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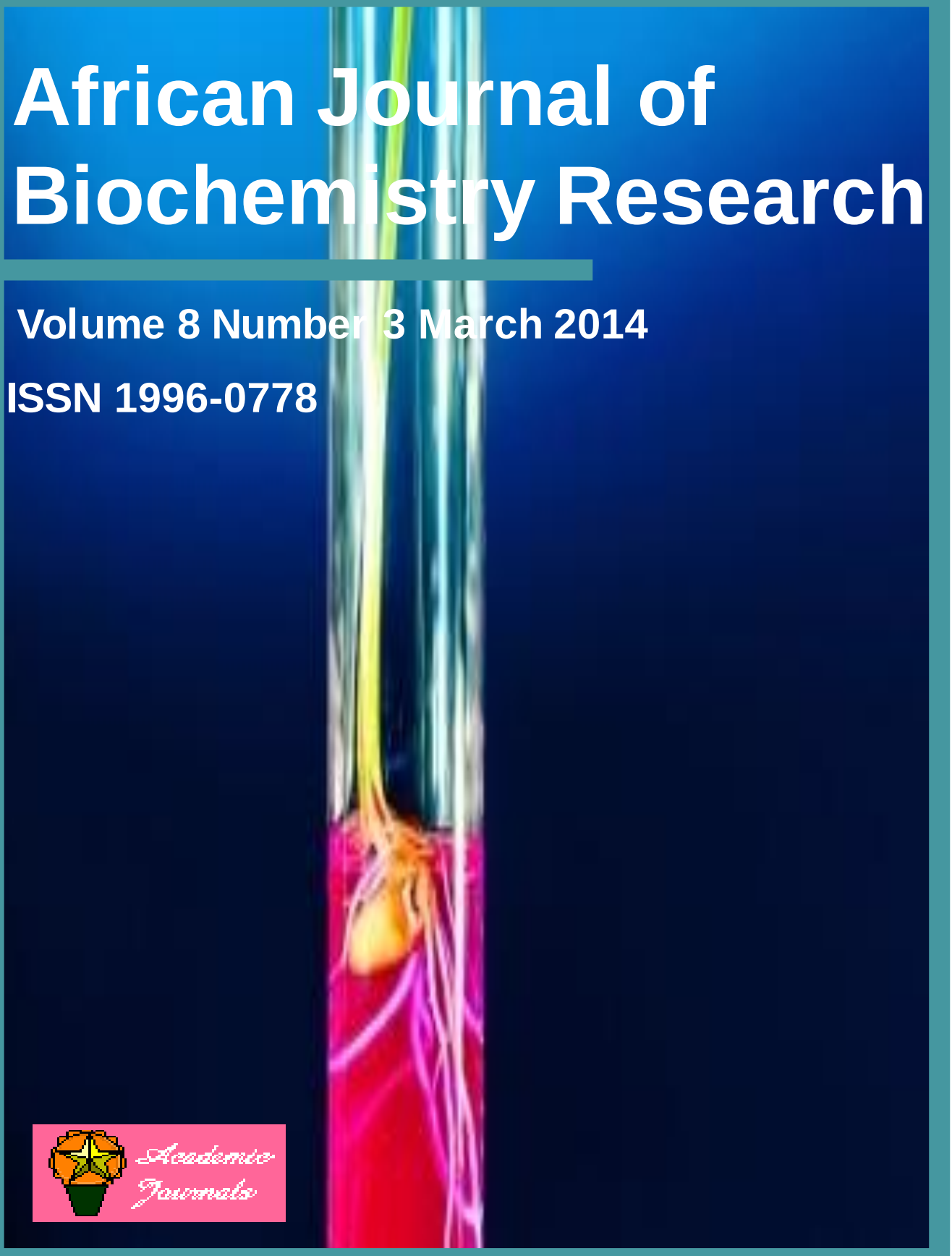

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African Journal of Biochemistry Research

*Full Length Research Paper*

# Usefulness of liver and kidney function parameters as biomarkers of 'heavy metals' exposure in a mammalian model *Mus musculus*

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**The toxicological evaluations of cadmium (Cd), iron (Fe), manganese (Mn), lead (Pb) and zinc (Zn) were carried out against Albino mice model, *Mus musculus.* On the basis of $LC_{50}$ value, Cd (0.47 mM) was found to be the most toxic followed by Zn (2.40 mM), Pb (2.42 mM), Fe (4.52 mM) and Mn (5.70 mM) as least toxic. The results of the sublethal concentration of the heavy metals ($1/10^{th}$ of 96 hr$LC_{50}$) on the liver function parameters and kidney markers showed that total glycerol (TG) levels increased significantly ($P < 0.05$) in Cd and Mn treated groups while Zn, Pb and Mn induced a significantly higher ($P < 0.05$) level of total protein. Mn and Fe induced significantly ($P < 0.05$) increase in the level of total bilirubin (T.BIL), urea and albumin (ALB) in exposed mice relative to the control group. The outcome of this study implied that sublethal responses of liver function parameters and kidney markers were found to have good potentials in serving as a combination of biomarkers for early detection and diagnosis of 'heavy metals' pollution in the mammalian model.**

**Key words:** Biomarkers, heavy metals, kidney markers, liver function parameters, *Mus musculus.*

## INTRODUCTION

Recently in Nigeria there was a report on lead (Pb) poisoning incident in Zamfara State (Dareta, Tunga Daji community) that claimed over 400 lives of children due to illegal mining of gold and exposure to contaminated soil and household dust in gold ore processing villages (MSF, 2012). This incidence has triggered a growing concern on the lethal effects of 'heavy metals' and also instigated greater research activities into biological effects of heavy metals pollutants in different parts of the world including Nigeria. Thus the need to acquire information on parameters such as kidney and liver function biomarkers that can be utilized in the early detection and diagnosis of heavy metals effects.

'Heavy metals' are toxic at relatively low concentrations and persist in the environment long after the source of emission has been removed. Thus could be classified as important sources of pollution (DeVagi and Arfiziah, 2009). 'Heavy metals' also bioaccumulate in one or several com-

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partments across food webs as shown by several scientific observations (Chukwu, 1991; Otitoloju and Don-Pedro, 2002; Otitoloju and Don-Pedro, 2004). The myriads of 'heavy metals' are grouped into essential and non essential. The essential elements play important roles as prosthetic groups in enzymes and key metabolic activities in living organisms, for example, iron (Fe), copper (Cu), manganese (Mn), cobalt (Co), vanadium (V), molybdenum (Mo) and zinc (Zn). The non-essential metals, such as arsenic (As), mercury (Hg), cadmium (Cd) and lead (Pb) are not needed in the physiological activities of living organisms hence they are usually toxic at relatively low concentrations (Falusi and Olanipekun, 2007; Raymond and Felix, 2011). The route of exposure of these 'heavy metals' to living forms are through air, water, soil, plants and food which can occur through dermal absorption (skin), inhalation (lungs) and ingestion (mouth). Presently, the amount of 'heavy metals' exposure is hundred times higher (Howard, 2002) than in the past thus living forms have become a “warehouse” of 'heavy metals'. Heavy metal bioaccumulation can be of public health significance especially, when it bioaccumulates in vital organs of man causing damages that can eventually lead to death. For example, calcium (Ca) when replaced by Pb in the bones can contribute to weakened bones and osteoporosis. Likewise Zn when displaced by Cd in the arteries cause inflammation and hardening of the arteries. Fe that replaces Zn and other minerals in the pancreas, adrenals and elsewhere can contribute to impaired blood sugar tolerance and diabetes. Cu that replacement by Zn in the brain is associated with migraine headaches, premenstrual syndrome, depression, anxiety, panic attacks and much more. Hg and Cu that replace selenium in various tissues impairs the conversion of thyroxine (T4) to triiodothyronine (T3), contributing to thyroid imbalances (Larissa et al., 2005). Exposure to ‘heavy metals’ result in acute and chronic toxicity. Therefore the toxic effects are multidirectional and manifested via changes in the physiological, chemical processes of the living things, impairment and dysfunction in the eliminative pathways (colon, liver, Kidney and skin). It has also been shown that the kidney and liver which are important organs of metabolism, detoxification and excretion of xenobiotics are especially vulnerable to damage when exposed to heavy metals (Alsaleh et al., 2006).

Blood enzymes are biomarkers of acute hepatic damage, thus their bioassay can serve as a diagnostic tool for assessing necrosis of the liver cells (Coppo et al., 2002). Many of these enzymes, such as a lactate dehydrogenase (LDH), aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) are released from the liver after its cellular damage and failure due to xenobiotics. The liver is the major site of intermediary metabolism and the synthesis of many important compounds, the site of conjugation and detoxification of potentially toxic substances and the site of storage of glycogen. Its role in detoxification of toxic substances makes it the organ for assessing the toxic effect of a particular substance. Literature abounds as regards the use of kidney damage and liver function parameters as indication of pathological effects of a sub-lethal concentration of heavy metals on animals such as on carp (Vinodhini and Narayanan, 2008), Wistar albino rats (Asagba and Eriyamremu, 2007; Suradkar et al., 2009; Samuel, 2010) and in humans (Orisakwe et al., 2007; Oyewole and Malomo, 2009).

The liver and kidney function markers tend to respond differently to different ‘heavy metals’ at different time therefore the response of a single or few biomarkers of the kidney and liver function cannot serve sufficiently as a tool for the diagnosis for accurate prediction of impairment in organism's health conditions. As a result the concurrent use of several biomarkers is important to minimize misinterpretation in cases of complex ‘heavy metals’ pollution situations.

In this study, the estimation of liver and kidney function parameters in *Mus musculus* was carried out to assess their importance as biological markers of environmental pollution related to heavy metals such as Cd, Fe, Mn, Pb and Zn.

## MATERIALS AND METHODS

### Test animals, acclimatization, selection of test animals and chemical

A total of seventy two male Albino mice, *Mus musculus* of similar sizes (19 - 24 g body weight and 10 - 15 weeks old) which served as test animals were purchased from the animal house in Nigerian Institute of Medical Research (NIMR) Yaba, Lagos, Nigeria. Mice were kept in ventilated plastic cages (length 20 cm, breath 12 cm by height 9 cm) with wood shavings under conventional conditions of natural light – dark cycle in farm house located in NIMR. The room temperature was at 30 ± 2°C and Relative Humidity - 70% ± 4%. The animals had free access to drinking fluid and a standard rodent laboratory chow (Coppens) purchased from Ladokun feeds Ltd, Ibadan. Acclimatization was for 14 days. ‘Heavy metals’ investigated in this work were obtained as metallic salts from Fisons Laboratory Reagents, Analar grades in Nigeria of the following types; zinc as $Zncl_2.3H_2O$, cadmium as $CdSO_4.8H_20$, lead as $PbSO_4$, manganese as $MnSO_4$ and ferric as $FeSO_4$. Active male mice of similar size and age (19 - 24 g body weight and 10 - 15 weeks old) were selected randomly into separate bioassay cages. This was because age and size were known to affect physiological responses of animals to chemicals.

### Ethics

The experiments carried out involved the utilization of whole and live mice. However, aiming for the protection and welfare of animals, studies were conducted in accordance with University of Lagos Ethics Committee guidelines for experiments with whole animals.

### Acute toxicity studies

Acute toxicity, preparation of test media for acute toxicity test, differential acute toxicity of heavy metals against mice and assessment of quantal response were determined as described by

Osuala et al. (2013).

