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Catalyse Optimal Analytical Condition Properties in Sub-Cellular Tissues of Freshwater Bivalve, *Lamellidens marginalis*

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Abstract
The Optimal test conditions for catalyze activity were determined from the cytosols of the foot, gills and digestive gland of freshwater mussel. Catalase properties were investigated in *L. marginalis* due to a lack of quantitative data on optimal analytical conditions of catalyze in subcellular fractions of mollusc tissues. The foot, gill, and digestive gland cytosols of mussels have an optimal temperature 45 °C and pH of 7.5 for catalyse activity. The digestive gland had the highest activation energy (R^2=0.3371,53.76 KJ/mol), followed by the gill (R^2=0.3889,55.56 KJ/mol) and foot (R^2=0.4162,56.58 KJ/mol). Some substrates other than H_2O_2 can inhibit catalyse in the present study. Based on the apparent Km values, in *L. marginalis* the cytosol of the digestive gland was observed at 7.39 mM H_2O_2, gills at 8.91 mM H_2O_2 and foot at 15.11 mM H_2O_2.

Keywords
Arrhenius plot, Catalyse, Energy activation, Energy inhibition, Lineweaver-Burk plot, *L. marginalis*

INTRODUCTION

Oxygen toxicity, formerly referred to as the toxic effect of oxygen under high pressure (hyperbaric oxygen), is now predominantly focused on exposure to oxy-radicals [1] generated in our daily lives. Oxygen is inherently dangerous, although the oxygen paradox is that higher eukaryotic organisms cannot exist without it [2].Biological sources of oxyradicals include radiolysis and photosensitization of pigments, chemical redox reactions, transition metals, oxido-reducing enzymes, subcellular organelles, stimulated phagocytes, and redox cycles of xenobiotics (foreign compounds) [3].The toxic effects of these oxyradicals include lipid peroxidation, nucleic acid damage and enzyme inactivation [4]. The extent to which oxyradical generation causes biological damage depends on the effectiveness of antioxidant defenses [5,6]. The self-cleansing of these oxyradicals is carried out by an antioxidant enzyme, a catalyse, which is mainly involved in the removal of H_2O_2 ($2H_2O_2 \rightarrow 2H_2O + O_2$). Many of the enzymes are inducible, exist in multiple forms, and have complementary subcellular localization [7].Catalase was mostly, if not entirely, peroxisomal, its activities being expressed mainly in the cytosol of mussels.Catalase properties were more consistent with a catalyse than a catalyse peroxidase.Catalase peroxidase is pH and temperature dependent and more sensitive to H_2O_2 [8, 9, 10]. The nature of the antioxidant defenses may be different for euryoxic and stenoxic organisms [11], and their subcellular localizations will be important factors in the consequences of pro- and antioxidant events occurring in a cell [12,13] all tissues of the mussel, but are present in the highest activities in the digestive gland [14], which is also the main site of uptake of foreign substances and oxyradical-producing biotransformation enzymes [15].

Catalase has been used in aquatic organisms as a biomarker to monitor pollution and assess oxyradical damage [16,17].Kinetic parameters of catalyse enzyme from gills, digestive gland and mantle of *Mytilus galloprovincialis* were determined [18]; digestive gland of *Mytilus edulis* [10]; *Elliptio complanata* [19]; *Ruditapes decussatus* [20]; Brown mussel *Perna perna* [21].Meager information is available concerning catalyse activity in crustacean species [14, 22].

Catalase catalyzes the decomposition of hydrogen peroxide to produce molecular oxygen and water [23].However, CAT exhibits low affinity for the substrate;therefore the effective decomposition of H_2O_2 occurs only at its high concentration [24].CAT is a sentive SOS biomarker and is considered one of the earliest responses to pollution exposure [25].CAT induction has been described by many authors on various species of mollusc [26, 27].Due to the importance of catalyse as an environmental biomarker, we performed the present study to determine the optimal analysis conditions for the investigation of catalyse in the subcellular fractions of freshwater mussel *Lamellidens marginalis*.

