity was determined after 4, 8, 16, 32, and 64 hours of exposure.

As a result, the activity of DTD was induced by Pb acetate in *Gammarus pulex*. In a period of 64 hours of exposure, the activity became increasingly high. The activity is induced approximately 12 fold at the 64 hours of exposure compared with control. It is also reported that DTD activity is induced 250 % by a single treatment of PbAc compared with control in both liver and kidney in rats (Arizono et al., 1996; Iannaccone et al., 1976).

Data regarding the effect of pH on DTD activity in *Gammarus pulex* indicate that activity is maximal in the pH range 7.0-8.8 decreasing at higher values (Fig. 2).

Similar to our results, it has reported that the activity of DTD in channel catfish (*Ictalurus punctatus*) was maximal in the pH range of 7.0-9.0 and declined at higher values (Hasspieler et al., 1992).

We have also determined the molecular weight of DTD as 66 kDa. from partially purified enzyme by using Sigma SDS molecular weight markers kit (MW-SDS-70) (Fig.3). It has been shown that DTD occurs as a 55 kDa flavoenzyme with two equal-size subunits and two molecules of FAD in mammals (Ernster, 1967).

Km value was estimated by Lineweaver-Burke analysis. Data from kinetic studies indicated a $K_m$ of 125mM NADH for *Gammarus* DTD. $K_m$ values for the organs of channel catfish were determined by Hasspieler and Guilio (1992). In their

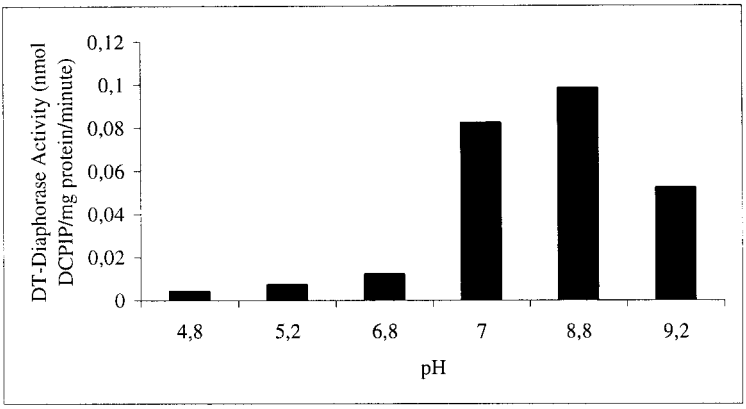


**Figure 2.** pH-activity profile of *Gammarus pulex* cytosolic DTD in the presence of different buffers.

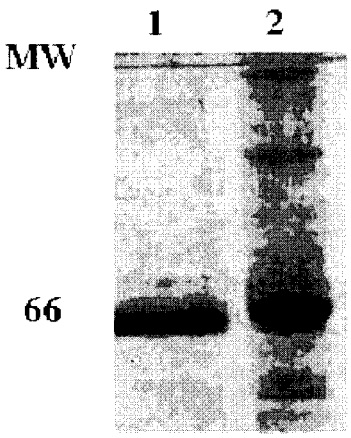


**Figure 3.** SDS-PAGE of *Gammarus* DTD.
The amount of protein applied was 20 μg.
Lane 1, marker (bovine plasma albumin),
Lane 2, *Gammarus* DTD.

study, $K_m$ values of stomach and posterior kidney DTD were 129 mM and 132 mM NADH, respectively. They have also determined the $K_m$ values for liver 84 mM and gill 62 mM DTD.

Heme oxygenase and metallothionein are known to be sensitive parameters which respond to metal exposure (Arizono et al., 1991). Some metals such as lead, cobalt and nickel have been identified as metals which don't have stable replacement ability to zinc in metallothionein. After repeated treatments with these metals, both heme oxygenase and metallothionein increased several fold higher than that observed from a single treatment.

Since no relationship between single and repeated treatment of PbAc was found in DTD activity by Sugiura et al., 1993, the induction mechanism of DTD by PbAc is thought to be different from that of heme oxygenase or metallothionein.

