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Study of Inhibitory Effect of Certain Chemicals on Phenoloxidase (PO) of *Antheraea assama* Ww.

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ABSTRACT

The enzyme phenoloxidase (PO) is an important component of insect defense mechanism. Successful inhibition of PO activity could be effective against insects and be used in their biological control. The present study was therefore conducted to observe inhibition of PO activity on the Muga silkworm Antheraea assama Ww. Five different inhibitors, Benzoic acid, Sodium azide, Sodium sulphite, Thiourea and Dithiothretol (DTT) were used at 1mM, 2mM, 3mM and 4mM concentration to see their inhibitory effect on L-DOPA oxidation by PO. It was found that with increasing concentration, all the tested inhibitors exhibited decrease in PO activity, maximum inhibition being recorded at 4mM concentration. Among the inhibitors, DTT resulted in maximum inhibition followed by Thiourea and Benzoic acid. Comparison of different inhibition on enzyme activity at 4mM concentration too proved DTT to be the best inhibitor (2.02±0.81) whereas Sodium sulphite exerted the least inhibitory effect (6.21±1.17).

Key words: *Antheraea assama, Benzoic acid, Sodium azide, Sodium sulphite, Dithiothretol*

INTRODUCTION

Insects are by far the most abundant animals in the world. One of the main reasons of their abundance is their success in adapting themselves to varying conditions, which is largely supplemented by their immuno competence against stress and challenges of various kinds.

Insect immunity consists of both cellular and humoral mechanisms[4,8]. Cellular immunity is mediated by haemocytes through phagocytosis, encapsulation and nodule formation[8]. Humoral mechanism on the other hand includes various Antimicrobial peptides (AMP), Pattern Recognition Proteins (PRP) and the Phenoloxidase (PO) system.

Among the innate immune system factors, PO is critical for the insect's defence mechanism[1]. It is involved in multiple processes, such as cuticular sclerotization, melanization and wound healing. Sclerotized cuticle have been reported to block pathogen entry into the body, while melanization destroy invading pathogens[2]. PO which is a copper-3-polypeptide[2] is usually synthesized and released into the haemolymph as the inactive zymogen Pro-phenoloxidase (PPO).

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Once activated (by challenges) PO catalyses the initial step of the melanin biosynthesis pathway by hydroxylation of monophenols into o-diphenols and then further oxidation of p-diphenols into o-quinones. o-quinones can non-specifically crosslink neighbouring molecules to form insoluble melanin [12]. Quinones generated by PO go through a cascade of enzymatic and non-enzymatic reactions leading to polymerization. Further, quinones crosslink with cuticular structural proteins and chitin which results in the hardening of the cuticle [6]. The hardened cuticle provides the first line of defence against external challenges, melanization around invading pathogens provide a further mechanism of immunity[15,7].

So, it can be hypothesised that enhanced PO activity could provide the insects with protection against invading pathogens and inhibition of PO activity could render them susceptible to external challenges. Therefore to test this hypothesis the present study aims to inhibit PO activity on a test insect using selected inhibitors (copper-chelators). Such inhibitions, if proven to affect the insects immune system, by inhibiting the PO activity could pave the way for the development of a novel method of insect control in future.

With this objective we have selected the Muga silkworm *Antheraea assama* Ww, a sericigenous insect, native to the North-East region of India[3] as our test insect. Because of its easy availability and bigger size, collection of adequate amount of histological samples for the study, will be easier.

MATERIALS AND METHODS

Insects: Healthy and disease free Muga silkworm (*A. assama* Westwood) larvae of 5th instar reared on Som plants (*Machilus bombycina* King) were collected from the Central Silk Board Farm, Boko, Guwahati, Assam.

Collection of haemolymph: Haemolymph from the larvae were collected by excising one of the prolegs and immediately diluted with an anticoagulant (containing trisodium citrate, P^H = 7.0) and with a buffer (TrisHcl, P^H = 7.1) in the ratio of 1:1:1 and homogenized in an ice cooled potter homogenizer and the lysate were centrifuged at 17000rpm for 30 minutes. Supernatant were stored at -20°C freezer.

Chemicals: TrisHcl buffer (pH = 7.1), trisodium citrate, 10% CPC (cetylpyridinium chloride), L-DOPA(dihydroxyphenylalanine) of 6mM concentration to be used as substrate along with the inhibitors Sodium Sulphate, Sodium Azide, Benzoic Acid, Thiourea and Dithiothretol were procured from HIMEDIA. All the chemicals were kept under proper storage conditions during the period of the work.

Inhibition Study: Five copper-chelators, viz. Benzoic acid, Sodium azide, Sodium sulphate, Thiourea and Dithiothretol, at different concentrations (1mM, 2mM, 3mM and 4mM) were tested for their inhibitory effect on L-DOPA oxidation by PO in the haemolymph of *Antheraea assama*, following Goudru *et al*.[5] with slight modifications. 6mM L-DOPA concentration was taken as substrate for the inhibition process as maximum PO activity occurs in this concentration[5].

A solution containing 20µl of haemolymph supernatant obtained from the stored sample after homogenization and centrifugation at 17000rpm, containing PPO was incubated for 5min with 10µl of 10% CPC to obtain the active enzyme PO. To this, 940µl of buffer with specific concentrations of one particular inhibitor was added and incubated for 10min at 30°C. After adding the substrate (6mM L-DOPA), the reaction was triggered and absorbance was recorded for 2min under standard assay conditions. Same procedure was followed for all the inhibitors at different specified concentrations (1mM, 2mM, 3mM and 4mM).