MATERIALS AND METHODS

1. Animal Collection

Freshwater mussels, *Lamellidens marginalis* (6.0 - 6.5 cm long, 2.0-2.5 g) were collected from the Cauvery River (Tiruchirappalli, India) and transported to the laboratory. The mussels were acclimated to laboratory conditions for five days before being sacrificed for the catalyse enzyme test. The mussels were starved for a day before killing to create an empty gut.

2. Preparation of Cytosolic Fraction

The cytosolic fraction was prepared from fresh digestive gland using the procedure [28]. All preparation procedures were carried out at 4^{o}C. The tissue was homogenized using a glass homogenizer in homogenizing buffer: 0.25 M sucrose containing 20 mM Tris-HCl, pH 7.6, 0.15 M KCl, and 1mM DDT. The homogenate was centrifuged at 500x g for 1 h and the pellet discarded. The supernatant was centrifuged at 12,000x g for 45 min and the resulting pellet resuspended in homogenizing buffer containing 1mM EDTA and 8.0 mM $CaCl_2$, and recentrifuged at 20, 000x g for 45 min. After the pellet was removed from the tube, the supernatant was used as cytosolic fraction.

3. Enzyme Assay

Catalase activity was assayed using the method [29]. The reaction mixture contained 50 mM phosphate buffer, pH 7.0, and 50 mM H_2O_2. The reaction rate was measured at 240 nm. One unit of catalyse activity was defined as 1 µm of H_2O_2 degraded/min/mg protein. Protein concentration was determined by the method [30] using bovine serum albumin as a standard.

3. Optimal Analytical Procedure

The enzyme was assayed in 100 mM phosphate buffer by varying the assay conditions of substrate concentration, pH (5.5–10), and temperature (20–55^{o}C), in different experiments. To determine the optimal activity, eight substrate concentrations of H_2O_2 (20–90 mM) were used, and apparent Michaelis constant (apparent Km) was calculated by a Lineweaver-Burk plot of 1/V vs. 1/S. The variation in the reaction rate with temperature was measured in several samples using Arrhenius plot.

RESULTS

The specific activity of catalyse measured in foot,gill and digestive gland cytosols of mussel varied within different ranges of pH (Figs.1-3). The maximum activity of catalyse was observed on cytosol of digestive gland at pH 7.5 (38.8±0.047 µmol/min/mg protein) followed by cytosol of gill at pH 7.5 (34.27±0.030 µmol/min/mg protein) and cytosol of foot at pH 7.5 (30.71±0.050 µmol/min/mg protein).The specific activity of catalyse gradually increased with increase in temperature(20–45^{o}C) from the cytosol of digestive gland (56.61 ± 0.062 µmol/min/mg protein) followed by cytosol of gill (50.76 ± 0.043 µmol/min/mg protein) and cytosol of foot (50.00 ± 0.041 µmol/min/mg protein) (Figs.4-6).An Arrhenius plot was used to determine the effect of temperature on catalyse activity, or the activation energy (Ea) of catalyse in mussels (Figs. 4.1, 5.1, and 6.1).The Ea for catalyse activity of cytosol of digestive gland was 53.76 KJ/mol with R^2 =0.3371 followed by cytosol of gill 55.56 KJ/mol with R^2 = 0.3889 and cytosol of foot 56.58 KJ/mol with R^2 =0.4162 in mussel.The effect of substrate (H_2O_2) on enzymatic activities is shown in Figures 7-9. Catalase activity slowly increased with increased concentration of substrate. The maximal activity of catalyse was observed between 60 mM concentration of substrate in digestive gland cytosol (51.31±0.304 µmol/min/mg protein), cytosol of gill (47.05±0.020 µmol/min/mg protein) and cytosol of foot (41.62±0.020 µmol/min/mg protein) respectively.The enzymatic activities were applied in Michaelis-Menten kinetics and apparent Vmax, Km values were determined. The apparent Vmax and Km of digestive gland cytosol was 49.26;7.39 mM H_2O_2 followed by cytosol of gill 46.94;8.91 mM H_2O_2 and cytosol of foot 43.85;15.11 mM H_2O_2 in *L. marginalis* (Figs. 7.1; 8.1 and 9.1).

