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Lead toxicity and biochemical characterization of δ-ALAD on endemic prawn, *Palaemonetes turcorum*

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Abstract

The aim of this studuy was to determine the toxic effect of lead (Pb) on *Palaemonetes turcorum*, which is endemic to Turkey, *in vivo* and its inhibitory effect on its δ-Aminolevulinic acid dehydratase (ALAD; E.C.4.2.1.24) *in vitro*, and characterize the enzyme's biochemical features. The LC_{50} (96 h) value, due to Pb toxicity and its confidence limits *in vivo*, were calculated by the EPA Probit Analysis Program. The inhibition effect of Pb on *Palaemonetes turcorum*'s ALAD was determined *in vitro*. The enzyme was partially purified with ammonium sulfate and a Sephadex G-200 gel medium. The LC_{50} value for Pb was found to be 21.98 ppm in *Palaemonetes turcorum*. It was also observed that the inhibition of the enzyme and the amount of Pb in the liver (hepatopancreas) had increased. It was found that the optimum pH of *Palaemonetes turcorum* ALAD was about 7.0 and the enzyme was resistant to heat up to 55°C. As a result of kinetic analyses, the V_{max} value and K_m value were found to be 22.83 nmol phorphobilinogen (PBG)/h/mg protein and 0.06 mmol/reaction mixtures, respectively. The enzyme was purified 51 fold with ammonium sulfate and a Sephadex G-200 gel medium, consecutively. As a result of the study held with SDS-PAGE, the enzyme's molecular mass was about 237.6 kDa.

Keywords: ALAD, lead, *Palaemonetes turcorum*, purification, toxicity.

δ-ALAD enziminin endemik karides *Palaemonetes turcorum*'da biyokimyasal karakterizasyonu ve kurşun toksisitesi

Özet

Bu çalışmada kurşunun yurdumuz için endemik olan *Palaemonetes turcorum*'a toksik etkisi, bu türe ait δ-Amino Levulinik Asit Dehidrataz (ALAD; Porfobilinojen sentaz; E.C.4.2.1.24) enzimi ile etkileşimi ve enzimin biyokimyasal özelliklerinin belirlenmesi amaçlanmıştır. Kurşunun *in vivo* toksik etkisine bağlı LC_{50} değeri (96 saat) EPA Probit analiz programı ile hesaplanmış ve *Palaemonetes turcorum* ALAD'ı üzerine olan inhibe edici etkisi de *in vitro* saptanmıştır. Enzim amonyum sülfat ve Sephadex G-200 jel ortamı ile yapılan işlemlerden sonra kısmen saflaştırılmıştır. *Palaemonetes turcorum*'da LC_{50} değeri 21,98 ppm olarak bulunmuştur. Zamana bağlı olarak enzim inhibisyonunun ve karaciğerdeki kurşun miktarının arttığı saptanmıştır. *Palaemonetes turcorum* ALAD'ının optimum pH'sı 7 civarında ve enzimin ısıya dayanıklı olduğu (55°C) bulunmuştur. Kinetik analizler sonucu V_{max} değeri 22,83 nmol PBG/sa/mg protein ve K_m değeri 0,06 mmol/reaksiyon karışımı olarak bulunmuştur. Enzim, ardışık olarak amonyum sülfatla çökeltme ve Sephadex G-200 jel ortamı ile ham ekstreye nazaran 51 kat saflaştırılmıştır. SDS-PAGE ile yapılan çalışma sonucunda saflaştırılan *Palaemonetes turcorum* ALAD'ının yaklaşık 237,6 kDa moleküler ağırlığa sahip olduğu gözlenmiştir.

Anahtar Kelimeler: ALAD, kurşun, *Palaemonetes turcorum*, saflaştırma, toksisite.

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INTRODUCTION

Lead (Pb) is one of the most important heavy metal pollutants found in the environment, including the aquatic environment. These pollutants are mainly the result of industrial and domestic waste materials. The use of metal-binding proteins to measure the effect of environmental heavy metal pollution on biological systems has been proposed for a couple of decades (Hennig 1986, Wang and Fowler 2007, Ji Won et al. 2008).

