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Effects of D-Galactose Induced Oxidative Stress and Ethanolic Extracts of *Bacopa monniera* and *Lactuca sativa* on Non-Specific Esterase in Midgut of Silkworm *Bombyx mori*

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Abstract

The non-specific esterase (NSE) activity was estimated in the midgut of simultaneously and independently treated larvae of silkworm *Bombyx mori* (Race-PM) with D-galactose and ethanolic extract of *Bacopa monniera* and *Lactuca sativa*. Treatments of only D-galactose showed non-significant ($p > 0.05$) decrease in NSE activity as compared with control group. Concomitant treatment of D-galactose and ethanolic extract of *B. monniera* and *L. sativa* showed non-significant change in non-specific esterase activity by as compared with control group. There was significant ($p < 0.05$) change in NSE activity in the groups treated with only ethanolic extract of *B. monniera* and *L. sativa.* The lipid peroxidation and fluorescence was measured to analyze the extent of oxidative stress. The results showed high degree of positive correlation ($r = 0.8088$) between oxidative stress and non-specific esterase activity.

Keywords

Lipid Peroxidation, Fluorescence, Aging and Correlation

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1. Introduction

Non-specific esterase (NSE) is a lysosomal enzyme and is co-localized in primary lysosomes and in a large diverse population of autophagic vacuoles (Willard *et al*., 1984; Jimenez and Gilliam, 1990). NSE enzymes are believed to participate in the intracellular hydrolysis of the ingested material (Bang, 1975; Cheng *et al*., 1974; Michelson, 1975). The nonspecific esterases are broadly distributed enzymes that catalyze the hydrolysis of ester and amide linkages. They fall into several different classes which can be distinguished by their substrate preferences and their sensitivity to inhibitors.

Esterase enzymes play an important role in conferring or contributing to insecticide resistance in insects. This has been shown in aphid *Myzus persiace* (Field and Devonshire, 1998), mosquitoes, *Culex quinquefasciatus* and *C. pipiens* (Guillemaud *et al*., 1997), blowfly, *Lucilia cuprina* (Campbell *et al*., 1998) and housefly *Musca domestica* (Claudianos *et al*., 1999; Taskin and Kence, 2004). The biochemical and physiological properties of esterases were previously studied (Healy *et al*., 1991; Oakeshott *et al*., 1993). In insects, the esterase enzyme patterns have shown high rates of intraspecific and interspecific variations (Nascimento and Bicudo, 2002).

Possibly, gene duplication followed by divergence of duplicated genes from the ancestral gene is the origin of at least part of this variability (Oakeshott *et al*., 1993; Nascimento and Bicudo, 2002). Acetylcholinesterase enzyme, a key enzyme in the insect central nervous system, terminates

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nerve impulses by catalyzing the hydrolysis of the neurotransmitter acetylcholine. The resistance associated with modification of acetylcholinesterase makes it less sensitive to inhibition by organophosphates and carbamate insecticides (Feyereisen, 1995). Several point mutations have been identified in *D. melanogaster* (Mutero *et al*., 1994; Menozzi *et al*., 2004) and *M. domestica* Ace (acetyl choline esterase) genes, encoding acetyl choline esterase (Kozaki *et al*., 2001).

In insects, esterases are involved in important physiological processes, including the catabolism of juvenile hormone (Zera *et al*., 1992, 2002), pesticide resistance (Whyard *et al*., 1995; Rosario-Cruz *et al*., 1997), digestion (Kerlin and Hughes, 1992; Argentine and James, 1995) and reproduction (Richmond and Senior, 1991; Karotam and Oakeshott, 1993). Esterases are main hydrolytic enzymes responsible for converting complex storage lipids into easily utilizable glycerides (Subramaniam, 1982).

Oxidative stress is caused by the imbalance between the reactive oxygen species production and elimination in the biological system. D-galactose is a naturally occurring sugar in the body; however, at high levels, it can cause the accumulation of reactive oxygen species, finally resulting in increased oxidative stress (Shan *et al*., 2009). D-galactose has been used to inject mice or rats for pharmacological studies. There are several evidences which shows that D-galactose cause aging-related changes including the increase of reactive oxygen species and the decrease of antioxidant enzyme activity (Liu *et al*., 2009; Wang *et al*., 2009). Administration of D-galactose showed increased lipid peroxidation and fluorescence, decreased antioxidant enzyme activity and reduced life span (Cui *et al*., 2004).