The absorbance was measured at 475nm and the enzyme activity was calculated by using the following formula-

$$b = \frac{\Delta A \times V}{E \times d \times v \times \Delta t} \times d.f$$

where, ∆A= change in absorbance

V= total volume of assay mixture

E=extinction coefficient ($3.6mM^{-1}cm^{-1}$)

d=light path

∆t=time for which the change was observed (in minute)

V=volume of sample (in µl)

d.f=dilution factor

Statistical Analysis: Experimental data for PO activity as affected by inhibitors were analyzed using one way analysis of variance (ANOVA). Significance of inhibition by the different inhibitors was tested at $p \leq 0.05$ determining the LSD.

RESULT

In the present study, inhibition of PO activity was observed with all the 5 different inhibitors viz. Benzoic acid, Sodium azide, Sodium sulphite, Thiourea and Dithiothretol at 1mM, 2mM, 3mM and 4mM concentration. The inhibition was determined in terms of their inhibitory effect on L-DOPA oxidation by PO. 6mM L-DOPA concentration was used as a substrate for the inhibition process as it has been reported that maximum PO activity occurs in this concentration[5].

Our results showed that in 6mM DOPA all the inhibitors at their different concentrations showed active inhibition of PO activity, albeit at different rates (Table 1). All the tested inhibitors exhibited a linear decrease in PO activity with increasing concentrations; being highest at 4mM (Table: 1). Dithiothretol (DTT) was observed to show the maximum inhibition followed by Thiourea and Benzoic acid.

Table 1: Effect of selected inhibitors on enzyme activity at different concentration

Inhibitors	1mM	2mM	3mM	4mM
Benzoic acid	7.02±1.82	6.42±0.90	5.85±1.09	5.76±0.54
Thiourea	5.53±1.68	4.68±1.29	4.41±1.16	4.05±1.33
Sodium sulphite	6.39±1.89	6.25±1.01	6.21±1.61	6.21±1.17
Sodium azide	7.52±2.16	7.47±1.16	6.07±1.82	5.80±1.58
Dithiothretol	5.85±1.55	3.29±1.15	3.19±0.54	2.02±0.81

* Enzyme activity (Mean ± SD)

Table 2: Comparison of the effects of different inhibitors on enzyme activity at 4mM concentration

Inhibitors	4mM conc.
Benzoic acid	5.76±0.54a
Thiourea	4.05±1.33a
Sodium sulphite	6.21±1.17ac
Sodium azide	5.80±1.58a
Dithiothretol	2.02±0.81b

*Same alphabets at the end of the numbers indicate no difference

DTT exhibited the best inhibitory effect particularly at 4mM concentration (2.02±0.81). At lower doses too DTT proved to be more effective as an inhibitor (5.85±1.55 at 1mM, 3.29±1.15 at 2mM and 3.19±0.54 at 3mM) than the others (Table 1). Thiourea too followed the same linear pattern of inhibition from lower to higher concentration; best effect

being at 4mM concentration (4.05±1.33). Benzoic acid did not show a reduction in enzyme activity with an increase in molar concentration although a gradual decrease in PO activity was evident from 1mM to 3mM concentration. Incidently no differences could be interpreted between 3mM and 4mM concentrations indicating an optimal range of inhibition not exceeding 4mM concentration. Sodium azide and Sodium sulphite showed the least inhibition among the five inhibitors used.

Oneway ANOVA performed on the data set obtained from the study helped in the comparison of PO inhibition potential of all the inhibitors at 4mM concentration (highest inhibition as suggested by the results). The comparison proved DTT to be the best inhibitor among the five tested (Table 2), followed by Thiourea, Sodium azide and Benzoic acid, which among them showed no significant variation in their inhibitory potential. Sodium sulphite proved to exert the least inhibitory effect among all the inhibitors.

DISCUSSION

The results suggest the inhibitory potential of all the inhibitors tested. All the tested inhibitors exhibited a linear decrease in PO activity with increasing concentrations. Similar trends in PO activity have also been reported by Popham *et al.*[13] working on *Heliothis virescens*. As insect PO is a typical copper-3-protein, it is not surprising that copper-chelators (the tested inhibitors) would prove to be good inhibitors. Similar justifications were offered by Lerner *et al*.[9] and Prabhakaran *et al*.[14] working on *Mycobacterium leprae*. The chelating properties of such inhibitors have also been reported by Li and Kubo[10]. The inhibitors used in the study, viz, DTT, Benzoic acid, Thiourea, Sodium azide and Sodium sulphite are all reported to be competitive inhibitors. Therefore they compete with the substrate for binding with the active sites of the enzyme (PO). Increased concentration of the same enables them to outcompete the substrate in terms of binding at the active sites. The substrate failed to bind to the active sites, enzyme-substrate complex formation reduces and as a result enzyme-substrate reaction goes down exhibiting less enzyme activity in turn.

Another explanation regarding inhibition of PO activity may be cited from the findings of Lu *et al.*[11]. According to their reports, PPO of most insects have one or two disulfide bonds at the c-terminus. Deletion of the disulphide bonds prove to decrease or inhibit PPO activity greatly. The same argument may also be cited in support of our results, although tentatively. It is probable that the inhibitor used in our study, in addition to blocking the active sites of the enzyme (PO), might also affect the disulfide bonds leading to the observed reduced activity of the enzyme. However to prove this hypothesis, further extensive research on inhibition mechanism of PO is absolutely essential.

CONCLUSION

The results of our study conclusively prove that PO inhibition by synthetic inhibitors is possible. This information in all probability has the potential to lead to the development of a novel way of environment friendly, non-toxic insect control mechanism in the near future.

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