Our study supports a concept that the DT-diaphorase has an important function in detoxification and is predictive of the development of new chemoprotective modalities based upon induction of the enzyme or enhancement of its activity.

## REFERENCES


Arizono K, Sugiura S, Miyazato S, Takiguchi M, Ariyoshi T (1996) DT-diaphorase induction by lead acetate in the liver of rats. Bull Environ Contam Toxicol 57:41-46

Arizono K, Okanari E, Ueno K, Ariyoshi T (1991) Heme oxygenase activity and cytochrome P-450 content associated with induced metallothionein in the liver of rats treated with various metals J Environ Sci Health 26: 941-951

Arthur JW (1980) Review of freshwater bioassay procedures for selected amphipods. ASTM STP American Society for Testing and Materials 715: 98-108

Bagnasco M, Camoinano A, Deflora S, Melodia F and Arillo A (1991) Enhanced liver metabolism of mutagens and carcinogens in fish living in polluted sea water. Mutat Res 262: 129-138

Benson AM, Hunkeler MJ, Talalay P (1980) Increase of NAD(P)H: quinone reductase by dietary antioxidants: Possible role in protection against carcinogenesis and toxicity. Proc Natl Acad Sci USA 77: 5216-5220

Burtis CA and Ashwood ER (1994) Tietz textbook of clinical chemistry-second edition 1220-1221

Chadzynski L (1986) Manual for the identification and abatement of environmental lead hazards. Division of Maternal and Child Health, US Public Health Service, USA.

Diamond JW, Hall JC, Pattie DM, Gruber D (1994) Use of an integrated monitoring approach to determine site specific effluent metal limits. Water Environ Res 66:733-743

Ernster L (1967) DT-diaphorase. Methods in Enzymol 10:309-317

Ernster L, Danielson L, Ljunggren M (1962) DT-diaphorase I. Purification from the soluble fraction of rat liver cytoplasm, and properties. Biochim Biophy Acta 58: 171-188

Hasspieler BM, Di Giulio RT (1992) DT-Diaphorase [NAD(P)H: (quinone acceptor) oxidoreductase] in channel catfish (*Ictalurus punctatus*): kinetics and distribution. Aquatic Toxicol 24: 143-152

Iannaccone A, Bascolo P, Bombardieri G (1976) Comparative effects of experimental lead poisoning on enzymatic activities of kidney and liver in rat. Life Sci 19: 427-432

Kutlu M, Sümer S (1998) Effects of lead on the activity of γ-Aminolevunilic acid dehydratase in *Gammarus pulex*. Bull Environ Contam Toxicol 61: 816-821

Lind C, Hochstein P, Ernester L (1982) DT-diaphorase as a quinone reductase: A cellular control device aganist semiquinone and superoxide radical formation. Arch Biochem Biophys 216: 175-185

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680

Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ (1951) Protein measurement with Folin phenol reagent. J Biol Chem 193: 265-275

Macek KJ, Buxton KS, Derr SK (1976) Chronic toxicity of Lindane to selected aquatic invertebrates and fishes. EPA/600/3-76-046, US Environmental Protection Agency.

McCahon CP, Pascoe D (1988) Culture techniques for three freshwater makroinvertebrates species and their use in toxicity tests. Chemosphere 17: 2471-2480

Munday R, Smith BL, Munday CM (1999) Effect of butylated- hyroxyanisole on the toxicity of 2-hydroxy-1,4 naphthoquinone to rats. Chemico-Biological Interactions 117:241-256

Riley RJ, Workman P (1992) DT-Diaphorase and cancer chemotherapy. Biochem Pharmacol 43: 1657-1660

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: A laboratory manual ,2nd eds Cold Spring Harbor Laboratory, Newyork

Sugiura S, Dhar SK, Arizono K, Ariyoshi T (1993) Induction of DT-diaphorase in the liver of rats treated with various metals. Japanese Toxicol Environ Health 39:1-7

Williams JB, Lu AYH, Cameron RG, Pickett CB (1986) Rat liver NAD(P)H: quinone reductase. J Biol Chem 261: 5524-5528