The inhibition of δ-aminolevulinic acid dehydratase (ALAD) (E.C.4.2.1.24) is a useful biomarker of Pb exposure and effect, in animal species and human (Rand 1995, Timbrell 2000). δ-ALAD and porphobilinogen synthase catalyses the formation of porphobilinogen by the condensation of two molecules of δ-aminolevulinic acid with the removal of two molecules of water (Sassa 1982). Porphobilinogen is the pyrole precursor utilized by all living systems for the biosynthesis of tetrapyroles,

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including hemes, chlorophylls, and corrins (Jordan 1989).

ALADs have been purified to homogeneity from a wide variety of sources such as bovine liver (Gibson et al. 1955), *human erythrocytes* (Anderson and Desnick 1979), *Rhodopseudomonas capsulatus* (Nandi and Shemin 1973), *Rhodobacter sphaeroides* (Nandi and Shemin 1968), *Escherichia coli* (Spencer and Jordan 1993, Mitchell and Jaffe 1993), *Streptomyces yokosukanensis* (Konuk et al. 2008), *Pseudomonas* sp. (Korcan et al. 2007), and *Citrobacter* sp. (Cigerci et al. 2008) in our lab, and spinach (Schneider and Liedgens 1981). Despite the similarity of the fundamental catalytic properties of all ALADs, differences in the enzyme primary structure, metal ion requirement, and thiol sensitivity have been observed between the various purified or partially purified enzymes (Jordan 1989).

The common prawn *Palaemonetes* spp. is an ecologically important and widely distributed shallow water species which has a potential economic importance (Kutlu and Sumer 1998).

In the present study, the aim was to determine the toxic effect of Pb on *Palaemonetes turcorum*, which is endemic to Turkey, *in vivo* and its inhibitory effect on its δ-Aminolevulinic acid dehydratase (ALAD; E.C.4.2.1.24) *in vitro*, and characterize the enzyme's biochemical features.

MATERIALS AND METHODS

Specimen collection

Specimens of *Palaemonetes turcorum* Holthuis, 1961 (Crustacae: Decapoda) were collected from a branch of the Sakarya River basin in the Yeşilhan region, located 36 km. South-East of Eskişehir, in Turkey. Animals were acclimatized under laboratory conditions for 7 days prior to use. They were maintained in an aquarium with aerated tap water with a pH of 7.5-8.2, a DO of 5.6 ± 0.2 mg/L, a total hardness of 260 mg/L (as $CaCO_3$), an alkalinity of 45.084 mg/L, and a temperature of 23 ± 1°C under a static system. The acclimatized animals were transferred from the stock tank into glass jars with a liter of water. The treatment conditions and water were the same as that for acclimatization.

Pb applications

The stock solution of Pb was prepared by dissolving lead acetate [$Pb(CH_3COO)_2.3H_2O$] (Fluka 153449) (analytical grade) in distilled water. Appropriate volumes of stock solution were added to get the final desired concentrations (1000 ppm). For the determination of LC_{50}, groups of 5 animals were exposed to eleven different Pb concentrations (0.25-100 ppm) for 96 hours. Control groups were maintained in conditioned tap water alone. Mortalities on each concentration were recorded. All experiments were carried out in three replications with good reproducibility.

The LC_{50} (96 h) value and its confidence limits were calculated by a EPA Probit Analysis Program (Kutlu and Sumer 1998). This value is based on mortalities after a 96 h exposure to the toxicant and a further 24 h in toxicant free water.