The alcoholic extract of *Bacopa monniera* (Bramhi) has been shown to be a potent antioxidant, free radical scavenger and anti-lipid peroxidative agent (Gajare *et al*., 2006; Bhattacharya *et al*., 2000). *B. monniera* contains tetracyclic triterpenoid saponins, bacosides A and B, hersaponin, alkaloids like herpestine, brahmine and flavonoids (Kirtikar and Basu, 1994). Ethanolic extract of *Lactuca sativa* (Lettuce) contains quercetin which is a potent antioxidant flavonoid. Quercetin showed increased antioxidant enzyme activity of mice brain (Su *et al*., 2007). Treatments of ethanolic extracts of *B. monniera* and *L. sativa* showed reduced lipid peroxidation and increased membrane integrity in silkworm *B. mori* (Gaikwad *et al*., 2010).

There is scanty literature available regarding the role of NSE during aging. Therefore, in present work simultaneous and independent treatment of D-galactose, ethanolic extracts of *B. monniera* and *L. sativa* in midgut of silkworm *B. mori* to study the pattern of NSE activity during aging process.

2. Material and Methods

2.1. Rearing of Silkworm

The silkworm larvae of race PM was reared in laboratory conditions at 28^0C and 80% humidity as suggested by Krishnaswami *et al*., (1978).

2.2. Preparation of Ethanolic Extract

The leaves of *B. monniera* and *L. sativa* were washed with distilled water and shade dried for 3 to 4 days. After complete drying the leaves were powdered and kept in ethanol for 72 hours. The alcohol was allowed to evaporate and extract was collected and stored in refrigerator for further use.

2.3. Administration of D-Galactose and Ethanolic Extracts

The larvae were divided into 6 groups containing 30 larvae in each group. The treatment was given during first three days of forth and fifth instars. Mulberry leaves of equal weight were dipped in various concentrations of D-galactose and ethanolic plant extracts (Table - 1) and air-dried. The larvae were fed on the dried leaves once in a day. On the forth day of fifth instar the midgut was removed and homogenized in 0.8% NaCl. Homogenate was centrifuged at 1000 g and supernatant was used as sample.

2.4. Estimation of Lipid Peroxidation

The lipid peroxidation was estimated by method of Buege and Aust (1987). The reaction mixture contained 1 ml of sample and 2 ml TCA-TBA-HCl reagent (15 % TCA + 0.375% TBA in 0.25M HCl). The tubes were kept in boiling water bath for 10 min. cooled and centrifuged. The absorbance of supernatant was measured at 532 nm on spectrophotometer. The amount of malondialdehyde (MDA) was calculated using extinction coefficient $1.56X10^5$/M MDA/cm^2.

2.5. Spectrofluorometric Analysis

Spectroflurometric analysis was performed by method of Dillard and Tappel (1984). 1 ml of tissue sample was taken in clean dry test tube. 6 ml of chloroform methanol mixture was added and the tubes were shaken for proper mixing. The mixture was centrifuged for 10 in at 1000g. 6 ml distilled water was added slowly in test tube and 4 ml of upper chloroform layer was removed to a test tube and 0.4 ml methanol was added. The fluorescence was determined on spectroflurometer and the results were expressed in intensity/mg protein.

2.6. Estimation of NSE

The NSE activity from sample was estimated by method described by Subramaniam (1982). The tissues were

homogenized in 0.8% saline and centrifuged at 1000g for 10 min. The pellet was discarded and supernatant (sample) was used for estimation of NSE activity. Assay mixture consisted of 0.5 ml tissue sample, 2.5 ml of substrate buffer containing 600 µg/ml α-naphthyl acetate in 50 mM phosphate buffer (pH 7.0), tubes were incubated at 40°C for 20 min. Finally 2.0 ml of arrest regent (1% fast blue RR and 1% sodium dodecyl sulfate in 1:5 proportion) was added. Blank tube contained 0.5 ml of distilled water instead of tissue homogenate and arrest reagent was added immediately. Assay control tube contained similar composition as blank but was incubated along with sample tubes to avoid increase in absorbance due to auto-hydrolysis of α-naphthyl acetate. Finally absorbance of control was subtracted from sample absorbance. The NSE activity was expressed as µg α-naphthol/mg protein/hr.

2.7. Estimation of Protein

The soluble protein from sample was estimated by Lowry's method (Lowry *et al*., 1951).