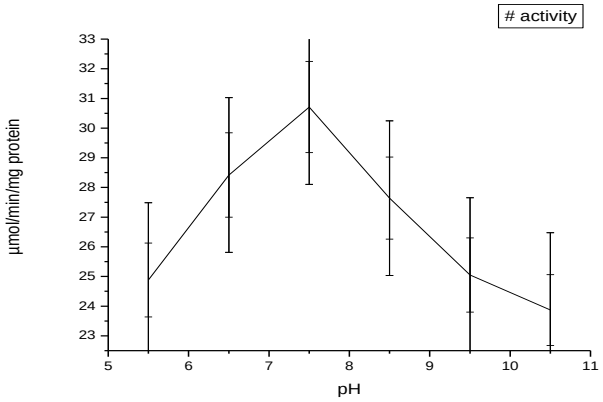

Fig. 1 Effect of pH on the activity of catalyse in foot cytosol of *L. marginalis.*

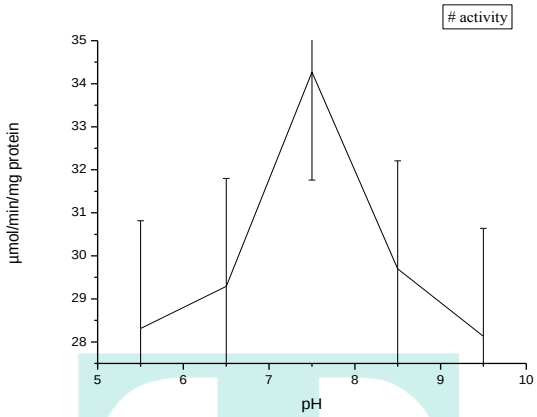

Fig. 2 Effect of pH on the activity of catalyse in gill cytosol of *L. marginalis*

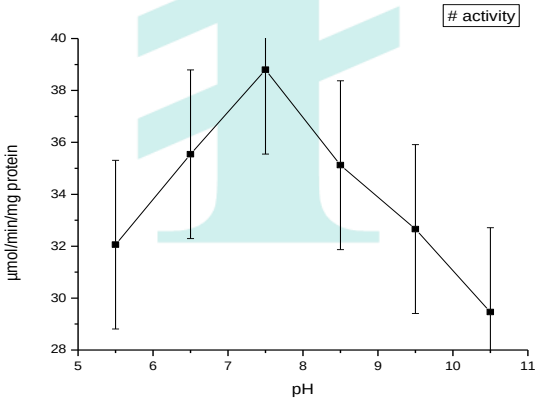

Fig. 3 Effect of pH on the activity of catalyse in digestive gland cytosol of *L. marginalis.*

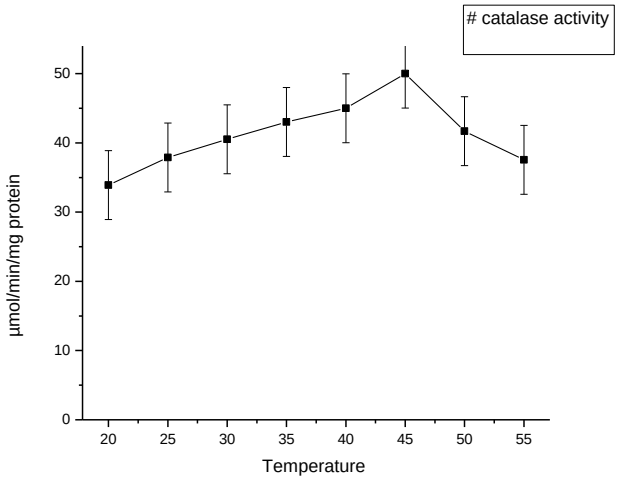

Fig. 4 Effect of temperature on the activity of catalyse in foot cytosol of *L. marginalis.*