Determination of ALAD activity

For the enzymatic studies, the on hepatopancreas (liver) was removed, homogenized on ice with a teflon pestle and Potter-Elvehjem glass homogenizer after the addition of 4 volumes of KCl containing a 0.1 M phosphate buffer (pH 6.8). The homogenate was centrifuged at 8000xg for 10 minutes at 4°C. The supernatant was used for measuring the ALAD activity (Coleman 1970). Determination of the activity of the ALAD enzyme was carried out by using the method described by Coleman (1970) and its modified pattern by Kutlu and Sumer (1998). The reaction mixture contained 0.1 M phosphate buffer (pH 7.0), 3.3 mM ALA, supernatant, and 0.3 M reduced glutathione. After applying nitrogen gas to the mixture, it was incubated at 37°C for 1 h. Then the reaction was stopped by adding TCA a 10% solution of (Trichloro acetic acid) containing 0.1 M $HgCl_2$. It was then centrifuged at 8000xg. After centrifuging, 0.5 mL of supernatant was added 1 mL of 5% TCA and 1.5 mL of modified Ehrlich reagent (containing 1 gr dimetilaminobenzaldehit (DAB), 30 mL acetic acid, 16 mL of 70% perchloric acid) and left for 5 min. Finally, the A555 was measured by spectrophotometer (Hitachi-160) and calculated by use of the PBG standard curve. ALAD activity was determined by measuring the rate of formation of product, porphobilinogen, and using the method of Coleman (1970). Protein concentrations were determined using the method described by Lowry (1951). One unit of enzyme activity was defined as the amount of enzyme that produced 1.0 nanomole of porphobilinogen in 1 h at 37°C. Specific activity was defined in terms of units per mg of protein.

The animals were exposed to a single toxicant concentration for a series of time periods (4, 8, 16, 32 and 64 h). Control groups were subjected to the

same procedures but had no toxicant added. At the end of the exposure times, tissues were homogenized as mentioned. After centrifugation, the ALAD activity was measured to quantify the enzyme inhibition.

Determination of accumulated Pb in tissues

Fresh hepatopancreas (liver) tissues were weighed; and placed in a burning tube, and them Two ml of nitric acid and 1 ml of perchloric acid was added to the sample tissues. Afterwards, the aqueous solutions of the tissues were obtained via the burning process. The samples were them transferred into polyethylene tubes and a final volume of 5 ml was obtained by adding ddH_2O (Karataş and Kalay 2002, Ayas et al.2009). The amount of Pb in all the experiments was determined by inductively coupled plasma, using the PerkinElmer 4300DV ICP-OES. Each experiment was repeated two times, and the results are given as averages.

Statistical analyses

All data was presented as a mean ± SD for parametric variables. Parametric variables were compared by using the one-way analysis of variance with post-hoc analysis using the Dunnet-C and Dunnet-t tests. Data was analyzed using the SPSS® for Windows (Version 11.0) computing program, and $p<0.05$ was considered statistically significant (Sokal and Rohlf 1969).

Characterization of ALAD (δ-aminolevulinic acid dehydratase)

For this purpose, different temperatures (from 15 to 85°C) were subjected to investigation. The incubations of the enzyme and reaction mixtures were carried out at 15, 25, 30, 37, 45, 55, 65, 75, and 85°C.

Appropriate buffers, detailed below, were prepared within the pH range of 4-9. The Tris-HCl buffer was used for pH values between 4-7, and a citrate buffer was used for pH values between 8-9. In order to determine the K_m value of the ALAD studied, a plot was also formed.

Partial Purification of ALAD

Precipitation with ammonium sulfate

In the purification technique of ALAD, sufficient solid ammonium sulfate was added to the crude enzymic preparation to obtain 25, 50, 75, and 100% saturation (136 g/L for 25%, 148 g/L for 50% from 25%, 161 g/L for 75% from 50%, and 176 g/L for 100% from 75% saturation). The enzymic activity was checked frequently to observe the precipitation from 25-50%. These intervals were then designed to obtain 20, 30, 40, and 50% saturation. The precipitated protein obtained from each saturation fraction was redissolved in 0.1 M K-phosphate buffer with a pH of 7.0. (Brown and Konuk 1995.)

Dialysis was used to remove ammonium sulfate and other low molecular weight substances present in the protein fractions. Each protein fraction from the fractional precipitation procedure was dialyzed overnight against a large excess of 0.1 M potassium phosphate buffer with a pH of 7.0.

Gel-filtration chromatography

The gel medium used in this study was Sephadex G-200. Since the gel medium was supplied as dry material, it was allowed to be swollen in a K-phosphate buffer overnight before being packed into a column (1.6 x 31.5 cm) at 4°C. Before application of the sample, four bed volumes of K-phosphate buffer (30 mM, pH 7.0) were used to wash the column at a rate of 10 mL/h. After sample application, elution was commenced with the same buffer and flow rate. Fractions, 3 mL elute in each collection tube, were then collected (Andrews 1964, Jerry and Freeman 1970). This was applied to all precipitated protein sections by ammonium sulfate saturation.

