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# Ghrelin effects on midgut tissue antioxidative defense and glutathione S-transferase activity in *Lymantria dispar* (Lepidoptera)

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**Abstract:** The aim of the study was to examine changes in Cu–Zn superoxide dismutase (SOD), catalase (CAT), their gel electrophoresis profiles, glutathione reductase (GR) activity, amount of glutathione (GSH), and the activity of glutathione S-transferase (GST) - phase II biotransformation enzyme in the midgut tissue of gypsy moth (*Lymantria dispar* L. (Lepidoptera: Lymantriidae)) larvae after ghrelin treatment. Four subpicomolar injections of ghrelin (0.3 pmol) or physiological saline (control) were applied every 24 h. The SOD, CAT, GR, GST activity, and amount of GSH were higher in the ghrelin-treated group than in the control. Electrophoresis gel bands of SOD and CAT had higher area and density in the treated group. The effects of ghrelin on the antioxidative defense and GST activity in insects were detected for the first time. The results provided evidence for possible application of insects as simple model systems in future studies of the role of ghrelin in the antioxidative protection of complex organisms.

**Key words:** Superoxide dismutase, catalase, glutathione reductase, glutathione, glutathione S-transferase

## 1. Introduction

Ghrelin, an appetite-stimulating hormone, is an important variable in the initiation of feeding behavior in invertebrates and vertebrates, including humans. Ghrelin, a 28-amino acid peptide, is an endogenous ligand for the growth hormone secretagogue receptor. There is widespread tissue expression of the ghrelin gene. The most important functions of ghrelin besides appetite stimulation (by activation of neuropeptide Y and agouti-related protein release) are positive energy balance, gastric motility and gastric acid secretion control, modulation of exocrine and endocrine gland functions, modulation of the cells proliferation, immune system control, etc. Ghrelin synthesized in digestive tract represents the main (80%) part of circulating ghrelin (Dornonville et al., 2001; Kojima et al., 2008).

Our previous publications have shown similarities between responses of insects to ghrelin influences and other animal taxa (Zwirska-Korczala et al., 2007; Kojima et al., 2008; Kheradmand et al., 2010; Perić-Mataruga et al., 2009, 2012a, 2012b). Ghrelin immunopositive activity was found in the endocrine cells of the midgut epithelium of *Lymantria dispar* (Linnaeus) (Perić-Mataruga et al., 2012). The gypsy moth, *L. dispar*, is one of the most destructive phytophagous pest insects of the northern hemisphere. It shows a tremendous capacity to feed on a wide range of trees and shrubs, totalling more than 500 plant species (Barbosa et al., 1971). Many secondary metabolites of host plants, in defense against *Lymantria dispar* L., may have strong prooxidative effects on midgut tissue and may provoke oxidative stress (Bi and Felton, 1995; Perić-Mataruga et al., 1997; Ilijin et al., 2014; Perić-Mataruga et al., 2014). That is an imbalance between the production of free radicals and the ability of the body to counteract or detoxify their harmful effects through neutralization by antioxidants. Moreover, reactive oxygen species (ROS) readily react with cellular macromolecules, causing damage to proteins, lipids, and nucleic acids (Halliwell and Gutteridge, 2007).

Thus, ROS production in insects, including *Lymantria dispar*, is strongly controlled by enzymatic and nonenzymatic antioxidants (Felton and Summers, 1995; Mathews et al., 1997; Perić-Mataruga et al., 1997). The *Lymantria dispar* midgut is the second-largest organ in larvae and the major site for elimination of toxic effects of plant allelochemicals, food digestion, transport of nutrients, regulation of ion balance, etc. *Lymantria dispar* needs strong and multilevel regulation of antioxidative

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protection in midgut tissue (Perić-Mataruga et al., 1997, 2000, 2003, 2006).