**Sublethal studies (chronic toxicity)**

Mice were divided into six experimental groups (n=12 per group) for the sublethal experiment and a control group (n=3) was also included. They were treated to salts of the heavy metals [$Pb(NO_3)_2$, $FeSO_4$, $CdSO_4$, $ZnSO_4$ and $MnSO_4$] and control group was administered distilled water only. All animals were fed with their feed (Coppens).

**Sub-lethal concentrations**

In all the series of experiments, the mice were exposed to only sub-lethal concentrations (fraction of 96 h $LC_{50}$ values derived from experiments carried out in acute toxicity test) (Osuala et al., 2013) of selected metal salts as specified below. A total of the sub lethal values for selected metals were as follows:

Pb 1/10 of $LC_{50}$ = 0.24 mM, Fe 1/10 of $LC_{50}$ = 0.45 mM
Cd 1/10 of $LC_{50}$ = 0.05 mM, Zn 1/10 of $LC_{50}$ = 0.24 mM
Mn 1/10 of $LC_{50}$ = 0.57 mM

The metallic salts of the corresponding heavy metals of 1000 mg each of the salt was weighed out and diluted in 20 ml of water. Solution of 0.1 ml was administered through cannular method to the mice everyday for 28 days. The control mice were fed the same volume of distilled water. Prior to sacrificing, 3 mice from each group were separated and fasted for 24 h before dissection. This procedure was maintained every 7 days for analysis under investigation.

**Collection of blood samples**

At the end of the $7^{th}$, $14^{th}$, $2^{th}$ and $28^{th}$ day treatment periods, the animals were fasted for 24 h after the last administration for the various exposure days. The animals were anaesthetized using i.p. Ketamine (500 mg $kg^{-1}$ i.p). Blood samples were collected from the orbital sinus.

**Collection and preparation of plasma samples for kidney and liver parameters**

The blood of mice blood was collected into ethylenediaminetetra-acetic acid (EDTA) bottle immediately. Blood was then centrifuged at 4,000 g for 10 min using bench top centrifuge (MSE Minor, England) to remove red blood cells and recover plasma. Plasma samples were separated and were collected using dry Pasteur pipette, labeled and stored in the refrigerator at -20°C for analyses. All samples were analysed within 24 h of sample collection.

**Biochemical measurements**

Albumin was measured based on Colorimetric assay with endpoint method as described by Tietz et al. (1994). Total protein content was assayed by the Biuret method as described by Plummer, (1971). Alkaline Phosphatase (ALP) was measured as described by Wright et al. (1972).

Alanine aminotransferase (ALT) and aspartate transaminase (AST) were measured as described by Reitman and Frankel (1957). Creatinine (CREA**)** was determined by Jaffe spectrophotometric method asdescribed by Pratt (1996) and Aitken et al. (2003). Total bilirubin was measured based on Jendrassik and Grof new liquid method as described by Tietz et al., (1994). Urea concentration was determined according to the method of Veniamin and Vakirtzi-Lemonias (1970). Uric acid assayed by the method described by Fossatti et al. (1980). Total cholesterol (T.CHOL) and Triglyceride (TG) were determined by enzymatic colorimetric method of Trinder (1969). High density lipoprotein (HDL) and Low density lipoprotein (LDL) - cholesterol were assayed using Randox diagnostic kits determined following the principle described by Trinder (1969).

**Statistical analysis**

The biochemical data were analyzed with one way analysis of variance (ANOVA). Differences at $P < 0.05$ were considered significant. This was used to compare several treatment means in appropriately designed experiments. Further analysis was carried out only when there was a significant difference at the 5% ($P < 0.05$) level of significance (taken as minimum requirement) based on Duncan multiple range test at 0.05 levels of significance using SPSS 10.0 computer software package (SPSS Inc., Chicago, U.S.A).

## RESULTS AND DISCUSSION

The results showed that there were significant ($P < 0.05$) differences in the liver function parameters and kidney function markers induced by test 'heavy metals', Cd, Mn, Zn, Pb and Fe (Tables 1 to 5). Cd significantly ($P < 0.05$) decreased plasma AST, ALT, ALP, Uric acid, Urea, ALB, CREA, T.PRO, HDL, LDL, T.BIL and T.CHOL compared to control except for TG where there was significant ($P < 0.05$) increase on the $7^{th}$ day of exposure but decreased with subsequent exposure days from $14^{th}$ -$28^{th}$ (Table 1). The significant decrease in TG levels induced by Cd after the $7^{th}$ day exposure period is in agreement with Prasenjit et al. (2008) who observed alteration in TG due to Cd toxicity. It was suggested that the decrease might be related to its enhanced utilization in corticosteroidogenesis and/or a decreased *de novo* synthesis. It could also be as a result of diminished lipoprotein formation or the diminished release of complete lipoproteins, which can lead to pathogenesis of fatty liver. TG is a chain of high-energy fatty acids that provide much of the energy for body cells to function. High TG levels are strongly linked to metabolic disorders that increase the risk of heart disease. Thus the subsequent increase could probably indicate the commencement of injury induced by Cd. 'Heavy metals' toxicity has been shown to increase the risk of atherosclerosis, mainly because the toxicity increases triglyceride levels (Ballatori, 2000). The mean value of T.PRO that was significantly decreased is suggestive of the fact that Cd is known to bind to the sulfhydryl groups of enzymes containing cysteine and thus forms complexes with amino acids and protein. It is possible that Cd influenced the conversion of tissue protein into soluble fractions reaching the blood for utilization in cell repair.

Exposure to Mn showed significant ($P < 0.05$) decrease in plasma AST, Uric acid, CREA, ALT, ALP, Urea, HDL, LDL, TG and T.CHOL compared to control except for ALB and T.PRO where there was significant ($P < 0.05$)

**Table 1.** Liver function parameters and kidney markers induced by Cd.

| Parameter | Control | Days of exposure | | | |
|---|---|---|---|---|---|
| | | 7 | 14 | 21 | 28 |
| AST (µ/L) | 79.10 ± 0.20$^{d}$ | 49.92 ± 0.01$^{c}$ | 35.17 ± 0.01$^{b}$ | 29.00 ± 0.00$^{a}$ | 35.14 ± 0.33$^{b}$ |
| T.BIL (µmol/L) | 0.17 ± 0.00$^{a}$ | 0.19 ± 0.04$^{a}$ | 0.47 ± 0.01$^{c}$ | 0.46 ± 0.02$^{c}$ | 0.36 ± 0.01$^{b}$ |
| Uric acid (µmol/L) | 82.89 ± 0.06$^{d}$ | 62.18 ± 0.91$^{c}$ | 24.42 ± 0.52$^{a}$ | 23.91 ± 0.41$^{a}$ | 28.85 ± 0.90$^{b}$ |
| CREA (µmol/L) | 9.07 ± 0.00$^{b}$ | 7.88 ± 0.26$^{a}$ | 7.90 ± 0.01$^{a}$ | 8.43 ± 0.04$^{a}$ | 8.33 ± 0.23$^{a}$ |
| ALT (µ/L) | 20.30 ± 0.00$^{c}$ | 14.52 ± 1.33$^{b}$ | 9.58 ± 0.43$^{a}$ | 11.98 ± 1.47$^{ab}$ | 14.67 ± 0.90$^{b}$ |
| UREA (µmol/L) | 1.98 ± 0.00$^{d}$ | 0.88 ± 0.06$^{a}$ | 1.60 ± 0.03$^{c}$ | 1.24 ± 0.00$^{b}$ | 1.30 ± 0.00$^{b}$ |
| ALB (g/L) | 6.51 ± 0.00$^{d}$ | 5.02 ± 0.12$^{a}$ | 6.21 ± 0.02$^{cd}$ | 5.89 ± 0.34$^{bc}$ | 5.56 ± 0.00$^{b}$ |
| T.PRO (g/L) | 12.29 ± 0.00$^{d}$ | 9.49 ± 0.22$^{a}$ | 10.48 ± 0.11$^{bc}$ | 11.14 ± 0.37$^{c}$ | 10.44 ± 0.16$^{b}$ |
| HDL (mmol/L) | 0.66 ± 0.00$^{c}$ | 0.31 ± 0.05$^{a}$ | 0.38 ± 0.00$^{ab}$ | 0.44 ± 0.03$^{b}$ | 0.42 ± 0.00$^{b}$ |
| LDL (mmol/L) | 0.28 ± 0.00$^{d}$ | 0.11 ± 0.00$^{c}$ | 0.07 ± 0.00$^{a}$ | 0.07 ± 0.00$^{a}$ | 0.09 ± 0.00$^{b}$ |
| T.CHOL (µmol/L) | 0.78 ± 0.00$^{d}$ | 0.45 ± 0.02$^{bc}$ | 0.36 ± 0.00$^{a}$ | 0.44 ± 0.02$^{b}$ | 0.48 ± 0.02$^{c}$ |
| TG (µmol/L) | 0.34 ± 0.00$^{a}$ | 0.53 ± 0.10$^{b}$ | 0.33 ± 0.01$^{a}$ | 0.22 ± 0.01$^{a}$ | 0.23 ± 0.00$^{a}$ |
| ALP (µ/L) | 20.47 ± 0.01$^{c}$ | 23.45 ± 1.47$^{c}$ | 15.63 ± 1.84$^{ab}$ | 12.73 ± 0.31$^{a}$ | 16.83 ± 0.94$^{b}$ |

Values are Mean ± SD, n=3. Values with different alphabetical superscripts along a row are significantly different at $P < 0.05$.

**Table 2.** Liver function parameters and kidney markers induced by Mn.

| Parameter | Control | Days of exposure | | | |
|---|---|---|---|---|---|
| | | 7 | 14 | 21 | 28 |
| AST (µ/L) | 79.10 ± 0.20$^{c}$ | 39.71 ± 3.40$^{a}$ | 67.24±1.16$^{b}$ | 82.37±0.71$^{c}$ | 35.55±1.23$^{a}$ |
| T.BIL (µmol/L) | 0.17 ± 0.00$^{a}$ | 1.00 ± 0.05$^{c}$ | 0.95 ± 0.08$^{c}$ | 0.88 ± 0.055$^{c}$ | 0.52±0.02$^{b}$ |
| Uric Acid (µmol/L) | 82.89 ± 0.06$^{d}$ | 35.75 ± 0.42$^{b}$ | 43.65 ± 2.63$^{c}$ | 29.13 ± 1.72$^{a}$ | 26.03 ± 0.06$^{a}$ |
| CREA (µmol/L) | 9.07 ± 0.00$^{d}$ | 8.46 ± 0.03$^{bc}$ | 8.14 ± 0.30$^{b}$ | 8.80 ± 0.01$^{c}$ | 7.22 ± 0.03$^{a}$ |
| ALT(µ/L) | 20.30 ± 0.00$^{d}$ | 10.14 ± 1.58$^{a}$ | 15.30 ± 0.32$^{bc}$ | 17.47 ± 1.31$^{cd}$ | 12.83 ± 0.20$^{ab}$ |
| UREA(µmol/L) | 1.98 ± 0.00$^{c}$ | 1.61 ± 0.06$^{b}$ | 1.10 ± 0.00$^{a}$ | 1.57 ± 0.02$^{b}$ | 1.65 ± 0.02$^{b}$ |
| ALB (g/L) | 6.51 ± 0.01$^{b}$ | 6.00 ± 0.00$^{a}$ | 5.63 ± 0.17$^{a}$ | 7.15 ± 0.26$^{c}$ | 6.87 ± 0.05$^{bc}$ |
| T.PRO (g/L) | 12.29 ± 0.00$^{b}$ | 11.95 ± 0.25$^{b}$ | 10.42 ± 0.24$^{a}$ | 13.90 ± 0.02$^{c}$ | 23.57 ± 0.09$^{d}$ |
| HDL(mmol/L) | 0.66 ± 0.00$^{c}$ | 0.30 ± 0.02$^{a}$ | 0.31 ± 0.01$^{a}$ | 0.39 ± 0.02$^{b}$ | 0.34 ± 0.01$^{a}$ |
| LDL(mmol/L) | 0.28 ± 0.00$^{c}$ | 0.07± 0.00$^{a}$ | 0.08 ± 0.01$^{b}$ | 0.07 ± 0.00$^{ab}$ | 0.07 ± 0.00$^{ab}$ |
| T.CHOL (µmol/L) | 0.78 ± 0.00$^{c}$ | 0.40 ± 0.02$^{a}$ | 0.42 ± 0.01$^{a}$ | 0.50 ± 0.03$^{b}$ | 0.39 ± 0.00$^{a}$ |
| TG (µmol/L) | 0.34±0.00$^{e}$ | 0.26 ± 0.02$^{d}$ | 0.23 ± 0.01$^{c}$ | 0.16 ± 0.00$^{b}$ | 0.19 ± 0.01$^{d}$ |
| ALP(µ/L) | 20.47 ± 0.01$^{b}$ | 14.32 ± 0.11$^{a}$ | 14.95 ± 2.18$^{a}$ | 17.58 ± 2.58$^{ab}$ | 13.50 ± 0.11$^{a}$ |

