Egypt. J. Chem. **Vol. 66**, No. 7 pp. 487 - 495 (2023)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Detection of Lysozyme and Antioxidant Enzymes in Normal and Immunized Haemolymph of the Silkworm, Bombyx moriDuring theImmature Stage

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Abstract

The activity of lysozyme(EC 3.2.1.17), as one of the immune system components of silkworm Bombyx mori L., was determined in the cell-free hemolymph of fifth larval instar by the lyso- plate assay using Micrococcus lysodeikticus. The lysozyme activity of the beginning newly larvae was 39 µg/ml, then increased to 248 µg/ml of hemolymph in mature larvae.The injection of Staphylococcus aureus and Escherichia coli into larvae resulted in an increase in lysozyme activity within 24 hours to 274 and 328 µg/ml, respectively. Antioxidant enzymes, including catalase(EC 1.11.1.6), peroxidase(EC 1.11.1.7), and ascorbate peroxidase(EC 1.11.1.11) have been detected also in hemolymph using isoenzyme native-polyacrylamide gel electrophoresis. The activities of catalase and ascorbate peroxidase isoenzymes were increased with time progress, especially at the wonder stage while, a slight increase in peroxidase activity was observed.

Keywords: Bombyx, Lysozyme, Antioxidant enzymes. Insect immunity.

1. Introduction

Insects and mammals, both have an ancient innate immune system, and while insects lack an adaptive immune system, they do have effective infection-fighting capabilities.Insects can be found in almost every known habitat and ecological niche, they are exposed to a wide range of infectious pathogens as a result of their variety.In insects, the innate immune system is made up of two central and several peripheral tissues. The fat body, which combines the functions of the vertebrate liver and adipose tissue, is the central tissues, while hemocytes are one of the impotent peripheral tissues.The fat body's primary job in the immune system is to release soluble substances into the hemolymph [1].

The basic strategy of insects defense mechanism by three types; physical barriers, cellular and humoral responses [2, 3]. The integument (body wall) as well as the peritrophic membrane, which covers the insect midgut, are both physical barriers. It protects against diseases by acting as a physical barrier. However, because this barrier is semipermeable, some microbes can pass through it [4, 5]. These structures provide the first line of defense against pathogens, the humoral and cellular immune responses are initiated when bacteria pass through these barriers.Invading microorganisms are first destroyed by hemocytes, and then the few germs that are not cleared by cells are eliminated by humoral responses [6].The humoral response is characterized by the production of antimicrobial peptides (AMPs) and the generation of reactive oxygen species (ROS) in the hemolymph [7, 8].All types of AMPs have been isolated in lepidopteran and dipteran insects (cecropins, defensins, attacin, gloverin, proline-rich peptides, and lysozymes) these peptides are mainly synthesized by the fat body and hemocytes then released into the hemolymph [9]. Lysozyme is a 1,4-N-acetylmuramidase that catalyzes the hydrolysis bond in the peptidoglycan component of bacterial cell

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EJCHEM use only: Received date 13 December 2021; revised date 26 December 2021; accepted date 28 December 2021
DOI: 10.21608/EJCHEM.2021.111076.5058

walls, causing cellular membrane integrity to be compromised and cell death [10].It can be found in insects, animals, plants, and microbes all across the world [11].Lysozymes have antifungal and antibacterial properties against both Gram-negative and Gram-positive bacteria [12].Lysozyme has been discovered as a normal component of the serum in lepidoptera[13, 14] and is thought to play an essential function in defence mechanisms. Injecting bacteria into an insect boosts lysozyme activity in the hemolymph [13].

ROS such as hydrogen peroxide and superoxide anion, drive signal transduction and mediate a variety of reactions, including cell proliferation and apoptosis [15], and are beneficial to an insect's innate immunity system [16, 17].ROS, on the other hand, are toxic to living organisms and cause oxidative damage to biomolecules including proteins, nucleic acids, and lipids when they exceed their physiological limits [18].Antioxidant defense plays an indispensable role to prevent the oxidative damage of ROS. In Bombyx mori, like many other insects, has antioxidative defence systems that are both enzymatic and non-enzymatic.The recent study aims to elaborate on the changes in lysozyme activity exist in the hemolymph of vaccinated and non-vaccinated Bombyx larvae along with the activity of the first line defense antioxidant enzymes include catalase, peroxidase and ascorbate peroxidase.

