Journal of Asia-Pacific Entomology 19 (2016) 1197–1202

Contents lists available at ScienceDirect

Journal of Asia-Pacific Entomology

journal homepage: www.elsevier.com/locate/jape

Spermidine alleviates oxidative stress in silk glands of *Bombyx mori*

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ARTICLE INFO

Article history:
Received 27 April 2016
Revised 21 October 2016
Accepted 28 October 2016
Available online 31 October 2016

Keywords:
Bombyx mori
Spermidine
Silk gland
Development
Antioxidant

ABSTRACT

Silk gland is a specialized organ for the synthesis of silk. Spermidine is a natural polyamine present in all living cells. It gained attention recently due to its role in longevity and growth promotion. Our earlier study reported enhancement in silk production after spermidine treatment during the 5th instar larval stage of *Bombyx mori* hybrid strain CSR2 × CSR4. Oxidative stress increases during the degeneration of silk glands in spinning stage. Spermidine plays an important role in relieving oxidative stress. Results showed that exogenous administration of natural polyamine, spermidine, significantly increased the metabolic activity and reduced the oxidative stress of the silk gland in the pre-pupal stage. Expression analysis also confirmed increase in metabolic activity of silk glands after spermidine treatment by showing enhancement in *MYC* gene expression. Finally, our results demonstrate that supplementation of spermidine enhanced cell viability and decreased oxidative stress of the silk glands. This work also opens up the idea of feeding antioxidants to silkworms which would be beneficial to the sericulture farmers.

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1. Introduction

Silk gland is an organ specialized for the synthesis and secretion of silk proteins (Zhang et al., 2006). Silk glands constitute approximately one-quarter of the worm mass and produce liquid silk comprising of complex proteins like fibroins, sericins, protease inhibitors and many other unknown proteins which show dynamic expression (Dong et al., 2013). Programmed cell death (PCD) is a very essential active process for the maintenance of animal homeostasis. In silkworms, larval organs such as the intersegmental muscles and silk glands degenerate and new adult structures develop (Lockshin and Williams, 1965; Terashima et al., 2000; Goncu and Parlak, 2008). Many apoptosis and autophagy related genes were identified in the silk glands of *Bombyx mori* (*B. mori*) (Zhang et al., 2010; Li et al., 2010). Apoptosis and autophagy are responsible for larval to pupal metamorphosis in the larvae of *B. mori* (Harinatha Reddy et al., 2014).

Efficient antioxidant scavenging system maintains the normal metabolism and protects the cells, and this potential decreases with age (Mehlhorn, 1994, 2003; Venugopal Reddy et al., 2016). Exogenous feeding of antioxidants caused longevity in *Callosobruchus maculatus* F (Garg and Mahajan, 1993). Spermidine (Spd) supplemented food was shown to increase life span of yeast, worm, fly and human cell lines (Eisenberg et al., 2009). Spd administration decreased oxidative damage, increased resistance to heat and H_2O_2 in yeast (Eisenberg et al., 2009). Spd was shown to promote longevity of many species in an autophagy dependent manner. Our study reported 31% enhancement in silk production by *B. mori* silk worms after Spd feeding (Gayathri et al., 2014).

In the present study, we validated the positive effect of Spd on *B. mori* (CSR2 × CSR4) silkworms. The effect of Spd on various parameters related to oxidative stress was measured in silk glands during 5th instar larval development and spinning stages. Metabolic activity, free radical status, total antioxidant potential along with superoxide dismutase (SOD) and Catalase (CAT) activity were investigated in the silk glands of *B. mori* during 5th instar and pre-pupal stages of development.