Values are Mean ± SD, n=3. Values with different alphabetical superscripts along a row are significantly different at $P < 0.05$.

increase on the 21$^{st}$ day of exposure but decreased with subsequent exposure on the 28$^{th}$ day whereas T.BIL increased significantly ($P < 0.05$) from 7$^{th}$ - 28$^{th}$ day exposure (Table 2). The marked increase in plasma bilirubin could probably be as a result of fulminant hepatotoxicity, ultrastructural and functional changes in bile canaliculi leading to cholestasis and also perhaps via formation of manganese-bilirubin complexes. It is also suggestive that Mn probably induced haemolysis of the red blood cell. *In vitro* studies demonstrate that Mn ions can penetrate the erythrocyte membrane and in the presence of reduced gluthione, cause irreversible membrane damage and hence haemolysis. Therefore, elevated level of T.BIL is a probable indicator of Mn toxicity and a potential biomarker of exposure. The observed significant increase in ALB and T.PRO which was observed in this study but subsequently decreased towards the end of exposure period was probably indicative of oxidative stress induced by Mn. T.PRO level is a frequent parameter of metal poisoning in any living organism. ALB is the protein with the highest concentration in plasma. The present study showed that ALB and T.PRO were significantly decreesed but on the other hand increased the level of T.BIL in the plasma. These results may also be attributed to the

**Table 3.** Liver function parameters and kidney markers induced by Zn.

| Parameter | Control | Days of exposure | | | |
|---|---|---|---|---|---|
| | | 7 | 14 | 21 | 28 |
| AST (µ/L) | 79.10 ± 0.20$^{b}$ | 52.71 ± 0.96$^{a}$ | 50.06 ± 3.53$^{a}$ | 49.52 ± 0.48$^{a}$ | 54.38 ± 4.74$^{a}$ |
| T.BIL (µmol/L) | 0.17 ± 0.00$^{a}$ | 0.66 ± 0.01$^{c}$ | 0.92 ± 0.04$^{e}$ | 0.82 ± 0.01$^{d}$ | 0.54 ± 0.02$^{b}$ |
| Uric Acid (µmol/L) | 82.89 ± 0.06$^{c}$ | 31.33 ± 0.58$^{a}$ | 30.48 ± 0.19$^{a}$ | 38.56 ± 0.24$^{b}$ | 37.89 ± 0.40$^{b}$ |
| CREA (µmol/L) | 9.07 ± 0.00 | 7.77 ± 0.19 | 7.95 ± 0.46 | 8.51 ± 0.60 | 8.74 ± 0.48 |
| ALT (µ/L) | 20.30 ± 0.00$^{d}$ | 14.21 ± 0.51$^{ab}$ | 13.85 ± 1.65$^{a}$ | 19.00 ± 0.59$^{cd}$ | 17.00 ± 0.90$^{bc}$ |
| UREA (µmol/L) | 1.98 ± 0.00$^{b}$ | 1.18 ± 0.23$^{a}$ | 1.28 ± 0.08$^{a}$ | 1.17 ± 0.07$^{a}$ | 1.23 ± 0.01$^{a}$ |
| ALB (g/L) | 6.51 ± 0.00$^{c}$ | 3.98 ± 0.34$^{a}$ | 5.71 ± 0.06$^{b}$ | 6.71 ± 0.04$^{c}$ | 7.00 ± 0.20$^{c}$ |
| T.PRO (g/L) | 12.27 ± 0.00$^{bc}$ | 7.62 ± 1.01$^{a}$ | 10.83 ± 0.32$^{b}$ | 13.31 ± 0.26$^{cd}$ | 14.43 ± 0.30$^{d}$ |
| HDL (mmol/L) | 0.66 ± 0.00$^{b}$ | 0.33 ± 0.07$^{a}$ | 0.33 ± 0.00$^{a}$ | 0.34 ± 0.05$^{a}$ | 0.41 ± 0.02$^{a}$ |
| LDL (mmol/L) | 0.28 ± 0.00$^{c}$ | 0.20 ± 0.09$^{bc}$ | 0.07 ± 0.00$^{ab}$ | 0.06 ± 0.01$^{a}$ | 0.10 ± 0.00$^{ab}$ |
| TCHOL (µmol/L) | 0.78 ± 0.00$^{c}$ | 0.33 ± 0.05$^{a}$ | 0.46 ± 0.01$^{b}$ | 0.4900 ± 0.01$^{b}$ | 0.52 ± 0.010$^{b}$ |
| TG (µmol/L) | 0.34 ± 0.03$^{d}$ | 0.18 ± 0.01$^{a}$ | 0.23 ± 0.01$^{c}$ | 0.17 ± 0.00$^{a}$ | 0.21 ± 0.00$^{b}$ |
| ALP (µ/L) | 20.47 ± 0.01$^{a}$ | 19.67 ± 1.68$^{a}$ | 27.0233 ± 0.68$^{b}$ | 29.56 ± 0.12$^{b}$ | 27.03 ± 1.17$^{b}$ |

Values are Mean ± SD, n=3. Values with different alphabetical superscripts along a row are significantly different at $P < 0.05$.

great demands and cellular damage that occurred in the tissues of Mn - exposed mice and manganese toxicity may probably cause protein and albumin breakdown. Bilirubin formed in the reticulo-endothelial system is transported to the liver bound albumin. In the liver, bilirubin is conjugated to glucuronic acid to form direct bilirubin. Conjugated bilirubin is excreted via the biliary system into the intestine where it is metabolised. Biliary excretion is the major elimination pathway for Mn, accounting for greater than or equal 95% of Mn excretion; consequently any existing liver damage may delay or decrease its elimination and increase the relative amount of bilirubin in plasma (Ballatori, 2000). Total bilirubin is elevated in obstructive conditions of the bile duct, hepatitis, cirrhosis, in hemolytic disorders and several inherited enzyme deficiencies. This finding is consistent with that of Veena et al. (2012). A study conducted by Goering (2003) showed that Mn increases the activity of 3-hydroxy-3-methylglutaryl coenzyme A, the rate limiting enzyme for cholesterol biosynthesis and that bilirubin decreases cholesterol 7α hydroxylase, which is important in the conversion of cholesterol into bile acids (Akoume et al., 2003).

Zinc significantly ($P < 0.05$) decreased plasma AST, ALT, Uric acid, Urea, ALB, CREA, HDL, LDL, TG and T.CHOL compared to control except for ALP from 14$^{th}$ - 28$^{th}$ day of exposure, T.PRO (21$^{st}$ - 28$^{th}$ day of exposure), T.BIL (7$^{th}$ - 28$^{th}$ day of exposure) where there was significant ($P < 0.05$) increase when compared to the control (Table 3). The consistent increase in the T.BIL concentration all through the exposure period in mice exposed to Zn is a probable indication of an adverse effect of the Zn on the haemoglobin metabolism. The implication of such an increase in bilirubin may result in jaundice. The significantly increased level of ALP observed from the 14$^{th}$ - 28$^{th}$ day in this study, is a similar finding reported by Leena et al. (2011). ALP is a 'marker' enzyme of damage for the plasma membrane and endoplasmic reticulum (Shahjahan et al., 2004). It is frequently used to assess the integrity of the plasma membrane. The increase in ALP activity following Zn exposure suggested disruption of the ordered lipid - bilayer of the membrane structure of the plasma. Rich sources of ALP are the bile canaliculi of the liver, osteoblasts in the bone, proximal tubules in the kidney and mucosal cells of the small intestine. Damage to any of these organs would lead to elevated activity of its isoform of ALP in the plasma. It seemed, therefore, that Zn induced the biosynthesis of ALP in the kidney due to the disruption of cellular integrity. This could also be attributed to the irritation of all particular liver cells by zinc or due to increased loss of intra cellular enzyme by different structures via cell membrane which appear to act as a stimulus to the synthesis of more enzymes. The initial decreased level of total protein probably reflected the compensatory processes occurring in mice after exposure to Zn. This is consistent with the findings of Leena et al. (2011). Subsequent increase in the T.PRO level from the 21$^{st}$ - 28$^{th}$ day of exposure, emphasizing the fact that T.PRO is a function of the health status which is one of the factors affecting the state of health of the animals (Igwebuike et al., 2008). Total protein level is a rough measure of protein status but reflects major functional changes in the kidney and liver functions.

Cadmium, Mn and Zn were shown in this study to have caused a decrease in plasma T.PRO which is indicative of metal toxicity. The heavy metals altered the protein concentration thus impairing the metabolism of protein. There are reports on toxicants showing decrease in protein concentration in fish (Ram and Sathyanesan, 1987; Singh et al., 1993). This also implied that the exposure