2. Materials and methods

2.1. Insects

Silkworm eggs of the local hybrid Bombyx mori, was provided by The Sericulture Research Department of the Plant Protection Research Institute, Agricultural Research Centre, Giza, Egypt, provided silkworm eggs of the local hybrid B. mori. Silkworms were raised on mulberry leaves in the laboratory at a temperature of 25-28°C and a relative humidity of 75-85 %. After the tenth day of the fifth larval instar, larvae began to wander, the developmental phases were represented by the periods (in days).

2.2.Larvae, immunization and hemolymph collection

The last instar of ten larvae were used in the third day. Each larva was immunized with log phase Escherichia coli and Staphylococcus aureus suspended in 10 µl of previously autoclaved 10 mM sodium phosphate buffer (pH 7.4).Hemolymph was collected immediately 24 hours after bacteria injection into sterile tubes containing a few phenylthiourea crystals and centrifuged at 10,000g for 10 minutes to eliminate hemocytes and cell debris. Cell-free hemolymph samples from 10 larvae were utilised right away or kept at −20 °C until used.

2.3.Lysozyme activity assay

For lysozyme activity, the lysoplate assay was utilised, as described by [19]. Four ml of 1% agarose gel were diluted in 0.07M sodium phosphate buffer, pH 6.2, containing Micrococcus lysodeikticus and layered on defatted 7.5 x 2.5 cm glass slides.The agarose gel was punched with 4mm diameter wells, and 10µl of lysozyme-containing samples or standards were applied.The slides were then incubated for 16-18 hours at 37°C in a humidified chamber before being washed in distilled water for 30 minutes, pressed under filter, totally air dried, flame fixed, and stained with Coomassie blue R-250 before being read. Serial two-fold dilutions of the original concentration of 0.5 g/ml of hen egg white lysozyme (Boehringer) were used to create the standard curve. The standards were stabilised in phosphate buffer saline containing 1% bovine serum albumin.

2.4.Catalase, peroxidase and ascorbate peroxidase activities detection

For detection of catalase (CAT), peroxidase (POX) and ascorbate peroxidase (APX) activities, native polyacrylamide gel electrophoresis (Native-PAGE, 7.5% T) was applied using the [20] discontinuous buffer systems at 4˚C and Bio-Rad Protean II electrophoresis Cell. Before loading onto the gels, samples were mixed with 10% glycerol (v/v) and 0.25 % bromophenol blue and10µl of each sample were added to each lane. The electrophoresis was run at a constant current of 35 mA at 4°C. After electrophoresis gel containing 0.5 % soluble starch was stained for catalase activity following the staining procedure of [21]. The gel was incubated for 30 seconds at room temperature (25 ˚C) in a solution containing 18 mmol/L sodium thiosulphate and 679 mmol/L H2O2.Then gel was washed with distilled water before being flooded with a potassium iodide solution containing 90 mmol/L and acidified with 0.5 % glacial acetic acid. On the brown background of the gel, negative bands of CAT enzymes were developed. For POX activity detection, after electrophoresis gels were soaked in a solution containing 2 mM O-dianisidine(Sigma) and 10 mM potassium phosphate (pH 7.2) for 45 min.The gels were subsequently immersed in a 0.006 % H2O2 solution, within 15 minutes, heavy brown bands developed.Gels were incubated for 24 h at 22°C before the reaction was considered negative [22]. For APX activity, after electrophoresis gels containing 1.1mol/L sorbitol and 1mmol/L Na-ascorbate were

stained using modified [23]. Gels were rinsed twice with 10 mmol/L potassium phosphate (pH 6.0) before being incubated for 15 minutes at room temperature with 4 mmol/L Na-ascorbate and 4 mmol/L H_2O_2 in the same buffer. Gels were rinsed in water and incubated with shaking for 3 minutes. After that, the gels were immersed in a solution containing 2.4 mmol/L potassium ferricyanide and 6.2 mmol/L ferric chloride.On a Prussian blue background, APX stained negatively.

3. Results

3.1. Changes in lysozyme activity

In Figure (1), the diameter of the clear zone, which ranges from 0.7 to 25 mm, is proportional to the logarithm of enzyme concentration, as shown in the standard curve [24]. The calculated R^2 of 0.73 indicates that the assay is highly reproducible.