Denature polyacrilamid gel electrophoresis (PAGE)

For this, the technique developed by Laemmli (1970) an SDS-PAGE was used. Then a 7.5% gel of gel was prepared including the molecular marker (Sigma, MW-M4048) alongside the proteins. The proteins were run at 30 mA for 1h. The silver staining was carried out according to the method of Blum et al. (1987). After staining the gels, they were visualized by the use of a Uvitec-UV illuminator system, and photographed by Fine Pix 19.

RESULTS

In the present paper, we report the toxic effect of Pb on *Palaemonetes turcorum in vivo* and the evaluation of ALAD, which plays an important role in the heme biosynthesis pathway and activity was extracted from this taxon *in vitro*.

The LC_{50} (96 h) value for Pb in *Palaemonetes turcorum* was found to be 21.98 ppm (9.44-47.55 ppm with 95% confidence limits).

As the Pb concentration in the incubation

mixture decreased, the ALAD activity of *Palaemonetes turcorum* increased (Fig. 1). The ALAD activity significantly decreased ($p<0.05$) in all treatment groups when compared to the control group. As shown in Fig. 2, the Pb contents of the *Palaemonetes turcorum* exposed to lower limit concentration (9.44 ppm) increased by the time of exposure. After a 64 h exposure period with lower limit concentration, it was determined that the Pb accumulation was approximately 600 μg/g in fresh weight. Pb accumulation in the tissues examined was observed to significantly increase at the 16th, 32nd, and 64th hours when compared to the control group.

The effect of incubation temperature and pH are shown in Fig. 3 and 4, respectively. Our findings showed that the ALAD of *Palaemonetes turcorum* has an optimal temperature at 55 °C (Fig. 3). The effect of pH on its activity was examined from a pH of 4.0 to 9.0 (Fig. 4). Optimal activity was obtained at a pH of 7.0.

When the Michaelis-Menten enzymatic velocity graph was drawn, the sigmoidal curve was obtained as happens in allosteric enzymes. V_{max} was found as 22.83 nmol/PBG/h/mg protein and a K_m value of 0.06 mmol/reaction mixtures (Fig. 5).

During the gel filtration, 25 fractions, each fraction containing 3 mL of elute from the column, were obtained and a maximum ALAD activity was observed at the 5th fraction. When the specific activity was calculated, a 51.64-fold purification was obtained at the fraction mentioned (Table 1). According to the SDS-PAGE run, the molecular weight of the enzyme was determined as 237.6 kDa (Fig. 6).

DISCUSSION

The effects of Pb on the heme biosynthesis pathway in organisms was known (Konuk et al. 2010). The inhibition of ALAD in different tissues by Pb was considered as sensitive for the intracellular toxicity of Pb and an end-point. In this study, *in vivo* inhibition of ALAD in *Palaemonetes turcorum*, which is an indicator of environmental pollution, with Pb was shown and the usage of ALAD of the *Palaemonetes turcorum* was studied. The biochemical features of the enzyme were determined by using fractionally purified ALAD from the *Palaemonetes turcorum*.

Pb is acutely toxic to aquatic invertebrates at concentrations between 0.1 and >40 mg/L for freshwater organisms and between 2.5 and >500

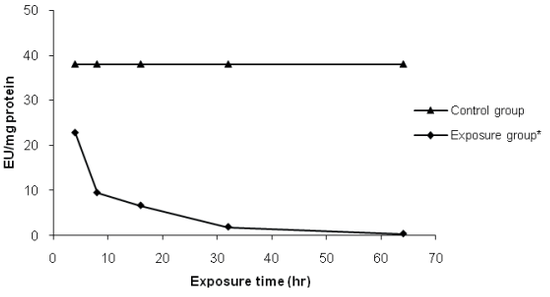

Fig. 1. The relationship between the reduction of ALAD activity and exposure time at lower concentration (9.44 ppm) level for *Palaemonetes turcorum*. All results are means of triplicated experiments. *; Significantly different from control ($p<0.05$).