**Table 4.** Liver function parameters and kidney markers induced by Pb.

| Parameter | Control | Days of exposure | | | |
|---|---|---|---|---|---|
| | | 7 | 14 | 21 | 28 |
| AST (µ/L) | 79.10 ± 0.20$^{b}$ | 24.33 ± 0.53$^{a}$ | 70.6 ± 2.52$^{b}$ | 76.93 ± 2.20$^{c}$ | 69.33 ± 2.67$^{b}$ |
| T.BIL (µmol/L) | 0.17 ± 0.00$^{a}$ | 0.17 ± 0.02$^{e}$ | 0.6000 ± 0.023$^{d}$ | 0.0567 ± 0.003$^{a}$ | 0.1167 ± 0.015$^{b}$ |
| Uric acid(µmol/L) | 82.89 ± 0.06$^{c}$ | 12.42 ± 0.12$^{a}$ | 19.1100 ± 0.779$^{a}$ | 42.3167 ± 4.893$^{b}$ | 42.1633 ± 4.400$^{b}$ |
| CREA (µmol/L) | 9.07 ± 0.00 | 7.24 ± 0.85$^{ab}$ | 6.6000 ± 0.237$^{a}$ | 8.1467 ± 0.209$^{bc}$ | 8.0100 ± 0.052$^{bc}$ |
| ALT(µ/L) | 20.30 ± 0.00$^{d}$ | 9.66 ± 0.20$^{a}$ | 20.06 ± 2.00$^{b}$ | 24.51 ± 1.46$^{b}$ | 20.02 ± 3.45$^{b}$ |
| UREA(µmol/L) | 1.98 ± 0.00$^{b}$ | 0.84 ± 0.15$^{a}$ | 1.10 ± 0.06$^{b}$ | 1.29 ± 0.01$^{bc}$ | 1.39 ± 0.02$^{c}$ |
| ALB (g/L) | 6.51 ± 0.00$^{c}$ | 3.6000 ± 0.615$^{a}$ | 4.6667 ± 0.220$^{b}$ | 6.7600 ± 0.012$^{c}$ | 6.4667 ± 0.081$^{c}$ |
| T.PRO (g/L) | 12.27 ± 0.00$^{bc}$ | 7.47 ± 0.75$^{a}$ | 9.68 ± 0.39$^{b}$ | 13.63 ± 0.16$^{d}$ | 13.46 ± 0.16$^{cd}$ |
| HDL(mmol/L) | 0.66 ± 0.00$^{b}$ | 0.18 ± 0.03$^{a}$ | 0.34 ± 0.02$^{b}$ | 0.42 ± 0.01$^{c}$ | 0.42 ± 0.01$^{c}$ |
| LDL(mmol/L) | 0.28 ± 0.00$^{c}$ | 0.05 ± 0.00$^{a}$ | 0.11 ± 0.01$^{b}$ | 0.2900 ± 0.01$^{d}$ | 0.22 ± 0.01$^{c}$ |
| TCHOL (µmol/L) | 0.78 ± 0.00$^{c}$ | 0.26 ± 0.04$^{a}$ | 0.46 ± 0.01$^{b}$ | 0.66 ± 0.01$^{c}$ | 0.66 ± 0.01$^{c}$ |
| TG (µmol/L) | 0.34 ± 0.03$^{d}$ | 0.08 ± 0.01$^{a}$ | 0.18 ± 0.01$^{b}$ | 0.30 ± 0.02$^{c}$ | 0.33 ± 0.01$^{c}$ |
| ALP(µ/L) | 20.47 ± 0.01$^{a}$ | 10.84 ± 0.23$^{a}$ | 14.69 ± 0.30$^{b}$ | 16.99 ± 0.81$^{c}$ | 14.35 ± 0.78$^{d}$ |

Values are Mean ± SD, n = 3. Values with different alphabetical superscripts along a row are significantly different at $P < 0.05$.

of heavy metals triggered the oxidative stress in the plasma by the generation of reactive oxygen species. The defensive surface proteins antagonise the toxic radicals resulting in elimination of protein from the liver cells. Additionally, the lowered level of T.PRO in plasma reflects the capacity of protein synthesis and denotes the osmolarity of the blood and liver impairments. Hence, would be a valuable indicator in the diagnosis of heavy metals toxicity in mammals. The observed decrease in total protein might also be due to several pathological processes induced by heavy metals including plasma dissolution, renal damage and protein elimination in the urine, a decrease in liver protein synthesis and alteration in hepatic blood flow and/or hemorrhage into the peritoneal cavity and intestine (Vinodhini and Narayanan, 2008). The present findings are in agreement with previous reports of decreased level of soluble protein, urea and creatinine reported by Al-Shinnawy (2009) and Ashok et al. (2011).

The results of Pb exposure to mice showed a signi-ficant ($P < 0.05$) decrease in AST, ALT, ALP, Uric acid, Urea, ALB, CREA, HDL, LDL, T.BIL, TG and T.CHOL compared with the control except for T.PRO where there was significant ($P < 0.05$) increase from on the 21$^{st}$ - 28$^{th}$ days of exposure (Table 4). The increase in T.PRO was also similarly observed in mice exposed to Mn which also could probably relate to the specific character of the heavy metals action. Lead may be rapidly absorbed and reach considerable amount in the blood (Haque et al., 2006). Han et al. (1996) and Moussa et al. (2001) both suggested that this element is strongly bound to macromolecules in the intracellular compartment because Pb binding protein have been observed to be deposited in kidney, liver, blood and brain. The significant decreased level of AST, ALT, ALP, uric acid, urea, ALB, CREA, HDL, LDL, T.BIL, T.PRO, TG and T.CHOL revealed in mice exposed to Pb with respect to control, are in agreement with some of the results obtained by Dhembare et al. (2011). The author reported that T.PRO and CREA contents declined in rats exposed to $KH_2PO_4$ but in disagreement with the findings of Veena et al. (2011), who observed that Pb caused a significant decrease in T.PRO and an increase in ALT, ALP, CREA and T.CHOL. The difference in their findings in relation to that of this study could be attributed to disparity in dosage. However, the result of this study also highlighted a significantly lowered ALB which is in agreement with the investigations of Ait et al. (2009). Albumin is the abundant protein in the human plasma representing 55 - 65% of T.PRO. It is synthesized in the liver at a rate that is dependent on protein intake subject to feed back regulation by plasma albumin level (Abd-El-Baset and Abd-El-reheem, 2009). Albumin is a useful indicator of the integrity of glomerular and other membranes. Its chief biological functions are to transport and store a wide variety of ligands to maintain the plasma oncotic pressure and to serve as a source of endogenous amino acids (Peter, 1975). Therefore from the point of view of this study, a decrease in ALB compared to the control was probably an indication of poor liver functions or impaired synthesis, either primary as in liver cells damage or secondary to diminished protein intake and reduce absorption of amino acids caused by malabsorption syndrome (Abd-El-reheem, 2008).

The results (Table 5) showed that Fe induced significant ($P < 0.05$) decrease in AST, ALP, Uric acid, CREA, HDL, LDL, TG and T. CHOL compared with the control except for T.BIL. Urea, ALB, T. PRO (7$^{th}$ - 28$^{th}$ day of exposure) and ALT increased significantly ($P < 0.05$) from 14$^{th}$ - 28$^{th}$ day of exposure where there were significant increases. The significantly increased urea level following exposure to Fe in mice is probably an indication of renal

**Table 5.** Liver function parameters and kidney markers induced by Fe.

| Parameter | Control | Days of exposure | | | |
|---|---|---|---|---|---|
| | | 7 | 14 | 21 | 28 |
| **Iron (Fe)** | | | | | |
| AST (µ/L) | 79.10 ± 0.20$^{b}$ | 54.47 ± 0.48$^{a}$ | 70.42 ± 0.06$^{b}$ | 73.15 ± 1.80$^{b}$ | 72.70 ± 1.08$^{b}$ |
| T. BIL (µmol/L) | 0.17 ± 0.00$^{a}$ | 0.87 ± 0.04$^{c}$ | 0.6333 ± 0.04$^{b}$ | 0.83 ± 0.06$^{c}$ | 0.61 ± 0.06$^{b}$ |
| Uric Acid (µmol/L) | 82.89 ± 0.06$^{c}$ | 28.27 ± 0.62$^{b}$ | 31.43 ± 1.66$^{c}$ | 20.18 ± 0.14$^{a}$ | 21.30 ± 0.59$^{a}$ |
| CREA (µmol/L) | 9.07 ± 0.00 | 8.46 ± 0.20$^{ab}$ | 7.70 ± 0.38$^{a}$ | 8.67 ± 0.27$^{b}$ | 9.75 ± 0.32$^{c}$ |
| ALT | 20.30 ± 0.00$^{d}$ | 17.58 ± 0.14$^{a}$ | 22.84 ± 0.93$^{c}$ | 31.75 ± 0.30$^{d}$ | 30.39 ± 0.09$^{d}$ |
| UREA | 1.98 ± 0.00$^{b}$ | 1.06 ± 0.00$^{a}$ | 0.9000 ± 0.07$^{a}$ | 3.30 ± 0.12$^{c}$ | 3.25 ±0.09$^{c}$ |
| ALB (g/L) | 6.51 ± 0.00$^{c}$ | 5.37 ± 0.04$^{a}$ | 6.09 ± 0.12$^{b}$ | 7.69 ± 0.29$^{c}$ | 7.83 ± 0.02$^{c}$ |
| T.PRO (mg) | 12.27 ± 0.00$^{bc}$ | 9.37 ± 0.42$^{a}$ | 9.8000 ± 0.43$^{a}$ | 13.63 ± 0.16$^{c}$ | 14.43 ± 0.13$^{c}$ |
| HDL | 0.66 ± 0.00$^{b}$ | 0.35 ± 0.01$^{a}$ | 0.36 ± 0.02$^{a}$ | 0.38 ± 0.00$^{ab}$ | 0.40 ± 0.00$^{b}$ |
| LDL | 0.28 ± 0.00$^{c}$ | 0.08 ± 0.01$^{b}$ | 0.05 ± 0.01$^{a}$ | 0.08 ± 0.01$^{b}$ | 0.09 ± 0.01$^{b}$ |
| TCHOL (µmol/L) | 0.78 ± 0.00$^{c}$ | 0.40 ± 0.05$^{a}$ | 0.44 ± 0.02$^{a}$ | 0.47 ± 0.01$^{a}$ | 0.48 ± 0.00$^{a}$ |
| TG (µmol/L) | 0.34 ± 0.03$^{d}$ | 0.21 ± 0.02$^{a}$ | 0.22 ± 0.01$^{b}$ | 0.19 ± 0.01$^{a}$ | 0.22 ± 0.01$^{b}$ |
| ALP | 20.47 ± 0.01$^{a}$ | 16.61 ± 1.18$^{a}$ | 19.61 ± 0.96$^{a}$ | 27.88 ± 1.26$^{c}$ | 25.72 ± 0.83$^{c}$ |

Values are Mean ± SD, n = 3. Values with different alphabetical superscripts along a row are significantly different at $P < 0.05$.

failure. The induced increase in the level of ALT following Fe exposure could also be indicative of an adaptive response to leakage into the blood stream due to the metal toxicity. ALT is a key metabolic liver enzyme (Adaramoye et al., 2008) released in damaged hepatocytes. Additionally, ALT has a part in transforming protein to glycogen, which is the major reserve fuel of the body during the stress-induced toxicity in the liver. The result indicated that under the influence of Fe inducing stress, the damage of tissues and organs may occur with concomitant elevation and liberation of ALT into the circulation. Fe is known to bind to the sulfydryl groups of enzymes which have the ability to alter the level of ALT activity in the tissues by disrupting their membrane. The 'heavy metals' enters the body by oral gavages and it is delivered to the liver through the portal blood circulation where the greatest part of it remains is stored. Only a small part of the Fe 'breaks the liver barrier' and consequently there will be a discharge of the cell content into the blood stream. The accumulated Fe in the liver can act by directly damaging the hepatocytes, primarily by destroying the permeability of the cell membrane which results in the increased release of cytosolic enzyme; ALT into the circulation. ALT is known to increase only in 'heavy metals' poisoning, toxic hepatosis and muscular dystrophy (Nduka, 1999). It has been reported that ALT is elevated significantly more than AST on heavy metals exposure, which indicates liver damage (Shalan et al., 2005). The increased liver and kidney markers such as ALT, Urea, ALB and T.PRO are suggestive of the fact that it is an indication of liver and kidney damage. This is in agreement with the findings of several researchers (Francielli et al., 2005; Asagba and Eriyamremu, 2007; Abou-Baker et al., 2011) and therefore potential tools as a biomarker of liver damage.

The observed changes in the liver and kidney parameters as early as the first week of exposure for each of the selected heavy metals is an indication that these parameters could serve as a good battery of biomarkers for early detection of pollution associated with heavy metals and their inclusion in monitoring programmes are thus recommended.