Ghrelin has been shown to increase the antioxidative defense system and to inhibit lipid peroxidation in many mammal tissues, including the gastrointestinal tract (Kheradmand et al., 2010). SOD belongs to the first line of defense against free radicals in *L. dispar*, catalyzing the dismutation of superoxide anion radical ($O_2^-$) into hydrogen peroxide ($H_2O_2$). The formed $H_2O_2$ is transformed into water and oxygen by catalase. The ubiquitous tripeptide L-γ-glutamyl-cysteinyl-glycine (glutathione), the most abundant soluble cellular thiol, is involved in processes essential for synthesis and degradation of proteins, formation of deoxyribonucleotides, regulation of enzymes, and protection of cells against ROS (Felton and Summers, 1995; Perić-Mataruga et al., 1997). Glutathione exists in two forms: reduced glutathione (GSH) and oxidized glutathione (GSSG). Increases in the whole amount of glutathione indicate the need for redox homeostasis maintenance. Glutathione reductase (GR), a flavoprotein enzyme, regenerates GSH from GSSG, with nicotinamide adenine dinucleotide phosphate (NADPH) as the source of reducing factor. Glutathione S-transferase (GST) is a glutathione-dependent enzyme because it uses glutathione as a cofactor. The phase II biotransformation enzyme GST catalyzes the conjugation of GSH to a wide variety of xenobiotics with an electrophilic site, yielding more water-soluble xenobiotics and facilitating their excretion. These glutathione-dependent enzymes participate in the detoxification of electrophilic decomposition products resulting from the attack of oxygen radicals on lipids and DNA, and prevent oxygen toxicity generated by redox active derivatives (Felton and Summers, 1995; Halliwell and Gutteridge, 2007).

Thus, we undertook this study to examine whether ghrelin correlates with antioxidative defense in the midgut tissue in order to clarify the possible effects of ghrelin on the activities of antioxidant enzymes SOD, CAT, GR, GSH - phase II biotransformation enzyme, and glutathione content in the midgut tissue of *Lymantria dispar*.

## 2. Materials and methods

*Lymantria dispar* egg masses were collected in an oak forest (near Belgrade; 44°42′N, 20°24′E). After hatching, larvae were reared on an artificial diet (O'Dell et al., 1984) at 23 °C, with a 16 h light:8 h dark photoperiod in petri dishes. Fifty newly molted 4th instar *L. dispar* larvae (the same size) were used per experimental group, which were ghrelin-treated and control.

The treated larvae received 0.3 pmol of ghrelin GS (n-octanoyl-S) LSPEHQKAQQRKESKKPPAKLQPR (Global Peptide Services, USA) in physiological saline every 24 h for four consecutive days. The control group received only physiological saline (Perić-Mataruga et al., 2009, 2012a, 2012b). Below, unless otherwise mentioned, all chemicals were supplied by Sigma-Aldrich (Germany).

Midgut homogenates of *L. dispar* larvae for detection of enzyme activity and glutathione content were done as described in Perić-Mataruga et al. (1997). SOD and CAT activity was determined according to standard methods (Misra and Fridovich, 1972; Beutler, 1982). GR activity was measured according to Glatzle et al. (1974) and activity of GST toward 1-chloro-2,4-dinitrobenzene was assayed according to Habig et al. (1974). Total glutathione amount was calculated in accordance with the method by Griffith (1980).

Electrophoretic separation of proteins in midgut tissue homogenates was performed in 12% nondenaturing polyacrylamide gels (Davis, 1964; Beauchamp and Fridovich, 1971). Immediately after electrophoresis, gels were prepared for determination of SOD by the photochemical method (Salin and McCord, 1971). The gels were incubated in the dark with riboflavin and nitro-blue-tetrazolium (NBT) and then exposed to UV light. Upon exposure to UV light, riboflavin produces $O_2$, which reduces NBT and creates dark blue formazan. Dark blue gels were obtained, with lighter bands indicating SOD activity. In order to ascertain whether SOD isoforms were Cu–Zn SOD, parallel groups of samples were incubated with KCN, a well-known Cu–Zn SOD inhibitor (Beauchamp and Fridovich, 1971). CAT was electrophoretically separated on 8% polyacrylamide gel according to the appropriate procedure. The gel was washed twice with distilled water, and then soaked in 50 mM $H_2O_2$ for 10 min, washed with distilled water, and then soaked in 2% $FeCl_3$ and 2% $K_3[Fe(CN)_6]$ to develop background color. No color would appear in the area where CAT had depleted $H_2O_2$ (Davis, 1964; Aebi, 1983). Bands were clearly expressed.

Protein band area and optical density in the region of the superoxide dismutase and CAT were analyzed using ImageJ 1.42q (NIH, USA).

All the results of enzyme activities and amount of glutathione were presented as mean values ± SEM. The statistical differences were assessed between the control and ghrelin-treated larvae by an independent sample t-test. Previously, all variables were tested for normal and homogeneous variances by Leven's statistic test. A P-value of less than 0.05 was considered to be statistically significant.