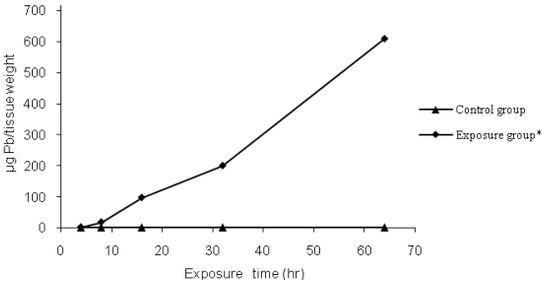

Fig. 2. The Pb contents of *Palaemonetes turcorum* exposed to Pb at lower concentration (9.44 ppm) for 64 h. All results are mean of triplicated experiments. *; Significantly different from control ($p<0.05$).

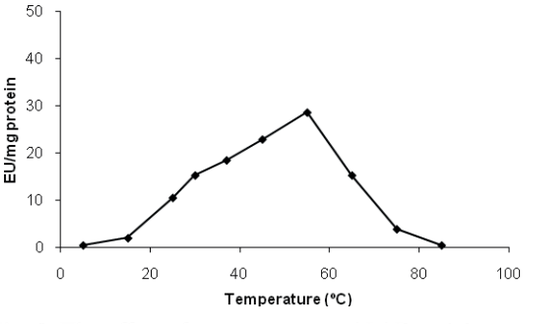

Fig. 3. The effect of temperature on ALAD activity.

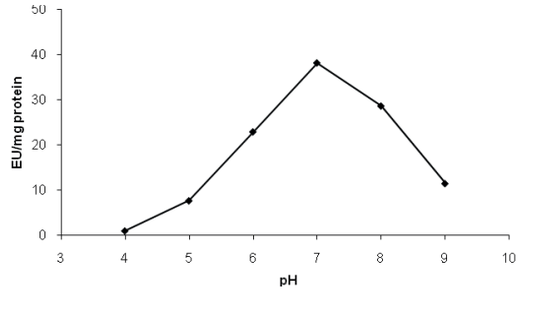

Fig. 4. The effect of pH on ALAD activity.

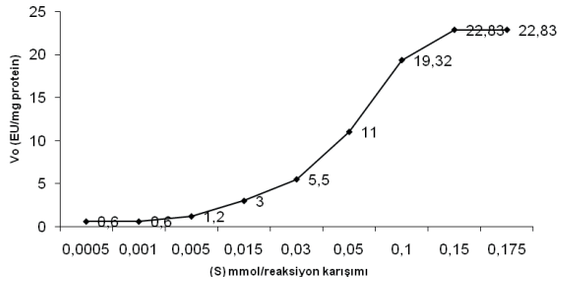

Fig. 5. The Michaelis-Menten curve of δ-ALAD in *Palaemonetes turcorum*.

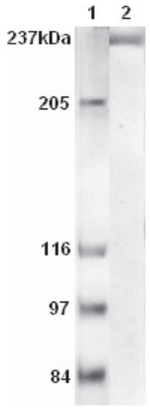

Fig. 6. The SDS-PAGE and molecular mass determination of the enzyme isolated. 1- Mw marker; 2- ALAD

mg/L for marine organisms (Anonymous 1999).

Our data indicated that Pb affects the metabolism of *Palaemonetes turcorum*. The mortality rates in *Palaemonetes turcorum* rose with increasing concentrations of Pb as well as time. A similar finding was reported by Kabila et al. (1996) in shrimp. They investigated the effect of Pb in *Macrobrachium malcolmsonii* (Monsson River prawn) and observed that there was a positive correlation between mortality and increasing Pb concentrations in the medium.

In comparison of the LC_{50} values with other shrimp exposed to lead, there is a wide range of Pb concentrations to give this value in the different species studied. Kabila et al. (1996) noted an LC_{50} of 91.5 ppm Pb for Monsson River prawn *Macrobrachium malcolmsonii*. Fafioye and Ogunsanwo (2007) reported that an LC_{50} value of Pb was 7.28 ppm in post larvae of *Penaeus monodon* while the LC_{50} value for Pb was 10.0 ppm for *Macrobrachium rosenbergii* post larvae.