Metabolic activity, free radical scavenging activity and total antioxidant potential showed maximum values on day 7 of 5th instar silk gland. These values decreased on day 9 of spinning stage. Upon Spd feeding, the metabolic and antioxidant levels in silk glands were found to be significantly high in treated group when compared to the control group during 5th instar stage and spinning stage. SOD and CAT activity also increased in the Spd treated group. From the present work it can be concluded that Spd treatment enhanced metabolic activity of silk gland and reduced the oxidative stress in pre-pupal (spinning) stage. Expression analysis also confirmed increase in metabolic activity of silk glands after Spd treatment by showing enhancement in *MYC* gene expression.

2. Materials and methods

2.1. Standard polyamine

Spermidine free base (RM 5438) was purchased from Himedia chemicals.

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http://dx.doi.org/10.1016/j.aspen.2016.10.014
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2.2. Collection of silkworms

The larvae of *B. mori*, cross-breed strain (CSR2 × CSR4) were obtained from Andhra Pradesh State Government Sericulture Center, Srikakulam, Andhra Pradesh, India. The larvae were brought in third instar stage and grown till 5th instar stage and were reared under standard conditions of 26 ± 2 °C with a relative humidity (RH) of 80–85%.

2.3. Experimental design

The 5th instar larvae on day 1 were divided into two groups, one group was taken as control and fed with normal leaf and the other group was fed with Spd (50 μM) sprayed leaves till day 7 of 5th instar. Concentration of Spd 50 μM was finalized for our present study as it gave significant increase in the gland weight compared to the other Spd concentrations (Gayathri et al., 2014). In control and treated groups, triplicates were kept with 30 worms in each. Larvae were dissected on every alternate day to isolate the silk glands. Isolated silk glands were subjected for weighing and the total gland weights were recorded for both control and treated groups. The larvae were allowed to spin cocoons for both control and treated groups, on day 13 the cocoons were harvested and subjected for cocoon weights. The experiment with the above setup was repeated three times with different egg layings of *B. mori* silkworms.

2.4. Cell viability assay (MTT assay)

MTT assay was carried out with silk glands as per the published protocol (Nath et al., 2005), but with few modifications. In brief, five randomly selected larvae of both control and treated groups were dissected in insect ringer solution (0.68% NaCl). Isolated silk glands were weighed and kept in microfuge tubes with 900 μl of insect ringer solution and 100 μl of MTT (5 mg/m1). Tubes were incubated for one hour in dark. After incubation, the glands were washed with insect ringer solution and extracted with 1 ml of 50% (v/v) tween-80 for 24 h in dark. After incubation the formazan product was collected and the total volume was made up to 3 ml with distilled water. Optical density was measured at 570 nm using colorimeter (Elico, CL 157, India).

2.5. DPPH and FRAP assays

DPPH assay was carried out in accordance with the published protocol (Zhao et al., 2006). Silk glands of control and treated groups were homogenized in 1 ml of chilled, 0.1 M phosphate buffer (Na_2HPO_4 and KH_2PO_4, pH 7.4) and centrifuged at 10,000 rpm for 30 min. To the 200 μl of gland homogenate, 800 μl of 0.1 mM DPPH (in ethanol), was added and incubated for 30 min in dark (A_s). For negative control, only ethanol was taken and for positive control, ethanol mixed with DPPH was taken (A_c). The absorbance was measured at 520 nm. From the absorbance readings, % Inhibition of free radicals was calculated as, % Inhibition = $(A_c - A_s) / A_c \times 100$.

Ferric reducing antioxidant power (FRAP) assay was carried in accordance with published protocol (Wong et al., 2006) with slight modification. Silk glands were homogenized in chilled 0.1 M phosphate buffer and centrifuged at 10,000 rpm for 30 min. 200 μl of homogenate was added to 3 ml of FRAP reagent (300 mM sodium acetate buffer, pH-3.6, 10 mM TPTZ, 20 mM $FeCl_3$) were mixed in the ratio 10:1:1 and incubated at 37 °C for 30 min. The absorbance of the reaction mixture was measured at 593 nm. The reaction mixture without sample was taken as blank. The change in absorbance was calculated as, Δ Absorbance = Final absorbance (absorbance with sample) − Blank absorbance (absorbance without sample).