## 3. Results

SOD activity in the midgut tissue of ghrelin-treated *Lymantria dispar* L. larvae (fourth instar) was higher (10.29 ± 0.80 U/mg protein) than in the control group (7.25 ± 0.42 U/mg protein), ($P < 0.001$) (Figure 1). Administration of ghrelin to the fourth instar larvae correlated with

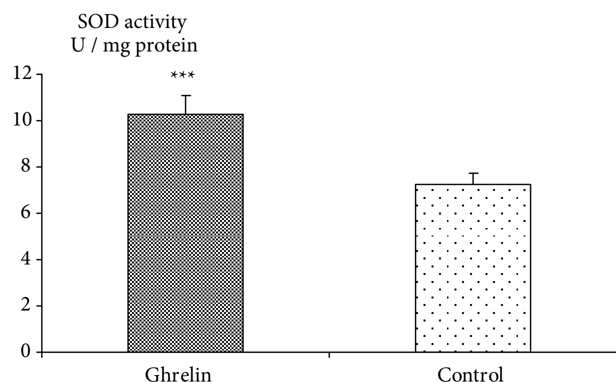


**Figure 1.** Effects of ghrelin on superoxide dismutase activity in midgut tissue of *Lymantria dispar*. Bars represent the mean values ± SEM, n = 50. An asterisk denotes significant differences between groups at P < 0.001.

significantly increased catalase activity (104.19 ± 3.31 U/mg protein) compared to the control group (79.06 ± 2.20 U/mg protein) (P < 0.001) (Figure 2).

Polyacrylamide gel bands stained for SOD activity in the midgut tissue of fourth larval instar of *Lymantria dispar* revealed higher relative density (51.89%) and area (33.76%) in the ghrelin-treated group compared to the control (Figure 3). Similar results were observed with gels stained for CAT activity.

The CAT bands had higher relative density (47.27%) and area (55.5%) in the ghrelin-treated group than in the control (Figure 4). Total glutathione was significantly higher in the ghrelin-treated group (0.432 ± 0.024 U/mg protein) of *L. dispar* larvae than in the control (0.31 ± 0.03 U/mg protein) (P < 0.01) (Figure 5). GR activity in midgut tissue was lower in the control group insects (0.4 ± 0.08 nmol NADPH/mg protein/min) than in ghrelin-treated insects (0.87 ± 0.09 nmol NADPH/mg protein/min) (P < 0.01) (Figure 6).

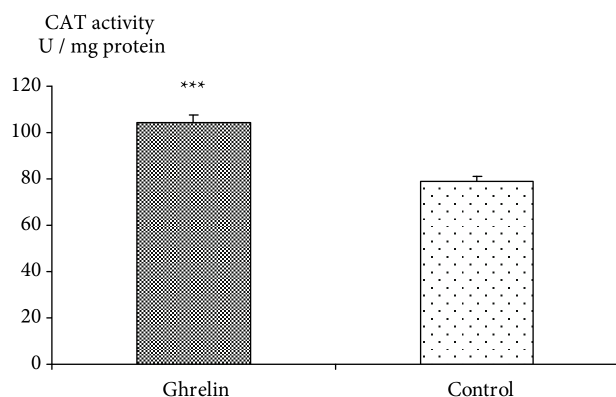


**Figure 2.** Effects of ghrelin on catalase activity in midgut tissue of *Lymantria dispar*. Bars represent the mean values ± SEM, n = 50. An asterisk denotes significant differences between groups at P < 0.001.

The ghrelin treatment was also associated with increased GST activity (1.0 ± 0.1 nmol GSH/mg protein/min) in comparison to the control (0.5 ± 0.08 nmol GSH/mg protein/min) (P < 0.01) (Figure 7).

## 4. Discussion

Ghrelin plays a crucial role in the regulation of appetite, food intake, and energy homeostasis in animals and humans. Our previous experiments showed the midgut as a place of ghrelin synthesis in *Lymantria dispar* (Perić-Mataruga et al., 2012b). Ghrelin, the obesity hormone, correlates to a significant increase in body weight; affects feeding, food intake, and locomotor behavior; shortens larval development; changes the nutritional indices; and increases midgut and fat body mass. Ghrelin treatment of the gypsy moth larvae increases the activity of the most important digestive enzymes (Perić-Mataruga et al., 2009, 2012a, 2012b). The increased body weight of *L. dispar* after ghrelin treatment was the consequence of increased food consumption and utilization, which was shown in ghrelin-treated larvae (Perić-Mataruga et al., 2012a). The efficiency of conversion of ingested food into biomass depends, among other factors, on the activity of digestive enzymes. Good antioxidative protection of the *Lymantria dispar* midgut structure and a wide range of digestive enzymes and nutrients are important after eating host plant leaves with potentially prooxidative effects (Perić-Mataruga et al., 1997, 2006, Mrdaković et al., 2013). Results of this paper showed that ghrelin affects antioxidative defense components in the midgut tissue and GST activity. Midgut tissue of *Lymantria dispar* larvae had increased activity and expression of SOD (Figures 1 and 3) and CAT (Figures 2 and 4). It is important for polyphagous and phytophagous insects, such as the gypsy moth, due to high prooxidative pressure after ingestion of food (leaves) with prooxidative potential, something that happens often in nature. SOD and CAT are classified as "primary antioxidants" and they work synergistically with the well-known "secondary antioxidants" such as glutathione and vitamins. Ghrelin increases mRNA levels of SOD in many tissues (Zvirska-Korczalaat al., 2007; Kheradmand et al., 2010). The strong expression of those enzymes could indicate a need for more efficient elimination of ROS produced after *L. dispar* overfeeding. The antioxidative enzyme activity in the midgut tissue and lumen prevents the peritrophic membrane and midgut epithelial cell damage caused by ROS that produce other sorts of free radicals and damage DNA, biomembrane, lipids, proteins and other macromolecules (Perić-Mataruga et al., 1997, 2000). Glutathione plays an important role in cellular defense against free radicals and their oxidant species. It functions by reaction with free radicals, followed by the formation of GSSG and other disulfides (Ahmad and Pardini, 1990;