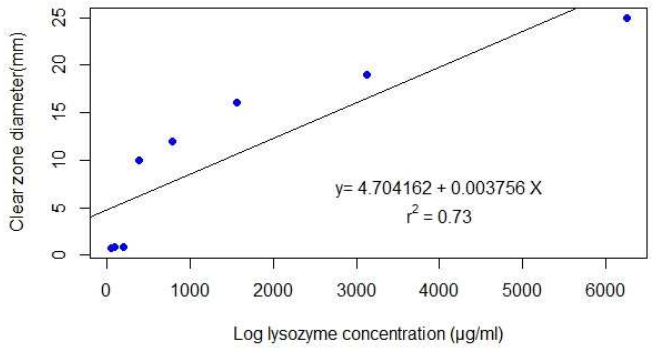

Figure 1: The relationship between the logarithm of known concentrations of chicken lysozyme and the clear zone diameter was measured in millimetres

The activity of lysozyme in non-immunized hemolymph was measured in developing silkworm larvae during the last instar (Fig. 2). At the beginning of the fifth larval instar, after the ecdysis immediately (zero time), the lysozyme activity was low and its concentration was 39.6 µg/ml until the eighth day, 78.3 µg/ml, then suddenly the activity sharply increased at the ninth day to reach approx. a level three times as high. The high level of activity was maintained until pupation, i.e., 248 µg / ml in concentration. After injection of Staphylococcus aureus (Gram-positive bacteria) the level of hemolymph lysozyme concentration elevated to 274 µg/ml, versus 328 µg/ml in case of Escherichia coli (Gram negative bacteria) injected into larvae as shown in (Fig.2).

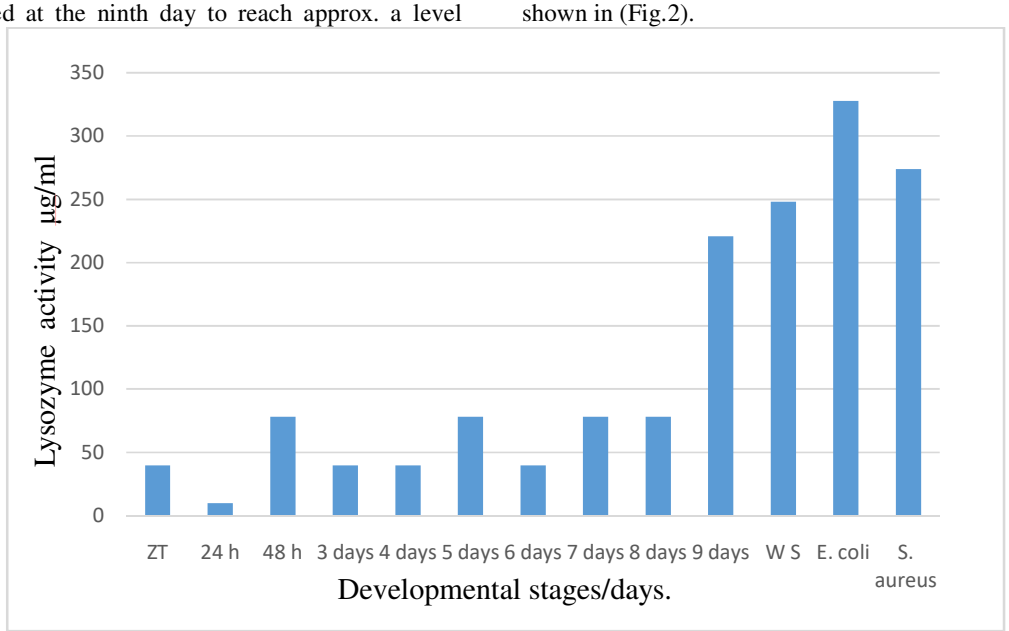

Figure 2: Lysozyme activity in the hemolymph of B. mori 5th instar, ZT, zero time; Days 1,2,3,4,5,6,7,8,9 and wonder stage, respectively. Induction of lysozyme activity by Escherichia coli and Staphylococcus aureus, respectively

3.2. Isoenzymes activity assay

In order to monitor the changes in physiological status of Bombyx mori larva due to different treatments and interval times, other molecular technique was applied. It is known as isoenzyme activity assay.

3.2.1. CAT activity

Figure (3) shows CAT isoenzyme activity assay on native-PAGE at different times. Patterns of separated enzyme proteins on gels revealed the presence of three CAT variants isoenzymes (a, b&c). The dominant isoenzyme was variant B in all samples. The intensity of each type isoenzyme band, i.e., the activity of isoenzyme, was varied considerably at different times. It was noticed that the activity of isoenzyme B was lower at zero hr, 24 hr, 48 hr, 3, 4, and 5 days, then increase in its activity was noticed until wonder stage. Isoenzymes A & C had the same behaviour as that of isoenzyme B. This result revealed that with time progress, there was an increase in CAT isoenzyme activities especially at wonder stage.