In the present investigation, the LC_{50} value (21.98 ppm) for 96 h of Pb exposure for *Palaemonetes turcorum* appears to have higher LC_{50} values than that of other shrimp examined. However, it should be considered that the individual responses of these an shrimp to the toxicants might be species-specific.

Table 1. The Purification steps of ALAD and its activities in the procedure.

Step	Total Protein (mg)	Total Activity (EU)	Specific Activity (EU/mg protein)	Recovery (%)	Purification grade (fold)
Homogenate	2.14	6.631	3.09	100	1
30-40% saturated $[(NH_4)_2SO_4]$	0.151	5.526	36.60	83.33	11,84
Column Sephadex G-200	0.0277	4.42	159.57	66.85	51.64

In *Gammarus pulex* (Kutlu and Sumer 1998), the LC_{50} value of Pb was found as 0.394 mg/L. It was found that after a 4-week application of 13 μg/L Pb in *Oncorhynchus mykiss* decreased the δ-ALAD activity of the red blood cell considerably (Hodson 1976).

It was reported that a 2-week application of Pb on *Oncorhynchus mykiss*, *Salmo turutta*, *Carassius aurratus*, and *Lepomis gibbosus*, with 10, 90, 470 and 90 μg/L concentrations, respectively, and obtained a decrease in the ALAD activity (Hodson et al. 1977). Similar results were also reported by Konuk et al. (2008), Korcan et al. (2007) and Ciğerci et al. (2008) from bacterial ALADs.

The IC50 value for the Pb inhibition of liver ALAD activity was found as 17.3 and 0.31-0.4 μM in *Ictalurus punctatus* and rats, respectively (Goering and Fowler 1987, Conner and Fowler 1994).

It was observed that 10, 25, 50 and 100 mg/kg Pb acetate applied to *Bufo arenarum* decreased the ALAD activity depending on the increase in the blood Pb concentration (Arrieta et al. 2000).

Leading to a 50%-inhibition in the enzyme activity, the Pb concentration values for *Biomphalaria glabrata* with and without pigment were found as 0.023 and 0.029 mg/L lead, respectively and for *Lumbricus variegatus* at 0.703 mg/L Pb concentration (Aisemberg et al. 2005).

The results obtained from the studies showed similarity with the ones showing the *in vivo* inhibition of ALAD enzyme with lead. Sassa (1982) reported that ALAD from rats showed maximum activity at 55°C. In Channel catfish the ALAD was found to be heat resistant and it reached the maximal activity at 65°C (Conner and Fowler 1994). In contrast, in *Gammarus pulex* the ALAD was reported to be heat sensitive and its maximum activity temperature was 37°C (Kutlu and Sumer

1998). In our study, the optimal temperature for *Palaemonetes turcorum* ALAD was observed at 55°C. As in rats and fish, the ALAD of *Palaemonetes turcorum* is resistant to temperature (Sassa 1982, Conner and Fowler 1994).

The optimal pH of the ALAD of *Palaemonetes turcorum* was found to be 7, which resembles the mammalian enzyme, for the optimal pH of the enzyme obtained from mammalian and birds is between 6.3-7.1. On the other hand, the enzymes obtained from yeast, *E. coli* and plants have a higher alkali optimal pH. Yeast, *E. coli* and plants have a pH of 9.8, 8.5, and 8, respectively (Senior et al. 1996).

When the Michaelis-Menten graph drawn, the sigmoidal curve was obtained also occurs in allosteric enzymes. This was reported previously by Conner and Fowler (1994).

According to the data obtained from purification studies, it was also observed that the molecular mass of ALAD was assumed to be c.a. 237.6 kDa. The molecular weight of ALADs ranges between 250-340 kDa in some organisms. The yeast and mammalian enzymes are 37 kDa and composed of 8 similar subunits. However, the plant and bacterial enzymes which are hexameric proteins are 50 and 40 kDa, respectively, and composed of 6 similar subunits (Boese et al. 1991).

In conclusion, if ALAD is used as a bioindicator in Pb exposure, the fundamental scientific data should be taken into consideration for the inhibition of enzyme by lead. ALAD's characteristics can vary from one species to another, and future experiments should be aimed to explain the similarities and differences of ALADs in various organisms. This study is the first where Crustacean samples were used and should be supported by other studies carried out on other organisms belonging to this group.

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