2.8. Statistical Analysis

Kruskal-Wallis test was implemented for data analysis and Karl Pearson's correlation coefficient method was used to study correlation between lipid peroxidation and NSE activity.

Table 1. Groups and treatments given.

Group	Treatments	Group	Treatments
Control	Distilled water	30L	30 mg/ml D-galactose + 2 mg/ml *L. sativa*
30D	30 mg/ml D-galactose	B	2 mg/ml *B. monniera*
30B	30 mg/ml D-galactose + 2 mg/ml *B. monniera*	L	2 mg/ml *L. sativa*

3. Results and Discussion

D-galactose is a reducing sugar and can be metabolized at normal concentration. However, at high levels, it is converted into aldose and hydroperoxide under the catalysis of galactose oxidase, resulting in the generation of a superoxide anion and oxygen-derived free radicals (Wu *et al*., 2008). In insects enzymes of defense mechanism includes superoxide dismutase, catalase, ascorbate peroxidase while, non-enzyme molecules are α-tocopherol, ascorbic acid and glutathione besides these dietary flavonoids also play crucial role in nullifying reactive oxygen species. Overwhelming reactive oxygen species cause deleterious effects on biological molecules resulting in lipid peroxidation, protein oxidation, DNA damage and lipofuscinogenesis (Sohal *et al*., 1995; Standtman, 1992, Finkel and Hallbrook, 2000).

Lipid peroxidation is one of the major manifestations of aging. In present investigation the lipid peroxidation was measured in midgut tissue for determination of extent of oxidative stress caused by D-galactose treatment. Results showed highly significant ($p < 0.001$) increase in the lipid peroxidation of D-galactose treated group as compared with the control group (Fig. 1). The simultaneous treatment of D-galactose and ethanolic extract of *B. monniera* showed decreased lipid peroxidation as compared with only D-galactose treated group showing protective effect of ethanolic extract of *B. monniera*. There was non-significant ($p > 0.05$) decrease in the group treated with D-galactose and ethanolic exctract of *L. sativa* as compared with the control group. The groups treated with only ethanolic extracts of *B. monniera* and *L. sativa* showed highly significant and moderately significant ($p < 0.01$) decrease in lipid peroxidation respectively as compared with control group.

Ethanolic extract of *B. monniera* contains number of antioxidant flavonoids like triterpenoid glycosides, bacosides and flavonol which are proven to have antioxidant properties (Chillara *et al*., 2005). Previous studies have detected significant levels of antioxidant activities and phenolic components in *L. sativa* (Caldwell, 2003; Cao *et al*., 1996; Chu *et al*., 2002; Vinson *et al*., 1998). The antioxidant extracts of *L. sativa* exhibited significant oxygen radical absorbance capacity (Caldwell, 2003; Cao *et al*., 1996), prevention of lipid oxidation in lower-density lipoproteins (Vinson *et al*., 1998), inhibitory effects on ethylene formation induced by oxyradicals (Chu *et al*., 2002), and hydroxyl radical absorbance capacity and inhibitory activity against Cu^{++} induced protein oxidation.

Lipofuscinogenesis is unavoidable process which progresses with aging. Lipofuscin granules accumulate in postmitotic cells causing hurdles in cellular processes. The lipofuscin granules contain non-degradable fluorescent products. The spectrofluorometric analysis of midgut homogenates showed excitation wavelength 360 nm and emission wavelength 524 nm. The intensity of fluorescence was higher in the midgut tissue of D-galactose treated group as compared with the control group (Fig. 2). The intensity of fluorescent products was low in the group treated with ethanolic extract of *B. monniera* and ethanolic extract of *L. sativa* treated groups showing their antioxidant properties. In earlier studies Kalamade *et al*., (2008) reported similar decrease in lipofuscinogenesis in mice brain when treated with ethanolic extract of *B. monniera*. Similarly ethanolic extract of *L. sativa* also showed decreased lipofuscin granules in nervous

tissue as well as midgut. *L. sativa* extract contain quercetin which is reported by several authors as potent antioxidant (Havsteen, 2002).