## Conflict of Interests

The author(s) have not declared any conflict of interests.

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*Full Length Research Paper*

# Properties of rhodanese from the liver of tilapia, *Oreochromis niloticus,* in Asejire Lake, Nigeria

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**The study investigates the purification and characterisation of rhodanese from the liver of the tilapia fish (*Oreochromis niloticus*) collected from Asejire Lake in Nigeria. This was with a view to understanding the biochemical basis of the survival of the fish in cyanide polluted water. Rhodanese was isolated and purified from liver tissue homogenate of tilapia using CM-Sephadex ion exchange chromatography and Sephadex G-75 gel filtration. The specific activity of the enzyme was 56.86 U/mg. The $K_m$ values for KCN and $Na_2S_2O_3$ as substrates were 0.1240 ± 0.0021 mM and 0.0516 ± 0.0097 mM, respectively. The apparent molecular weight was estimated by gel filtration on a Sephacyl S-400 column to be 35,460 Da. The optimal activity was found at pH 6.5 and the temperature optimum was 40°C. The rhodanese enzyme showed that the activity of the enzyme was not affected by $MgCl_2$, KCl, $NH_4Cl$, $MnCl_2$ and $CaCl_2$ while $AlCl_3$, inhibited the enzyme.**

**Key words:** Cyanide, detoxification, tilapia, liver, rhodanese.

## INTRODUCTION

Cyanide is a potent health hazard for human and ecosystem (Gupta et al., 2010). The toxic effect of cyanide (CN) is predominantly attributed to the inhibition of cytochrome c oxidase, a terminal oxidase of the mitochondrial respiratory pathway resulting in a condition of histotoxic anoxia.

Tolerance of organisms to cyanide contaminated environment and/or feed are due to a number of cyanide detoxification pathways, among the enzymatic pathways are 3-mercaptopyruvate sulphurtransferase (EC 2.8.1.2) and rhodanese (thiosulphate: cyanide sulphurtransferase, EC 2.8.1.1) which are widely distributed in living organisms. A major mechanism which removes $CN^-$ from the body by enzymatic conversion to the less toxic thiocyanate (SCN) in the presence of a sulfur donor is by rhodanese (Rh) (Way, 1983; Petrikovics et al., 2010).

Rhodaneses are highly conserved and widespread enzymes, currently regarded as one of the most effective mechanism evolved for cyanide detoxification. *In vitro*, rhodanese catalyzes the irreversible transfer of a sulphur atom from a suitable donor (i.e. thiosulphate) to cyanide, leading to formation of less toxic sulphite and thiocyanate (Gupta et al., 2010). The enzyme has been purified and characterised from a number of animal tissues (Lee et al., 1995; Agboola and Okonji, 2004; Akinsiku et al., 2010) some of which are actively exposed to cyanide contaminated environments or food. The specific activity of rhodanese in many animal tissues is present in Table 1

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**Table 1.** Specific activity of rhodanese (U/mg protein) in selected tissues of human and some domestic animals.

| Tissue | Specie | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Sheep[a] | Cattle[a] | Goat[b] | Camel[a] | Horse[a] | Pigc | Dog[a] | Chicken[d] | Human[e] |
| Liver | 5.21 | 4.95 | 2.93 | 1.7 | 1.7 | 0.56 | 0.18 | 0.31 | 0.15 |
| Rumen | | | | 0.28 | | | | | |
| Epithelial Layer | 16.3 | 10.3 | 3.31 | | | | | | |
| Muscular Layer | 0.09 | 0.05 | 0.01 | | | | | | |
| Kidney | | | | 0.08 | 1.4 | | 0.06 | 0.08 | 0.96 |
| Cortex | 1.72 | 0.82 | 0.94 | | | 0.4 | | | |
| Medullar | 0.24 | 0.05 | 0.25 | | | 0.1 | | | |
| Brain | 0.48 | 0.36 | 0.47 | 0.13 | 0.15 | ND | 0.32 | ND | 0.03 |
| Lung | 0.24 | 0.13 | 0.36 | 0.24 | 0.03 | 0.04 | 0.02 | 0.01 | 0.02 |
| Stomach | | | | | 0.1 | | 0.07 | | 0.01 |
| Abomasal Fundus | 0.09 | 0.04 | 0.35 | 0.07 | | 0.13 | | | |
| Abomassal Pylorus | 0.1 | 0.04 | 0.31 | 0.02 | | 0.006 | | | |
| Muscular layer of proventriculus | | | | | | | | 0.09 | |
| Epithelial layer of proventriculus | | | | | | | | 0.59 | |
| Muscle | 0.04 | 0.02 | 0.08 | 0.05 | 0.15 | 0.07 | 0.04 | ND | 0.01 |

*ND = Not determined; [a]Aminlari et al., 1989; [b]Nazifi, 2003; [c]Aminlari et al., 2002; [d]Aminlari et al., 1997; [e]Aminlari et al., 2007.

Asejire Lake (07º 21'N 04º 05'E), the source of the tilapia used in this study, is a manmade lake on River Osun, has an impounded area of 2342 ha and located about 30 km east of Ibadan, Southwest Nigeria. River Osun is one of the series of West African rivers which do not drain into Niger system but discharge into coastal lagoons and creeks bordering the Atlantic Ocean (Ayoade et al., 2006).

River Osun constantly received cyanide contaminants through agricultural and industrial activities (Olajire and Imeokparia, 2001) yet the river and lake support aquatic life (Ayoade et al., 2006; Adeyemo and Akomolafe, 2011). Tilapia *Oreochromis niloticus* is the most widely harvest freshwater fish in the Lake.

The survival nature of the fish is suspected to be due to the biotransformation of cyanide to thiocyanate by rhodanese. In early report from our laboratory we described the physicochemical and kinetic characterization of rhodanese from the liver of *Clarias gariepinus* Burchell in Asejire lake (Akinsiku et al., 2010), this paper describes the isolation and characterization of rhodanese from the cytosolic fraction of tilapia liver from the lake.

## MATERIALS AND METHODS

CM-Sephadex C-50, Sephadex G-75 and Sephacyl S-400 were obtained from Pharmacia Fine Chemical, Uppsala, Sweden. All other reagents were of analytical grade and were obtained from either Sigma or BDH. The tilapia fishes, *Oreochromis niloticus*, were obtained from Asejire Lake located in the outskirt of Ibadan, Oyo State, Nigeria.

### Enzyme and protein assays

The activity was expressed in rhodanese unit (RU). One rhodanese unit was taken as the amount of enzyme, which under the given condition produced an optical density reading of 1.08 at 460 nm per min (Sorbo, 1953). Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin (BSA) as the standard.

### Enzyme isolation and homogenisation

Scalpels were used to open up the abdomen. The liver was quickly removed and stored in the freezer until required. The frozen liver was thawed at room temperature and rinsed with cold saline (0.9% NaCl, pH 7.4) to remove blood and other impurities. Fifty grams (50 g) of the liver (obtained from 15 fishes) was minced with a pair of scissors into smaller pieces and homogenized in two volumes of 0.1 M acetate glycine buffer, pH 7.8 containing 1 mM ε-amino-n-caprioc acid, and 10 mM sodium thiosulphate for 5 min with a Warring Blender. The homogenate was centrifuged for 60 min at 20,000 rpm. The crude enzyme was assayed for protein and enzyme.

### C+M-Sephadex ion exchange chromatography

CM-Sephadex C-50 was pretreated according to Agboola and Okonji (2004)**.** The resin was packed into a 2.5 × 40 column and equilibrated with 50 mM citrate buffer, pH 5.0 containing 10 mM $Na_2S_2O_3$. The protein from the preceding step was layered on the column. The column was first washed with 200 ml of the equilibration buffer to wash the unbound protein, followed by elution with a 250 ml linear gradient of 0-0.5 M NaCl in same buffer. Fractions of 4 ml were collected at a rate of 24 ml/h. The protein profile was monitored at 280 nm. The fractions were also assayed for enzyme. The active fractions were pooled and dialysed against 50% glycerol.

### Gel filtration on sephadex G-75

Sephadex G-75 gel filtration resin was prepared by swelling 40 g in boiling distilled water for 3 h. The resin was then washed with several changes of 0.1 M phosphate buffer containing 10 mM

**Table 2.** Purification processes.

| Fraction | Total activity (mg) | Total protein (mg) | Specific activity (U/mg protein) | Percentage Yield | Purification fold |
|---|---|---|---|---|---|
| Crude extract | 3113.43 | 2700.00 | 1.15 | 100 | 1.00 |
| CM Sephadex ion exchange | 2087.60 | 123.16 | 16.95 | 67.00 | 14.74 |
| Sephadex-G75 Gel filtration | 457.01 | 8.02 | 56.98 | 14.68 | 49.55 |

Each purification procedure is as described in the materials and methods section. Protein concentration was determined using Bradford and activity was determined by the rate of formation of thiocyanate. 1U of activity is defined as the amount of enzyme, which under the given conditions, produced an absorbance reading of 1.08 at 460 nm per min (Sorbo, 1951).

sodium thiosulphate, pH 7.2 and then packed into 2.5 × 70 cm glass column and equilibrated with the same buffer.

Forty millilitres (40 ml) of the dialyzed protein from the ion-exchange step was layered on the column. Fractions (4 ml) were collected from the column at a rate of 15 ml per hour. Protein profile was monitored at 280 nm and assayed for rhodanese activity. The active fractions were pooled and dialysed against several changes of 50% glycerol for storage.

#### Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis and subunit molecular weight determination in the absence of sodium dodecyl sulphate (SDS) was carried out according to the procedure of Weber and Osborn (1975) on 7.5% acrylamide gel. Destaining was carried out with a solution containing 25 ml of methanol, 37.5 ml of glacial acetic acid made up to 500 ml with distilled water.

SDS-polyacrylamide gel electrophoresis in 10% acrylamide gel was used for the determination of the subunit molecular weight. Standard proteins that were used for the calibration of the gel were bovine serum albumin (66,000 Da), ovalbumin (45,000 Da), pepsin (34,700 Da), trypsinogen (24,000 Da) and β-lactoglobulin (20,100 Da). The gels were run at 8 mA per gel at room temperature and stopped after 5 h. The staining and destaining were performed as described earlier. $R_f$ values of the standards calculated were then plotted against the logarithms of their molecular weight. The molecular weight of the enzyme preparation was then intrapolated from the curve.

#### Determination of apparent molecular weight

The native molecular weight was determined on a Sephacyl S-400 column (1.5 × 100 cm) using standard proteins (BSA (67,000; 3 mg/ml), α-chymotrypsinogen (25,000; 3 mg/ml) and ovalbumin (45,000; 3 mg/ml)). 3 ml of each standard protein was layered on the column. The column was eluted with 10 mM phosphate buffer, pH 7.0 at a flow rate of 17 ml/h. The void volume ($V_o$) of the column was determined by the elution volume of Blue Dextran (2 mg/ml). 4 ml of the pure enzyme preparation was then passed through the same column and the elution volume ($V_e$) estimated as describe earlier. Partition coefficient ($K_{av}$ ) was calculated from $V_o$ and ($V_e$). A plot of logarithm of the molecular weight of the standard proteins against $K_{av}$ was made, the molecular weights of the enzyme preparation was then intrapolated from the curve.