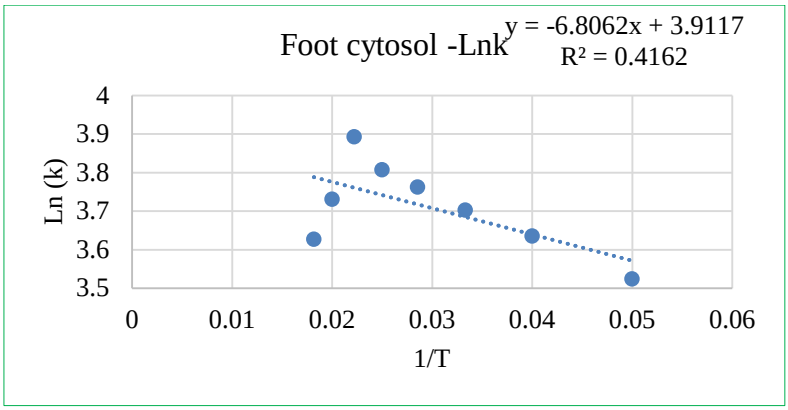

Fig. 4.1 Arrhenius plot of the effect of temperature on catalyse activity in foot cytosol.
*Each point represents the mean of three determinations

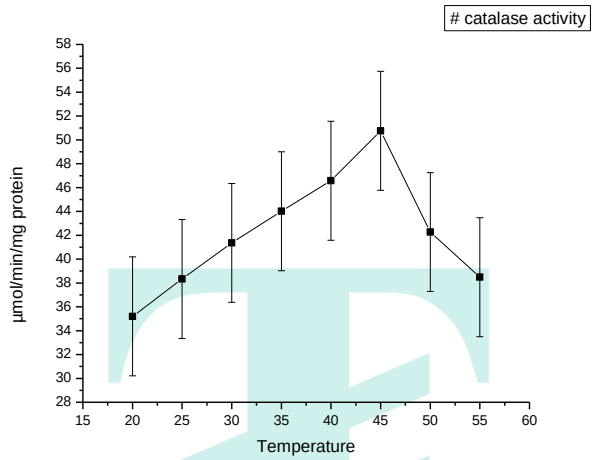

Fig. 5 Effect of temperature on the activity of catalyse in gill cytosol of *L. marginalis.*

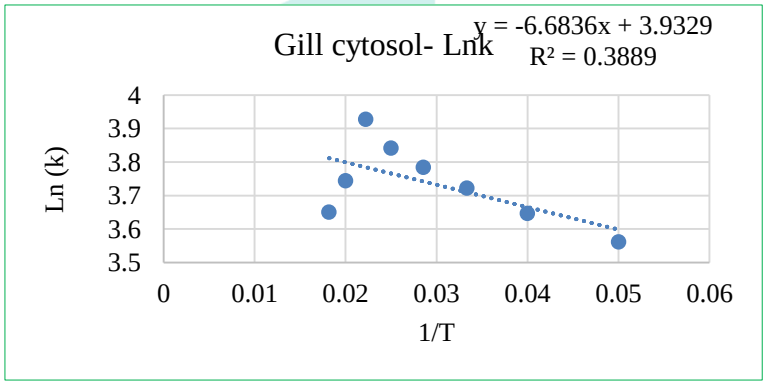

Fig. 5.1 Arrhenius plot of the effect of temperature on catalyse activity in gill cytosol
*Each point represents the mean of three determinations

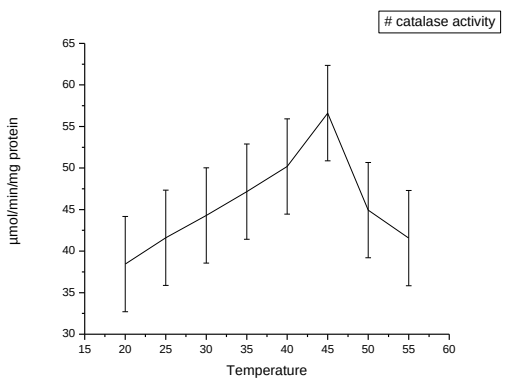

Fig. 6 Effect of temperature on the activity of catalyse in digestive gland cytosol of *L. marginalis.*