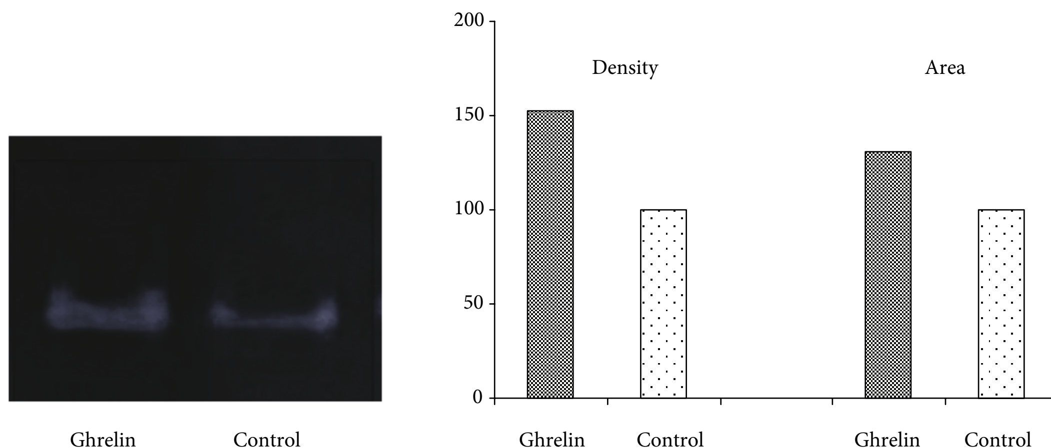


**Figure 3.** The profiles of superoxide dismutase electrophoresis gel bands of ghrelin-treated *Lymantria dispar* larvae (midgut tissue) and the control. Density and area of superoxide dismutase electrophoresis gel bands. Difference was expressed as the percentage of increase in the ghrelin-treated group compared to the control (100%).

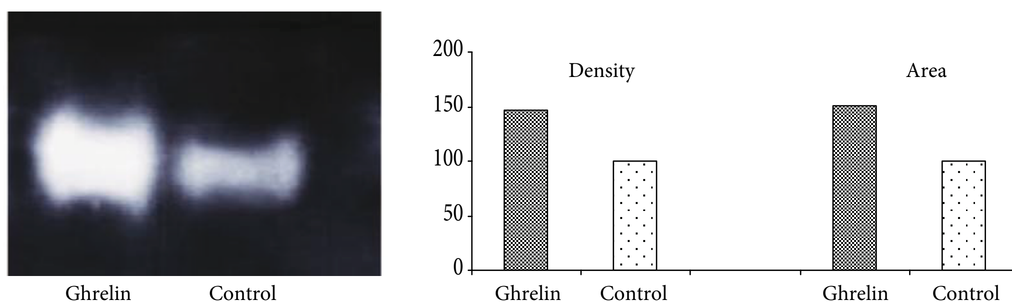


**Figure 4.** The profiles of catalase electrophoresis gel bands of the ghrelin-treated *Lymantria dispar* larvae (midgut tissue) and the control. Density and area of catalase electrophoresis gel bands. Difference was expressed as the percentage increase in the treated group compared to the control (100%).