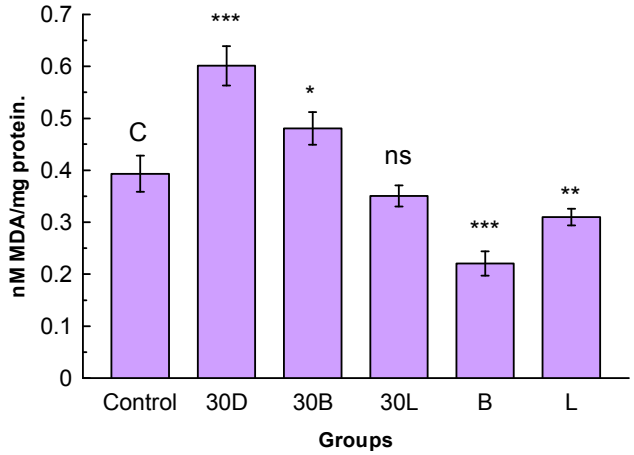

Fig. 1. Lipid peroxidation in midgut.

Data are Mean ± S.D. (n = 3); *, **, *** and ns indicates significance level $p < 0.05$, $p < 0.01$, $p < 0.001$ and $p > 0.05$ respectively.

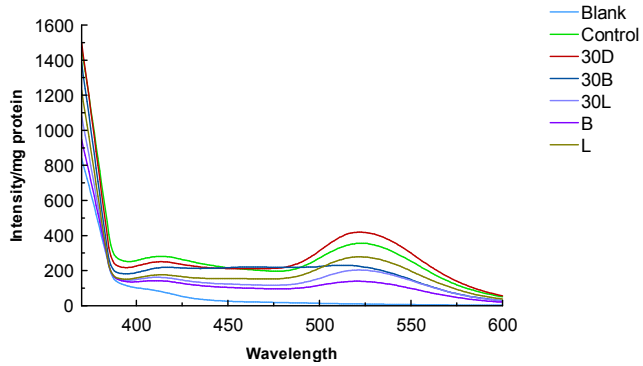

Fig. 2. Fluorescence in midgut.

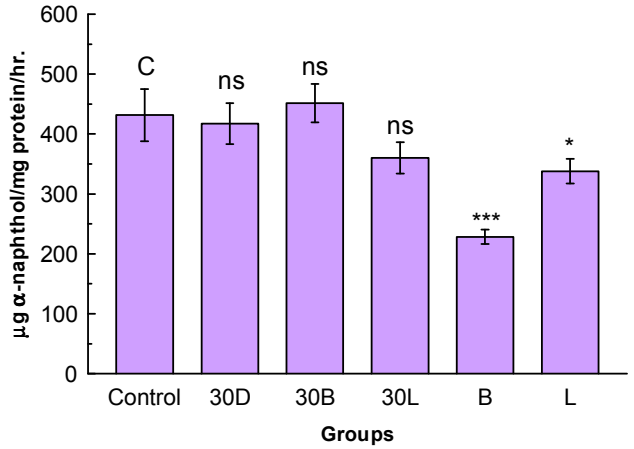

Fig. 3. NSE activity in midgut.

Data are Mean ± S.D. (n = 3); *, *** and ns indicates significance level $p < 0.05$, $p < 0.001$ and $p > 0.05$ respectively

The NSE activity was decreased in the midgut of D-galactose treated group and the group treated simultaneously with D-galactose and ethanolic extract of *L. sativa* however the decrease was non-significant ($p > 0.05$) as compared with control group (Fig. 3). In the group treated simultaneously with D-galactose and ethanolic extract of *B. monniera* showed non-significant increase in NSE activity as compared with control group. Treatment with only ethanolic extracts of *B. monniera* and *L. sativa* showed highly significant ($p < 0.001$) and moderately significant ($p < 0.01$) decrease in NSE activity as compared with control groups respectively. Statistical analysis for correlation between lipid peroxidation and NSE activity showed high degree of positive correlation ($r = 0.8088$).

Juvenile hormones are a group of acyclic sequiterpenoids that regulate many aspects of insect physiology. Juvenile hormones play crucial role in development, reproduction diapause and polyphenisms. NSE plays an important role in the metamorphosis of an insect by complete degradation of juvenile hormones (Riddiford, 1994; Wyatt and Davey, 1996; Nijhout, 1994). In *Drosophila melanogaster* 8 to 16% decrease in esterase activity was observed when the activity was measured on 10, 25 and 57 day of life span (Burcombe, 1972), while in *Callosobruchus maculatus* NSE activity was found to increase throughout life (Sharma and Sharma, 1981). Our results show there is increase in NSE activity as aging advances.

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