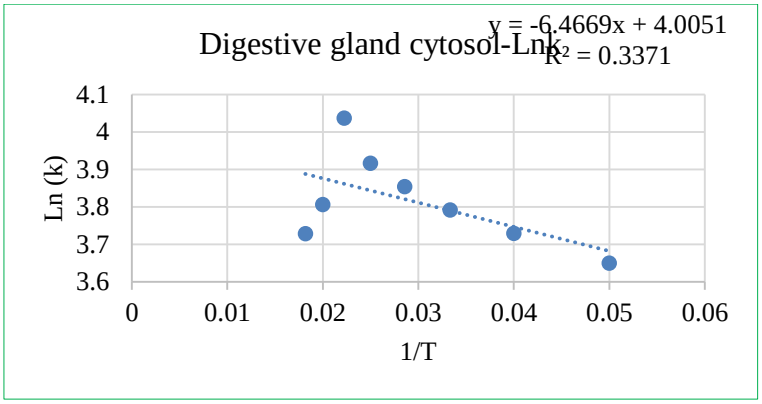

Fig. 6.1 Arrhenius plot of the effect of temperature on catalyse activity in digestive gland cytosol
*Each point represents the mean of three determinations

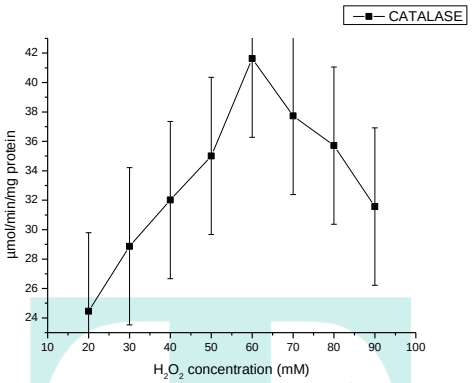

Fig. 7 Effect of substrate on the activity of catalyse in foot cytosol

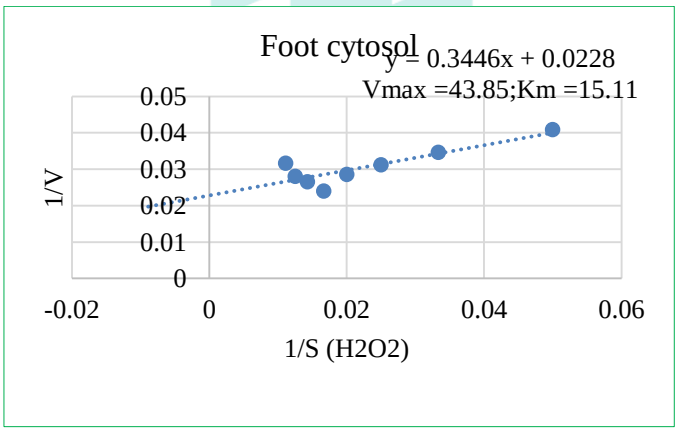

Fig.7.1 Lineweaver-Burk plot for catalyse activity in *L.marginalis* foot cytosol
*Each point represents the mean of three determinations

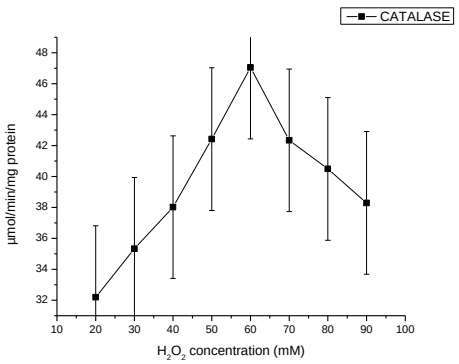

Fig. 8 Effect of substrate on the activity of catalyse in gill cytosol.