#### Determination of kinetic parameters

The kinetic parameters ($K_m$ and $V_{max}$) of the enzyme were determined by varying concentrations of KCN between 12.5 and 50 mM at fixed concentration of 50 mM $Na_2S_2O_3$. Also, the concentration of $Na_2S_2O_3$ was varied between 12.5 and 50 mM at fixed concentration of 50 mM KCN. The kinetic parameters were estimated from the double reciprocal plots (Lineweaver and Burk, 1934).

#### Effect of pH on the enzyme activity

The enzyme was assayed using the buffers of different pH values: 50 mM citrate buffer (pH 4.0-6.5), 10 mM phosphate buffer (pH 7.0-8.5), and 50 mM borate buffer (pH 9.0-11). A reaction mixture of 1 ml contained 0.5 ml of the respective buffer, 0.2 ml of 0.25 mM KCN, 0.2 ml of 0.25 mM of $Na_2S_2O_3$ and 0.1 ml of enzyme solution.

#### Effect of temperature on the enzyme activity

The enzyme was assayed at temperatures between 0 and 70°C. The assay mixture was first incubated at the indicated temperature for 10 min before initiating reaction by the addition of an aliquot of the enzyme which had been equilibrated at the same temperature. The residual enzyme was then assayed.

#### Determination of heat stability

The heat stability of the enzyme was also determined by incubating 0.6 ml of the enzyme for 1 h at 30, 40, 50, 60 and 70°C, respectively. 0.1 ml was withdrawn at 10 min interval and assayed for residual activity. The activity at 30, 40, 50, 60 and 70°C was expressed as a percentage of activity of the enzyme incubated at 30°C which was the control.

#### Effect of chloride salts on the enzyme activity

The salts include $MnCl_2$, $NH_4Cl$, $CaCl_2$, $AlCl_3$ and $MgCl_2$ at the final concentrations of 0.5 mM and 1.0 mM in a typical enzyme assay reaction mixture.

## RESULTS

The summary of the result of the purification of rhodanese from the liver of tilapia is summarized in Table 2. The specific activity of the pure enzyme was 56.86 U/mg protein. The elution profile of the enzyme in ion-exchange chromatography on CM-Sephadex C-50 is shown in Figure 1. The peak was obtained in the breakthrough volume without the salt gradient elution. The elution profile on Sephadex G-75 is shown in Figure 2. Only one protein band was observed after gel electrophoresis of the purified rhodanese either in the presence or absence of sodium dodecyl suphate (figures not shown).

The calibration curve on Sephacyl S-400 for the determination of the native molecular weight is shown in Figure 3. The molecular weight of rhodanese from the liver of tilapia after the gel filtration was estimated to be

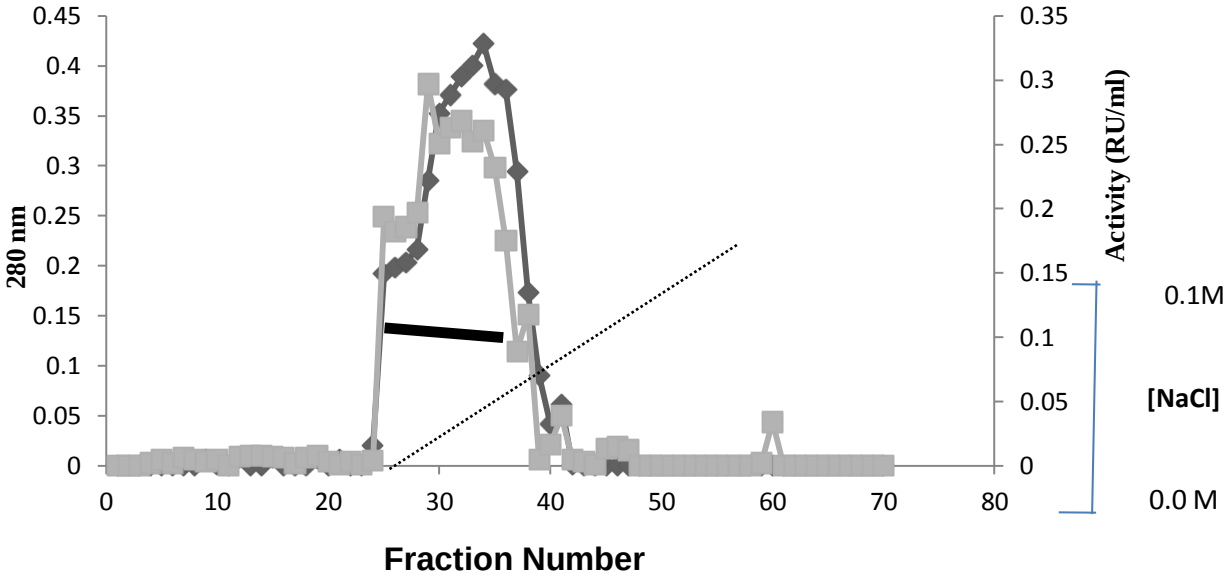


**Figure 1**. CM-Sephadex C-50 ion exchange chromatography elution profile of tilapia rhodanese. The column was equilibrated with 50 mM citrate buffer, pH 5.0 containing 10 mM $Na_2SO_3$. The dialysed protein was then layered on the column. The column was washed firstly with 200 ml buffer followed by elution with a 250 ml linear gradient of 0-0.5 mM NaCl in elution buffer. 4 ml fractions were collected at a rate of 24 ml/h. Protein profile was monitored using Bradford at 595 nm. ▬ Pooled Fraction; ····· Linear NaCl Gradient; Enzyme activity; ▢ $OD_{280}$

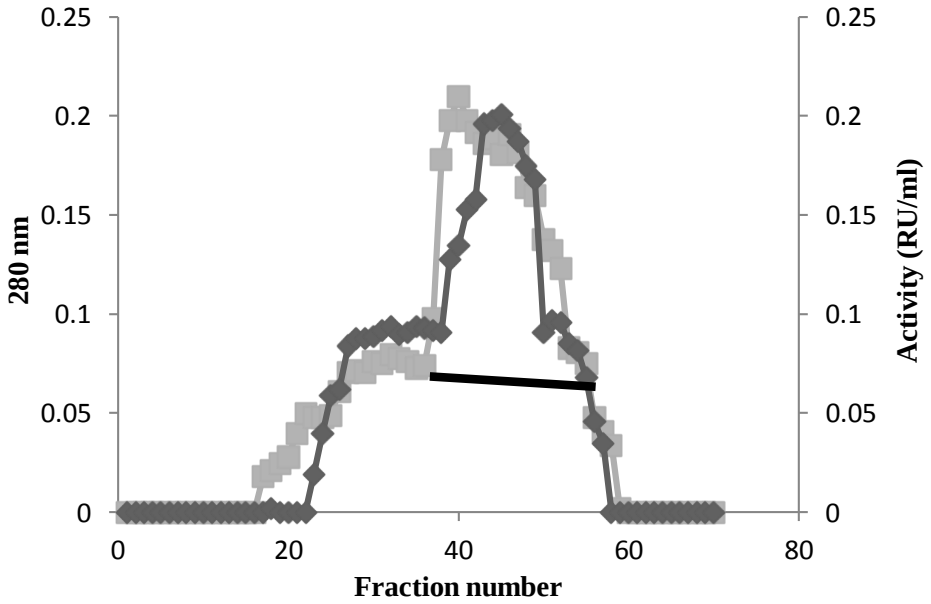


**Figure 2.** Sephadex G-75 Gel chromatography elution profile of Tilapia rhodanese. 40 ml of the dialysed protein from the ion exchange step was layered on a 1.5 × 40 cm column. 4 ml fractions were collected at a flow rate of 15 ml/h. Protein concentration was determined using Bradford at 595 nm. ▬, Pooled fraction; , Enzyme activity; ◆, $OD_{280}$

35,460 ± 450 daltons. The calibration curve obtained for polyacrylamide gel electrophoresis on 10% acrylamide gel electrophoresis is shown in Figure 4. The molecular weight was estimated to be 33,700 ± 714 daltons.

The double reciprocal plot of the change in KCN concentrations of fixed $Na_2S_2O_3$ is shown in Figure 5a, while that of varying concentrations of $Na_2S_2O_3$ at fixed KCN concentration is shown in Figure 5b. Table 3 shows the results of the kinetic parameters. The influences of pH on the rate of enzyme activity are shown in Figure 6. There were steady increases in enzyme activities until pH 6.5.

The effect of temperature on the activity of rhodanese

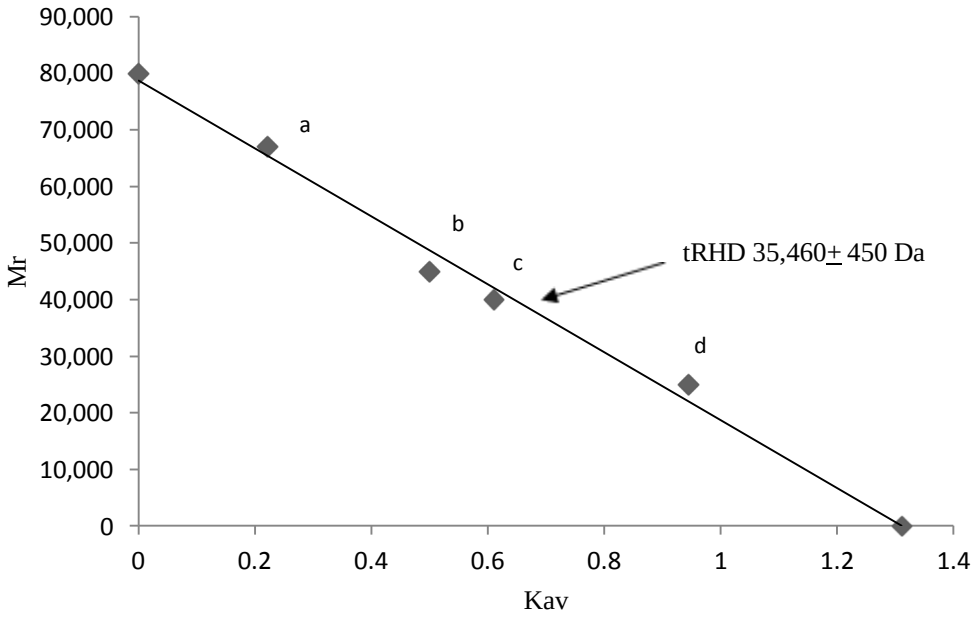


**Figure 3.** Calibration curve for molecular weight determination by gel filtration on sephacyl S-400. 3 ml of standard proteins was applied to the column (1.5 × 100 cm) and eluted with 10 mM phosphate buffer, pH 7.0 at a flow rate of 17 ml/h. Blue dextran (2 mg/ml) elution was used to determine the void volume. The standard proteins are (**a**) bovine serum albumin (66 kDa), (**b**) ovine albumin (45 kDa), (**c**) peroxidase (40 kDa), (**d**) chymotrypsinogen (25 kDa) and (**e**) β-lactoglobulin (20.1 kDa). The arrow indicates the position of the rhodanese enzyme (tRHD).