2.6. SOD and CAT assay

Superoxide dismutase assay was carried out in accordance with the published protocol (Beauchamp and Fridovich, 1971). Silk glands were homogenized in chilled 0.1 M phosphate buffer and centrifuged at 10,000 rpm for 30 min. 10 μl of homogenate was added to 3 ml of reaction mixture (A_s), (13 mM Methionine, 2 μM Riboflavin, 0.1 mM EDTA, 75 μm NBT in 50 mM PBS). The reaction mixture without extract was used as control (A_c). The tubes along with reaction mixture and sample were exposed to 400 W bulbs. % Inhibition was calculated as, % Inhibition = $(A_c - A_s) / A_c \times 100$.

CAT activity was carried out in accordance with the published protocol (Madhusudhan et al., 2012). Silk glands were homogenized in pre ice cooled 0.1 M phosphate buffer and centrifuged at 10,000 rpm for 30 min. The reaction mixture containing 1 ml of 0.1 M phosphate buffer, pH -7.0 along with crude extract were taken in to spectrophotometer sample cuvette and 10 μl of H_2O_2 substrate was added and reaction mixture was then immediately scanned at 240 nm every 12 s for 1 min. Decrease in absorbance at 240 nm based on decomposition of hydrogen peroxide was measured. Enzyme activity of different days in silk gland was measured from the standard plot. The CAT activity was expressed as Activity (U)/min.

2.7. RNA extraction and RT-PCR

The expression of *MYC*, *ATG8* and *CAT* gene was carried out by RT-PCR. Total RNA was extracted from 0.1 g of posterior silk gland of control and treated worms on day 7 of 5th instar larval stage by TRIzol method. The RNA samples (1 μg concentration) were reverse transcribed into cDNA using (iScript cDNA synthesis Kit, Bio-rad). At least three replicates were performed on each sample. In each run of PCR, housekeeping gene actin was used as the reference, 5′ TTCGCAAATCACTTTCGGCT 3′ and 5′ CCGAGCCCGACTACAAACAG 3′ were used as forward and reverse primers for *MYC* gene, 5′ CAT GCC ATG GAT GAA ATT CCA ATA CAA 3′ and 5′-GCG TCG ACT TAA TTT CCA TAG ACA T-3′ were used as forward and reverse primers for the *ATG8* gene amplification 5′ AAGGAGCTGGAGCTTTTGGG 3′ and 5′ AACCTCGAGGGTCACGAACA 3′ were used as forward and reverse primers for *CAT* gene and 5′ ATCCAGCAGCTCCCTCGAGAAGTCT 3′ and 5′ ACAATGGAGGGACCAGACTCGTCGT-3′ primers were used for actin gene amplification (control).

2.8. Statistical analysis of the data

The experiments were replicated and all the experimental data was subjected to statistical analysis by following *t*-test; two sample assuming equal variances (P (T < = t) one-tail = 0.05) and by two-way ANOVA with time and treatment as fixed effects.

3. Results

3.1. Effect of Spd on silk gland and cocoon weights of control and treated groups

Larvae feed vigorously during 5th instar stage, and the data showed an increase in the larval silk gland weights till day 7 of 5th instar in control and Spd treated groups. A sudden decrease in the silk gland weights was observed on day 9 of pre-pupal stage in both control and treated groups (Fig. 1A). This is expected, as after day 7, silkworms stop feeding and enter into a non-feeding wandering stage. Moreover silk secreted in the gland also extrudes out during pre-pupal stage. Treated group showed significant enhancement in silk gland weights on day 3 and day 5 of 5th instar stage and day 9 of pre-pupal stage. Results demonstrate that Spd feeding (50 μM) has positive effect on glands weights of *B. mori* worms. Interestingly, the gland weight remained significantly high on day 9 of the pre-pupal stage in the Spd treated group. Cocoon weights correlate with the result of the Spd treatment to gland weight,

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