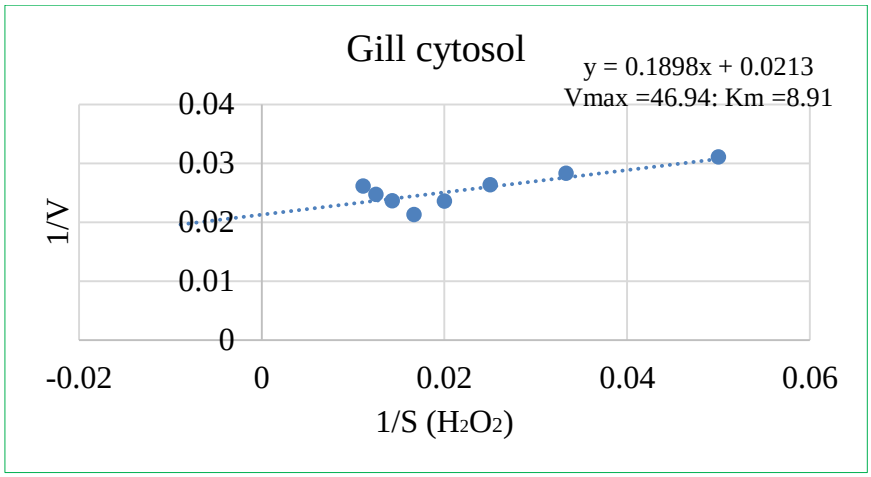

Fig. 8.1 Lineweaver-Burk plot for catalyse activity in *L. marginalis* gill cytosol.
*Each point represents the mean of three determinations

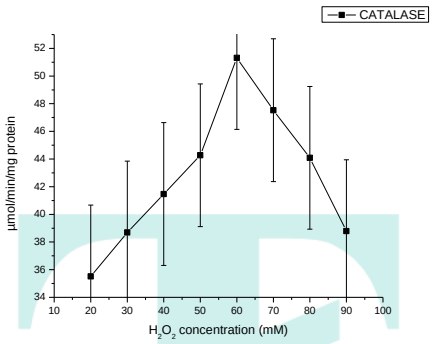

Fig. 9 Effect of substrate on the activity of catalyse in digestive gland cytosol

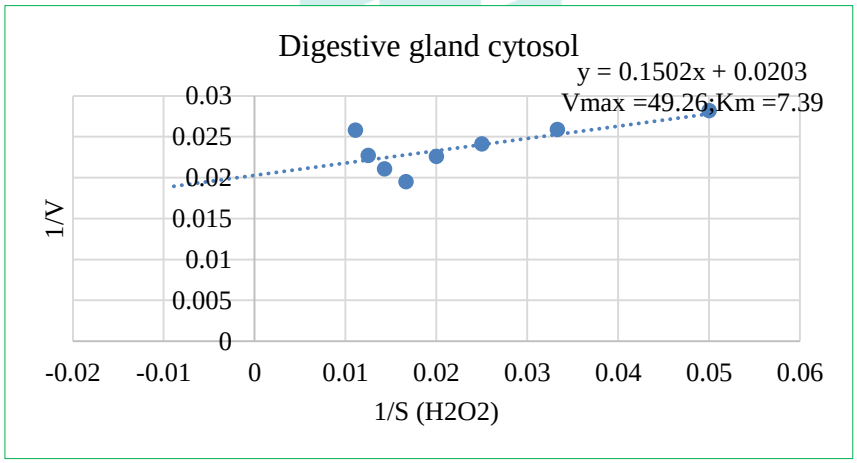

Fig. 9.1 Lineweaver-Burk plot for catalyse activity in *L. marginalis* digestive gland cytosol
*Each point represents the mean of three determinations