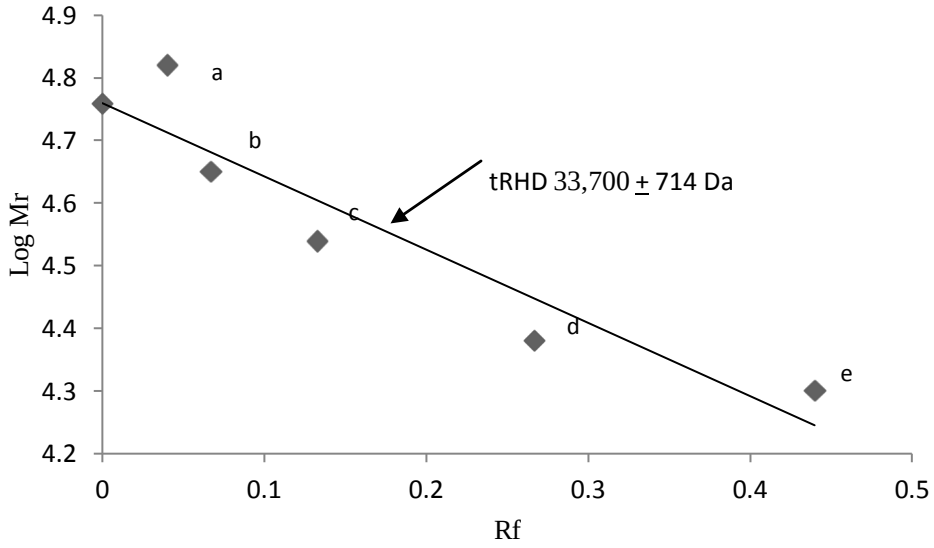


**Figure 4.** Calibration Curve for sub unit molecular weight determination on SDS-PAGE. The subunit molecular weight was determined by interpolation. The standard proteins are (**a**) bovine serum albumin (66 kDa), (**b**) ovine albumin (45 kDa), (**c**) peroxidase (40 kDa), (**d**) chymotrypsinogen (25 kDa) and (**e**) β-lactoglobulin (20.1 kDa). The arrow indicates the position of the rhodanese enzyme (tRHD).

from the tilapia liver is shown in Figure 7. Figure 8 shows the heat stability of the enzyme from the tilapia liver. The result of the effect of various chloride salts on the activity of catfish liver rhodanese (Table 4) showed that $MgCl_2$, KCl, $NH_4Cl$, $MnCl_2$ and $CaCl_2$ had little or no effect on the activity of the enzyme. Both concentrations of $AlCl_3$ have

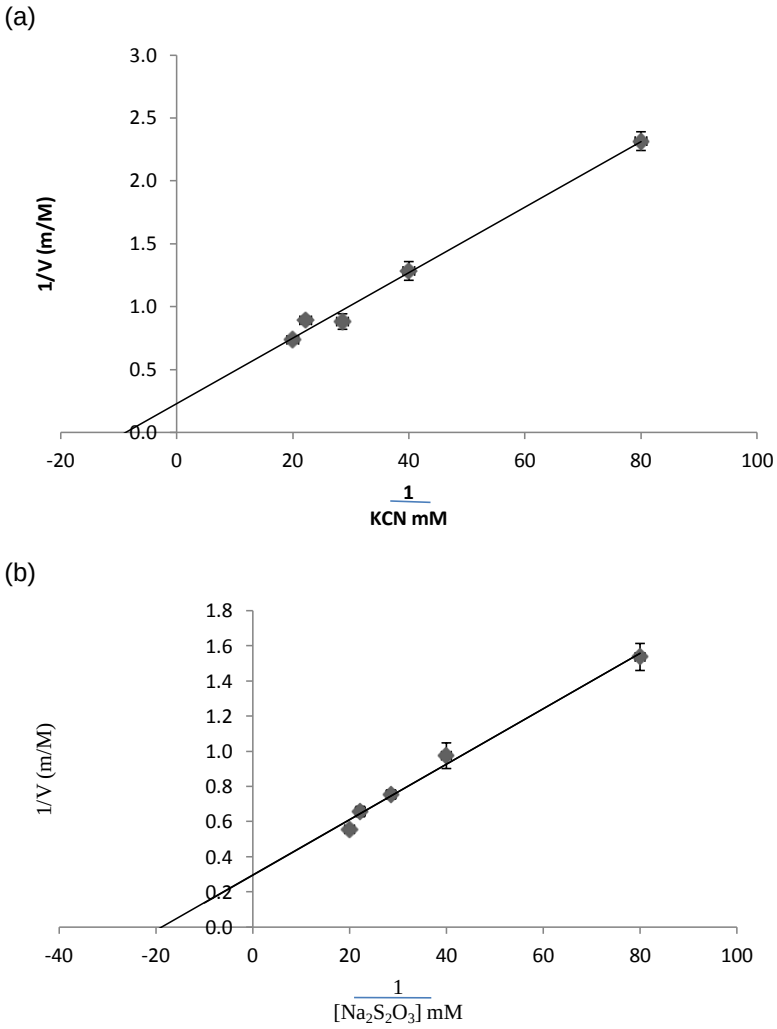


**Figure 5.** Lineweaver-Burk plot for the determination of $K_m$ and $V_{max.}$ **(a)** The concentration of $Na_2S_2O_3$ was varied between 12.5 mM and 50 mM at fixed concentration of 50 mM KCN while the concentration of KCN was determined at fixed concentrations of 50 mM $Na_2S_2O_3$. **(b)**. The reaction mixture contained 25 mM borate buffer pH 9 and 0.1 ml of enzyme in a total reaction volume of 3.0 ml at room temperature.

**Table 3.** Kinetics Parameters of KCN and $Na_2SO_4$ as Substrates of Rhodanese from Tilapia Fish liver.

| Parameter | KCN | $Na_2S_2O_3$ |
|---|---|---|
| $K_m$ (mM) | 0.1240 ± 0.0201 | 0.0516 ± 0.0097 |
| $V_{max}$ (RU/ml) | 4.395 ± 0.3800 | 3.4208 ± 0.3690 |

The above kinetic parameters $K_m$ and $V_{max}$ were determined from the double reciprocal plots and were a mean of triplicate determinations

the inhibition effect on the enzyme.

## DISCUSSION

Tilapia (*Oreochromis niloticus*) is more tolerant than most commonly freshwater fish to high salinity, high water temperature, low dissolved oxygen, and high ammonia concentrations (Popma and Masser, 1999). Their habitants such as lakes, streams, rivers are constantly subject to contaminations from various sources such as effluents from manufacturing companies (Bruton, 1979). Cyanides are present in many industrial wastewaters,

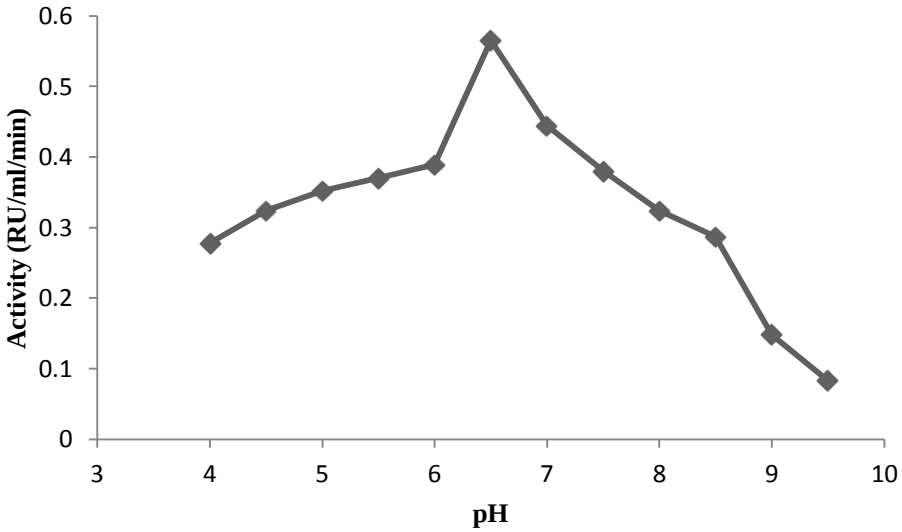


**Figure 6.** Effect of pH on the tilapia liver rhodanese. The assay mixture contained the appropriate buffers: 50 mM citrate buffer (pH 4.0-6.5), 10 mM phosphate buffer (pH 7.0-8.5), 0.2 ml of 0.25 KCN, 0.2 ml of 0.25 mm of $Na_2S_2O_3$ and 0.1 ml of enzyme inn final reaction volume of 3.0 ml.

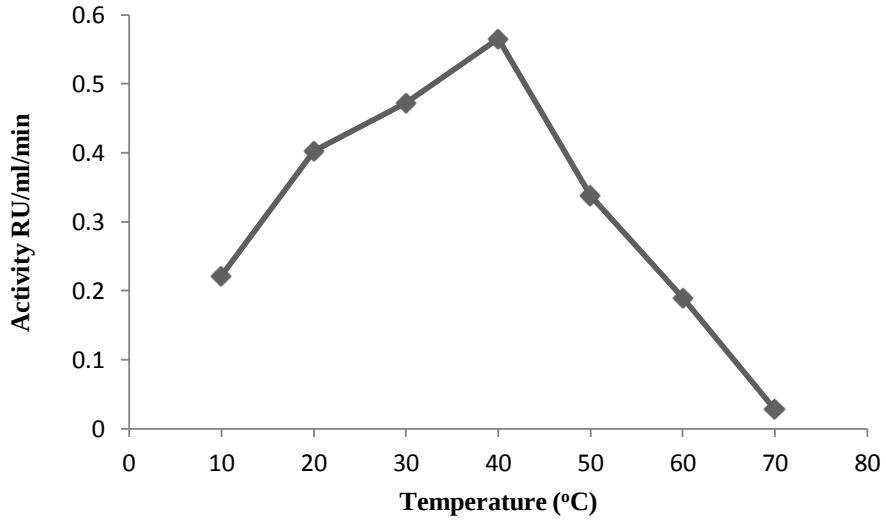


**Figure 7.** Activity-temperature profile showing optimum temperature of rhodanese. The assay mixture was first incubated at the incubated temperature for 10 min before initiating the reaction by the addition of an aliquot of the enzyme that had been previously equilibrated at same temperature.

especially effluents from iron and steel processing plants, petroleum refineries, and metal-plating plants, and constitute a hazard to aquatic ecosystems in certain waste-receiving waters (Eisler and Wiemeyer, 2004).

Study on feeding behaviour and nutrition requirements of *O. niloticus* by Popma and Masser (1999) shown that Tilapia ingest a wide variety of natural food organisms, including plankton, some aquatic macrophytes, planktonic and benthic aquatic invertebrates, larval fish, detritus, insects, even small fish fry and decomposing organic matter. With heavy supplemental feeding, natural food organisms typically account for 30 to 50% of tilapia growth. Most of these food items contain cyanogenic glycosides which upon hydrolysis release cyanide or hydrocyanic acid (HCN).