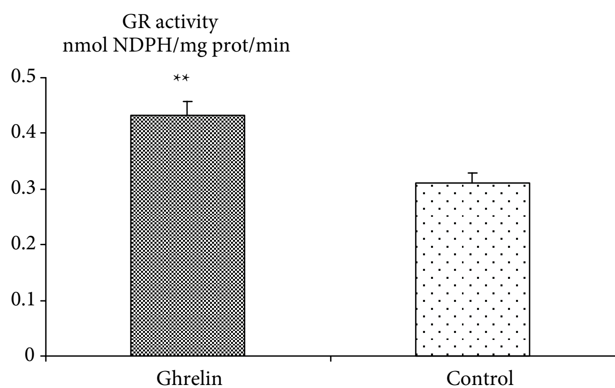


**Figure 5.** Effects of ghrelin on the amount of glutathione in the midgut tissue of *Lymantria dispar*. Bars represent the mean values ± SEM, n = 50. An asterisk denotes significant differences between groups at P < 0.01.

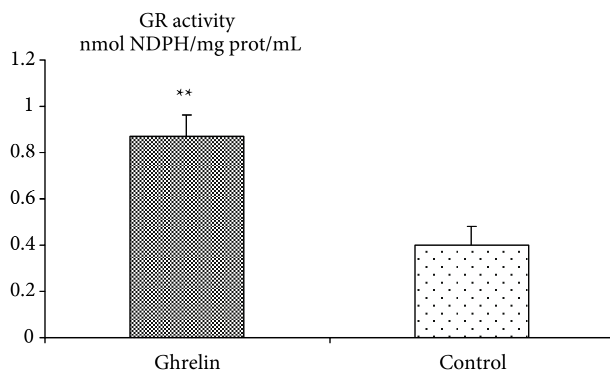


**Figure 6.** Effects of ghrelin on the glutathione reductase activity in midgut tissue of *Lymantria dispar*. Bars represent the mean values ± SEM, n = 50. An asterisk denotes significant differences between groups, P < 0.01.

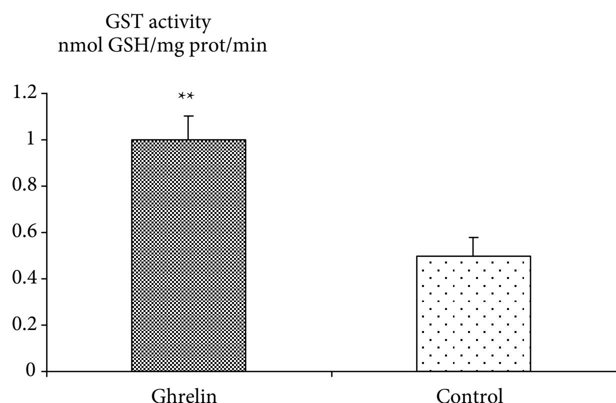


**Figure 7.** Effects of ghrelin on the glutathione S-transferase activity in midgut tissue of *Lymantria dispar*. Bars represent the mean values ± SEM, n = 50. An asterisk denotes significant differences between groups at P < 0.01.

Felton and Summers, 1995; Halliwell and Gutteridge, 2007). Glutathione content was higher in midgut tissue of ghrelin-treated *Lymantria dispar* larvae (Figure 5) and it helped with maintaining the redox homeostasis in insect midgut cells. GSH prevents damage to important cellular components caused by ROS, such as free radicals and peroxides. Scientific publications indicate that insects with a higher resistance to trophic stressors are characterized by an increased level of glutathione and higher SOD, CAT, GR, and GST constitutive activities in comparison to stress-susceptible organisms (Bi and Felton, 1995; Felton and Summers, 1995; Perić-Mataruga 1997, 2000; Mittapalli et al., 2007). In our experiments, we found that GR and GST activity was higher in ghrelin-treated *Lymantria dispar* larvae than in the control group (Figures 6 and 7). GR reduces GSSG and is thus irreplaceable in the regeneration of GSH necessary for the operation of many cell enzymes, including GST. The phase II biotransformation enzyme GST catalyzes the conjugation of GSH to a wide variety of xenobiotics, including prooxidants with an electrophilic site, yielding more water-soluble allelochemicals and facilitating their excretion. In the light of these results, it is reasonable to assume that ghrelin correlates to the protection of midgut tissue against prooxidative effects of host plant allelochemicals.

The present study demonstrated for the first time that ghrelin can promote antioxidant enzyme and GST activities and increase GSH content. Ghrelin increases feeding in the gypsy moth and simultaneously participates in improving antioxidative defense and GST activity by protecting the midgut structure from potentially prooxidative plant defense allelochemicals.

In conclusion, these results demonstrate the correlation between antioxidative strategy and ghrelin in the midgut tissue of *Lymantria dispar*. The results provided evidence for the application of insects as simple model systems in future studies of the underlying role of ghrelin in free radical protection in complex organisms.

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