DISCUSSION

The present investigation demonstrates the appropriate analytical procedures and kinetic constants for determining catalyse activities on cytosols of digestive gland, gill and foot of the fresh water mussel, *L. marginalis*. Detection of catalyse activity with H_2O_2 substrate reveals the abundance of catalyse activity in *L. marginalis* and exhibits a sharp optimal pH at 7.5 (Figs. 1-3). Similar trend [10] was observed an optimal pH of 7.0 for catalyse in *Mytilus edulis* ;and also observed an optimal pH of 6.5 and 7.0 for catalyse in arthropods [31]. There are two types of catalyse. One catalyzes only catalytic reactions and the other (catalyse peroxidase) catalyzes both catalytic and peroxidase reactions. The activity of catalase-peroxidase is pH dependent and is more sensitive to temperature and H_2O_2 [8,9].Catalase of *L. marginalis* also exhibits an optimal pH of 7.5, and is more sensitive to temperature and substrate concentration (Figs. 1-3;4-4.1;5-5.1;6-6.1;7-7.1;8-8.1and 9-9.1). This implies that, in *L. marginalis*, the enzyme occurs predominantly as catalase-peroxidases and that it is involved in both catalytic and peroxidase reactions. The effect of temperature on *L. marginalis* catalyse activity was measured and linear response activity was observed with increasing temperature up to 45^{o}C. Hence, the optimal temperature for catalyse enzyme in *L. marginalis* was 45^{o}C. In *M. edulis*, the optimal temperature was 45^{o}C for catalyse enzyme [10].

The values of Ea were determined from the reaction velocities at different temperatures by an Arrhenius plot for the catalyse enzyme in *L. marginalis*. The Ea for catalyse of digestive gland cytosol was 53.76 KJ/mol;R^2 = 0.3371 followed by cytosol of gill 55.56 KJ/mol;R^2 = 0.3889 and cytosol of foot 56.58 KJ/mol;R^2 =0.4162 (Figs.4-4.1,5-51and 6-6.1). The effect of substrate (H_2O_2) on enzymatic activities is shown in Figures 7-9. The Ea for the decomposition of H_2O_2 catalyzed by catalyse is low compared to the Ei. Similarly [8,10] observed a low Ea for catalyse in mussels. This may be due to inhibition of catalyse by other substrates such as 3-amino,1,2,4- triazole [32] and cumene hydroperoxide. Inhibition of catalyse activity in invertebrates by 3- amino-1,2,4-triazole was observed [9]. The cumene hydroperoxide-mediated peroxide metabolism was investigated in *M. malcolmsonii* [33]. In the present study,cumen hydroperoxide may also inhibit catalyse reactions in *L. marginalis*.

One of the most important characteristics of enzymes is substrate affinity, which usually is assessed as apparent Km with respect to a specific substrate. In *L. marginalis*, a gradual increase in catalyse activity was noted in response to increased concentration of substrate. The optimal substrate concentration was 70mM H_2O_2 concentration and the apparent Km of digestive gland cytosol was 7.39 mM H_2O_2 followed by gill 8.91 mM H_2O_2 and foot 15.11 mM H_2O_2 in *L. marginalis* (Figs.7.1;8.1and 9.1).Additionally, we found that the Vmax value of catalyse activity in mussel digestive gland cytosol was 49.26, followed by gill cytosol at 46.94, and foot cytosol at 43.85.Same trend was also observed an apparent Km for H_2O_2 of 68.4 mM in marine mussels [10]. On the other hand, the apparent Km for H_2O_2 was 8.6 mM for *Hyalomma dromedarii* [31]. The specific activity of catalyse observed in present study was greater than the specific activity of glutathione peroxidase [33], which implies that in *L. marginalis*, catalyse is predominantly involved in removing H_2O_2 rather than glutathione peroxidase. The properties of catalyse in *L. marginalis* determines the optimal assay condition of 60 mM H_2O_2, 45° C,pH 7.5, and 3 ml assay volume. Because studies of catalyse in molluscs are scanty, hope this preliminary findings on *L. marginalis* will be useful for the further analysis of catalyse in bivalve molluscs.

CONCLUSION

The specific activity of catalyse observed in the present study was greater than the specific activity of glutathione peroxidase, which implies that in *L. marginalis*, catalyse is predominantly involved in removing H_2O_2 rather than glutathione peroxidase. The properties of catalyse in *L. marginalis* determines the optimal assay conditions of 60 mM H_2O_2, 45°C,pH 7.5, and 3 ml assay volume. Because studies of catalyse in molluscs are scanty, we hope these preliminary findings on *L. marginalis* will be useful for the further analysis of catalyse activity in bivalve molluscs.

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DECLARATION OF CONFLICT

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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