The Asejire Lake, the source of tilapia for this study also received cyanide from cassava processing activities around the lake. Rhodanese and 3-mercaptopyruvate sulphur transferase represent the chief enzymes of cyanide detoxification (Nagahara et al., 1999; Aminlari et

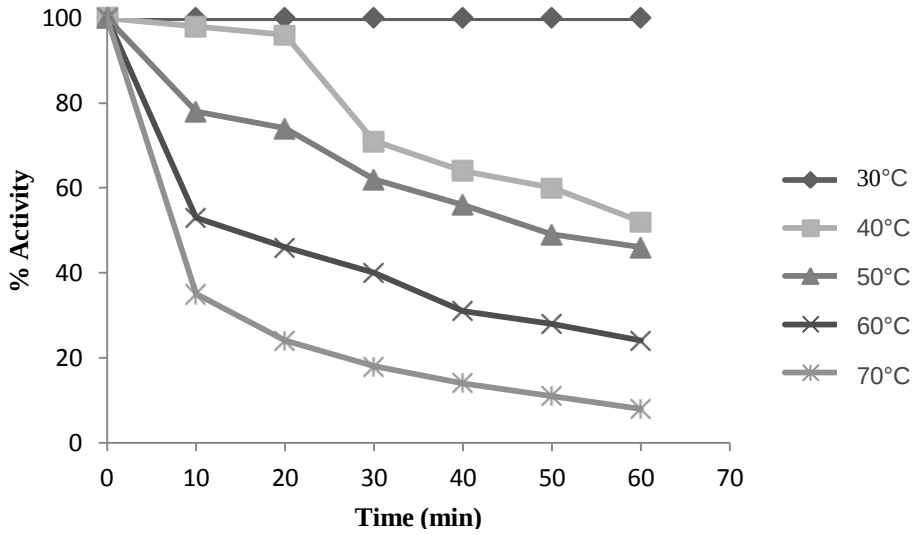


**Figure 8.** Effect of temperature on heat stability of tilapia rhodanese. 0.6 ml of enzyme was incubated at temperature ranges indicated above and aliquots of 0.1 ml was withdrawn at respective temperatures and assay was carried out for residual enzyme activity in 50 mM borate buffer. Percent residual activity is compared to the activity of the native enzyme at 30°C.

**Table 4.** Effect of chloride salts on tilapia liver rhodanese.

| Parameter | Percentage enzyme residual activity | |
|---|---|---|
| Salt | 0.5 mM | 1.0 mM |
| None | 100 | 100 |
| $MgCl_2$ | 92 ± 6.14 | 90 ± 3.25 |
| KCl | 91 ± 2.07 | 89 ± 3.32 |
| $NH_4Cl$ | 84 ± 1.25 | 79 ± 2.20 |
| $MnCl_2$ | 83 ± 2.01 | 86 ± 3.25 |
| $CaCl_2$ | 74 ±2.98 | 77 ± 2.82 |
| $AlCl_3$ | 67 ± 5.45 | 67 ± 0.19 |

Enzyme assay were carried out as described in methods with each assay mixture containing the salts at final concentration of 0.5 and 1.0 mM. The values are a mean of at least triplicate determinations

al., 2002).

The purification procedures involved ion-exchange chromatography on CM-Sephadex C-50 and gel filtration on Sephadex G-75. Our initial attempts to use ammonium suphate fractionation resulted into a great lost of enzyme activities hence we omitted the purification process.

The specific activity of rhodanese from the liver of tilapia was found to be 56.98 RU/mg of protein. Sorbo (1953) and Himwich and Saunders (1948) obtained 256 RU/mg and 131 RU/mg, respectively for bovine liver rhodanese. Lee et al. (1995) obtained 1,070 RU/mg of protein for purified mouse liver rhodanese. Agboola and Okonji (2004) obtained 136.6 RU/mg protein for fruit bat liver rhodanese, Akinsiku et al. (2010) obtained 73 and 72 RU/mg for cRHD I and cRHD II, respectively for catfish liver rhodanese. Tayefi-Nasrabadi and Rahmani (2012) obtained 0.206 RU/mg protein for rhodanese from liver Rainbow Trout (*Oncorhynchus mykiss*).

The apparent relative molecular weight of 35,460 Da compares very well with that reported for the enzyme from other sources. It is in line with the reports that rhodanese has molecular weight value approximately between 33000 and 37000 daltons (Nagahara et al., 1999). The subunit molecular weight was 33,700±714 daltons. This result suggests that the enzyme is a monomeric protein. While Jarabak and Westley (1974) and Lee et al. (1995) showed that purified mouse and human liver rhodaneses are monomeric proteins, other workers have shown that they are dimers of two identical subunits of molecular weights 18,000-19,000 daltons (Blumenthal and Heinrikson, 1971).

The apparent $K_m$ values, KCN and $Na_2S_2O_3$, were 0.1240 and 0.0516 mM, respectively. These values are lower than those of rainbow trout (KCN = 36.81mM; $Na_2SO_4$ = 19.84 mM) (Tayefi-Nasrabadi and Rahmani, 2012), african catfish liver (KCN = 25.40 mM; $Na_2SO_4$ = 18.60 mM) (Akinsiku et al., 2010), fruit bat liver (KCN = 13.36 mM; $Na_2SO_4$ = 19.15 mM) (Agboola and Okonji, 2004), mouse liver (KCN = 12.50 mM; $Na_2SO_4$ = 8.30 mM) (Lee et al., 1995), bovine liver (KCN = 19.0 mM; $Na_2SO_4$ = 6.7 mM) (Sorbo, 1953), and human liver (KCN = 9.50 mM; $Na_2SO_4$ = 4.50 mM) (Jarabak and Westley, 1974), indicating that the affinity of tilapia enzyme for these substrates is more than that of the other enzymes and that it would catalyze the detoxification reaction with high efficiency. As reported by Agboola and Okonji (2004) less effective enzymatic system may be due to a

lower exposure to cyanide and high affinity in some mammalian animals is due to continue exposure through their diet. This high affinity of tilapia rhodanese is supported by feeding and nutrition requirements of the fish that include wide range of natural foods which are cyanogenic (Popma and Masser, 1999). Madalla (2008) reported that (*Oreochromis niloticus* L) survived on diet which containing 40% and 10% of hydrogen cyanide from cassava leaf meal and cassava root meal respectively. It should be noted that cyanide detoxification is a secondary benefit of rhodanese, which has a number of major physiological roles, including the supply of sulphide for the formation of iron-sulphur centres for the electron transport chain (Cerletti, 1986), participation in energy metabolism (Ogata et al., 1989), and function as a thioredoxin oxidase (Nandi et al., 2000).

An optimum temperature of 40°C was obtained for tilapia liver rhodanese. This result is in good agreement with the results reported for rhodaneses from different sources. Sorbo (1953) reported an optimum temperature of 50°C for bovine liver rhodanese. Ezzi et al. (2003) obtained a wide temperature optimum of 35-55°C for rhodanese enzyme in all different *Trichoderma* strains. Agboola and Okonji (2004) reported 35°C for the rhodanese in the cytosolic fraction of fruit bat liver. Also, Akinsiku et al. (2010) reported 40°C for the rhodanese in the liver of catfish (*C. gariepinus*) from Asejire Lake, same source of tilapia for this study. Tayefi-Nasrabadi and Rahmani (2012) reported 25°C for rhodanese from the liver of rainbow trout. Akinsiku et al. (2010) posited that with the level of pollution in this water, there will be various metabolic activities going on in it which likely results in the release of heat and the ambient temperature of 40°C which might have conditioned the enzyme to function at higher temperature. The heat stability experiment showed that the enzyme was stable up to 60°C for about 30 min which means that the enzyme is thermostable.

An optimum pH of 6.5 was obtained for rhodanese from the liver of the tilapia from Asejire Lake. Same pH value was obtained by Akinsiku et al. (2010) for the rhodanese of catfish from the same lake. They posited that the value is as a result of the acidity of the water. Pollutants in water include wide spectrum of chemicals and pathogens which sometimes alters the acidity, conductivity and temperature of the water. The water sample collected from Asejire Lake showed a pH of 5.7, 6.3 and 6.5 at different points of the Lake (Lameed and Obadara, 2006).

Studies on effect of chloride salts on the enzyme showed that the activity of the enzyme was not affected by $MgCl_2$, KCl, $NH_4Cl$, $MnCl_2$ and $CaCl_2$ while $AlCl_3$ inhibited the enzyme greatly. Metal ions showing inhibitions are those that have strong affinity for cysteinyl and histidyl side chains of proteins (Ulmer and Vallee, 1972). The properties of rhodanese from the liver of tilapia are similar to those from other animal sources and might properly function in the same manner.

## Conflict of interests

The author(s) have not declared any conflict of interests.

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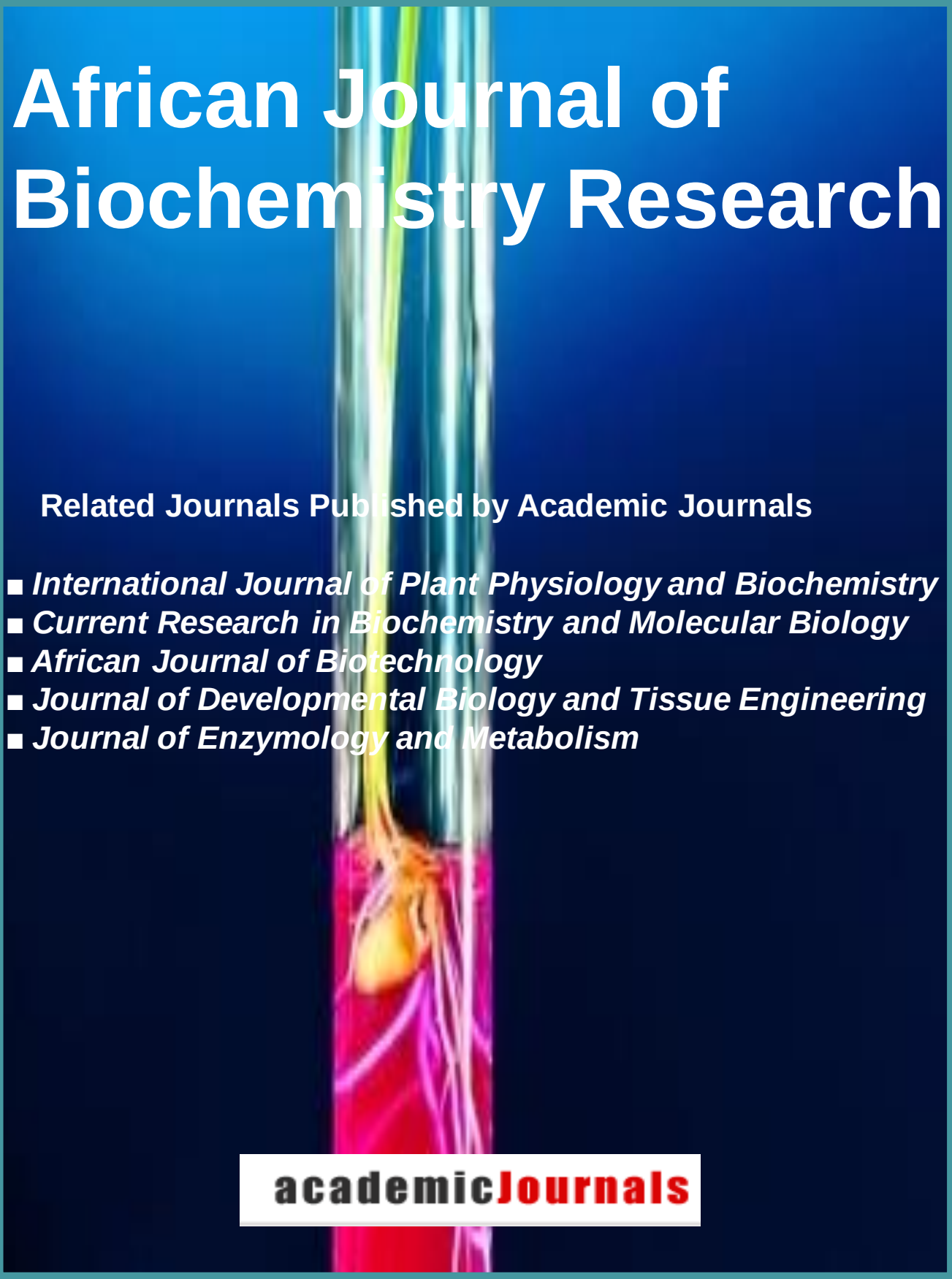

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