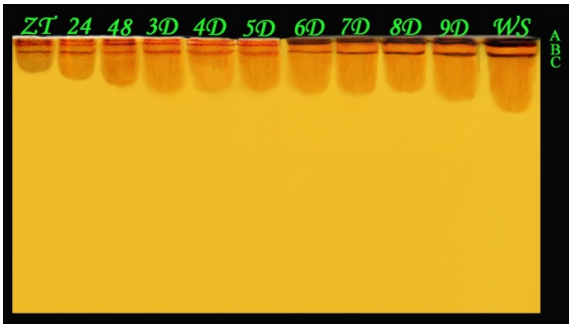

Figure 3: Catalase isoenzymes activity patterns of the hemolymph of B. mori 5th instar, lanes 1-11: ZT, zero time; Days 1,2,3,4,5,6,7,8,9 and wonder stage, respectively

3.2.2. APX activity

APX isoenzyme activity assay on native-PAGE at various intervals are shown in Fig. (4). Two APX variants isoenzymes (a &b) were identified on the gel. The isoenzymes A activity was lower at both zero time and 24 hr then increase in activity at 2th day was noticed until the wonder stage.It was clear that isoenzyme B activity begins at 6th day and increased with time progress, especially during the wonder stage. It was noticed also that, in case of CAT and APX isoenzymes, variant B was dominant at this time, which means that this type of isoenzyme might play an important role and could be used as a biomarker for monitoring changes occurred at that stage.

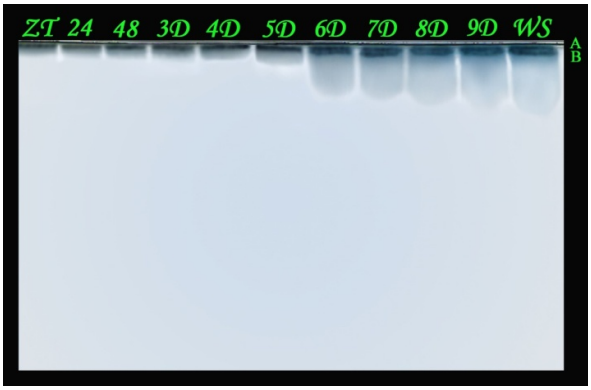

Figure 4: Ascorbate peroxidase activity patterns of the hemolymph of B. mori 5th instar, lanes 1-11: ZT, zero time; Days 1,2,3,4,5,6,7,8,9 and wonder stage, respectively.

3.2.3. POX activity

Figure (5) shows POX isoenzyme activity assay on native-PAGE at different times. In all patterns, three POX isoenzymes were recognized on the gel. They were named A, B & C. It was noticed that the intensity, i.e., activity of POX isoenzyme A & B bands were higher in all samples than isoenzyme C. The dominant isoenzyme was POX variant A. No distinguished differences among POX A, B &C isoenzymes activities through interval times, however, there was a slight increase in POX isoenzyme activities especially at wonder stage.

Figure 5: Peroxidase activity patterns of the hemolymph of B. mori 5th instar, lanes 1-11: ZT, zero time; Days 1,2,3,4,5,6,7,8,9 and wonder stage, respectively.

4. Discussion

4.1. Detection of lysozymes in the hemolymph

Many researchers have been studied the defence mechanisms of insects since the beginning of the century. The results of the present study showed that the lysozyme activity of non-immunized Bombyx hemolymph ranged from 39.6 to 248 µg/ml, confirming the findings of certain researchers who discovered that non-vaccinated hemolymph from different insects can kill bacteria [25, 26, 27]. According to [28], the lytic zone formed by the silkworm enzyme is substantially greater than that produced by the egg-white lysozyme, when examined at the same concentration at 280 nm.[13] showed the purified Bombyx lytic enzyme to be a small basic protein similar to hen egg-white lysozyme with properties such as stability to heat, acidic pH but have specific activity about six times that of egg-white lysozyme.Except for Manducasexta larvae [29], which had very low lysozyme activity, the activity levels are similar to those reported with the hemolymph of various lepidopteran insects [13, 28, 30, 31]. From the beginning to late 5th larval instar (at day 8), lysozyme activity in Bombyx hemolymph was maintained at a steady level, then suddenly increased to three times its previous level before the spinning stage. This level of activity is comparable to what has been reported [30], it is important to note that the rapid rise in activity happens right before the wandering period, which is when the risk of damage and infection is greatest. When no bactericidal activity is found in the normal, non-vaccinated hemolymph, substantial increases in lytic activity can be rapidly induced by injecting bacterial vaccine, either specific or unrelated, endotoxin, saline, or India ink into the larvae or pupae [32, 33, 34, 35, 36]. Injection of Escherichia coli into the third day of the last instar elevated lysozyme activity to a level of 328 µg/ml, this result agreed with [28].They discovered that E. coli must be killed by at least two elements, the lysosome-like enzyme is one, while the anionic cofactor with a lower molecular weight is the other. [34] demonstrated that the factor in wax moth (Galleria mellonella) larval hemolymph that kills Pseudomonas aeruginosa, Gram-negative, rapidly increased by injection of homologous vaccine, that it reaches a maximum value around 20 to 24 hrs and then rapidly drops by 36 to 48 hrs. The most significant functions of lysozyme, which is abundant in normal hemolymph at the last larval stage, is to release peptidoglycan fragments from invading bacteria that have been destroyed by phagocytosis or direct lysozyme activity, according to [37]. These generated peptidoglycan fragments operate as a signal molecule for immune gene activation. The lytic zone produced by the injection of Staphylococcus aureus (Gram-positive bacteria) in this paper was increased to 248 µg/ml. Confirming

with [38] who investigated the lysozyme activity dramatically increased by the injection of Gram-positive bacteria in the hemolymph of silkworm pupae. Also, lysozyme activity was induced in the hemolymph of wax moth and silkworm prepupae after injection of bacterial vaccination, according to [13].

4.2. Antioxidant enzymes activities

In this study, the antioxidant enzymes, such as CAT, POX, and APX activities of all developmental stages in the hemolymph of last instar were monitored by polyacrylamide gel electrophoresis. The electrophoretic separation of CAT and APX patterns in this work had a similar characteristic that increased with the interval times and reached the peaked at the wonder stage. However, the peroxidase activity patterns remained consistent throughout the last larval instar, with a minor rise at the wonder stage; the explanation for this stability is unknown. In insects, several enzymes such as CAT, POX and APX play significant roles in immunity, avoiding free-radical-associated damage, and protecting cells from harmful environmental effects [39, 40]. The physiological and metabolic transformations occurring in insect tissues are reflected in the variations in haemolymph composition [41]. Studies on the expression and activity of antioxidant enzymes in insects reveal that the cellular redox state controls a wide range of physiological activities, including survival, growth, cell damage, fecundity, adult life span, and fertility [42, 43]. (ROS), particularly H_2O_2, are crucial regulators of cell differentiation, development, and diapause, as well as a variety of physiological functions. H_2O_2 plays a dual role in the cellular environment, it serves as a pro-oxidant due to its oxidative properties, and it modifies signalling pathways due to its activity as a second messenger [44]. Though the role of ROS in the development process has been widely documented, most studies have been limited to in vitro effects of ROS or based on cell culture data, with significantly fewer investigations carried out on developing embryos or larvae. Even so, a few researches imply that oxidants and antioxidants play a role in insect growth and diapause.The presence of an antioxidant defence system protects the cell from ROS-mediated oxidative assaults under normal conditions, an array of antioxidant enzymes, make up the antioxidant defence system [45]. Silkworms, like other insects, have antioxidative defence systems that are both enzymatic and non-enzymatic in nature, catalases, peroxidases and ascorbate peroxidase are the most important antioxidant enzymes, whereas glutathione, tocopherols, and ascorbic acid are non-enzymatic components [46, 47]. H_2O_2 on the other hand, must be detoxified by catalase or peroxidases because it might change into a highly reactive hydroxyl radical (OH) in the presence of reduced metal atoms [48]. Catalase is a tetrameric enzyme that transforms H_2O_2 to water and oxygen without producing additional reactive oxygen species. It is a powerful enzyme that can degrade millions of hydrogen peroxide molecules in a single second [48]. Ascorbate peroxidase catalyses the oxidation of ascorbic acid while simultaneously reducing hydrogen peroxide H_2O_2 [49, 50]. While ascorbate can destroy H_2O_2 non-enzymatically but, enzymatic elimination of H_2O_2 would be more efficient. The ascorbate peroxidase was purified from the larvae of Helicoverpazea and investigated its activity relatively high in the fat body [51]. As part of the immunological response to infections, high quantities of ROS can be created suddenly [52]. Catalases, according to [53], plays critical role for tolerance to oxidative stress induced during fungal growth. The catalase activity was reduced in pebrine-infected silkworms [54]. The level of catalase activity changed in silkworm Bombyx mori under thermal stress condition according to [55, 56]. The catalase activity and total protein concentrations in the haemolymph of silkworm Bombyx mori infected with a fungal disease had lower trends than the control groups [57, 58].

5. Conflict of Interest Statement

The authors affirm that they have no known financial or interpersonal conflicts that would have appeared to have an impact on the research presented in this study.

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