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Research Article

Quantitative estimation of Proteins in different tissues of V instar larvae, Cocoon and Yielded silk of Tasar silkworm, A. mylitta D (Daba BV) treated by polyamines (Spermidine, Spermine, and Putrescine)

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ABSTRACT

Tasar silkworm, Antheraea mylitta Drury, is a silk-producing lepidopteran insect (family: Saturniidae) that lives in wild conditions on food plants, Terminalia arjuna. Silk is in fibrous form and made up of two proteins, fibroin and sericin, secreted from the silk glands during the last stage of larvae (V instar). The quantitative development of silk production depends on quality food intake and effective utilization. Polyamines (PAs) are polycationic organic compounds naturally present in the living cells and also synthetic. Numerous investigations explored the effectiveness of a wide variety of polyamines in different organisms. PAs have been involved in Chromatin stabilization, avoid to reaching early senescence, apoptosis and preventing the cells from oxidative damage, etc., In this present study, Tasar silkworms, A. mylitta, Daba BV ecorace was cultured and treated by PAs, Spermidine (Spd), Spermine (Spm), and Putrescine (Put), each with 50 µM, 100 µM and 150 µM concentrations by the leaves of food plant T. arjuna. Tissues extracted from the haemolymph, fat body, silk gland, and digestive epithelia of V instar larvae and fibroin, sericin mixture from the silk fibre cocoon, dialyzed through 10 K MWCO cellulose membrane and estimated the quantity levels of proteins by Lowry's method. The results explained that the Spd 50 µM, 100 µM, and Spm 50 µM, 100 µM treated larval tissues showed higher levels of protein content than control and the remaining larvae fed with Spd 150 µM, Spm 150 µM and all Put at all three concentrations showed the declined outcomes, even than control.

1. Introduction

1.1 Proteins and their role in animal cell

Proteins are macromolecules synthesized inside the living cells and participate in metabolic activities. Storage proteins in various tissues, transporter proteins, defence proteins such as antibodies, enzymatic proteins involve in cellular metabolism, hormones for control, coordination and as messengers, membrane proteins the exchange of materials, etc., are different types of proteins and their role living organisms (Peterson 2011). Proteins are built by amino acids attached by peptide bonds in a long chain. 2 Silkworm body proteins

Tissues present in the silkworm, fat body, Hemolymph, digestive tract, silk gland, etc comprise a huge number of proteins with many vital functions. The fat body and haemolymph synthesize proteins and are transported to various tissues and some are storage proteins. Digestive epithelial cells synthesize enzymes to digest food materials. Silk gland luminal epithelial cells prepare fibroin and Sericin proteins which are used to prepare silk fiber. Reproductive organs, testes, and ovaries synthesize gonadal proteins and participate in gametogenesis.

Storage proteins sp1 and sp2 present in the fat body and haemolymph were increased during the larval period of B. mori (Tojo, Nagata et al. 1980). Ecdysis protein levels are highest during the larval and pupal periods and involve development and metamorphosis (OGAWA and TOJo 1981). Cercopin and vitellogenin antibacterial proteins present in the haemolymph involve in the defence mechanism in the silkworm B. mori (Morishima, Suginaka, et al. 1990), (Singh, Pakkianathan et al. 2013). Microvitellogenin protein were appeared in the eggs of Chinese oak Tasar silkworm Antheraea peryi and their levels were increased during the development (Liu, Chen, et al. 2015). Ecdysis hormone levels increased during the diapause completed pupa of A. mylitta and promote the metamorphosis

(Kumar, Subrahmanyam, et al. 2008). Fibroin protein and Sericin proteins are synthesized in the Posterior silk gland and Middle silk gland during the V instar larval period and secrete silk at the end of this stage in mulberry silkworm (Takasu, Hata et al. 2010), (Sarangi 1985). The silk fibre is made up of fibroin and Sericin were used in the textile industry (Padamwar and Pawar 2004), cancer therapy (Elahi, Ali et al. 2021), Biomedical applications (Kunz, Brancalhão et al. 2016), regenerative medicine (Wang, Zhang et al. 2014), drug delivery (Wenk, Merkle et al. 2011), biomaterials for corneal epithelial cells development (Yoon, Kim et al. 2014).

1.2 Polyamines and their role in animal cells

Polyamine as potential molecules involved in many cellular metabolic activities including DNA, RNA modulation, gene expression, transcription and translation (Thomas, Tajmir-Riahi et al. 2016). PAs increased the protein content in the haemolymph and silk glands of B. mori strain (Yerra and Mamillapalli 2016), enhanced testicular development (Mysarla, Yerra et al. 2016). The growth and development of tissues depend on protein in the body. PAs involve in gonadal development. Polyamines increased the growth of body organs in cricket cockroach (Hamana, Suzuki, et al. 1989). PAs deficiency alters the metamorphosis in *Manduca sextra*, an insect of the Sphingidae family (Birnbaum, Whelan, et al. 1988). PAs increased hormone levels in *Plutella xylostella*, a diamondback moth (Zhang, Wang, et al. 2011).

1.3 Dialysis of tissue homogenates to extract purified protein solution:

The extracted tissues and the homogenate solution were contaminated with unnecessary materials and impurities. To remove the unwanted materials from the solution which has to contain only proteins were kept for dialysis (Savoie and Gauthier 1986).

Dialyzing the mixture of the homogenate from the fat body, gut walls, silk glands, Sericin (Ribeiro, de Moraes et al. 2014), and fibroin extracts through various membranes such as 3.5 K & 10K MWCO against the ddHH2O or Urea, etc., (a gradient of organic solvent concentration across the membrane) to remove the impurities from the solution and to retain the proteins inside the membrane (Jeraci, Lewis et al. 1989).

The objectives of this study aimed to to explore the quanity of proteins during the life cycle of Tasar silkworm, A. mylitta D., Daba BV ecorace. Studying the differential expression of proteins provide the information to develop strategies for increasing silk productivity.

2. Material and Methods

2.1 Selection of silkworm, treated by Polyamines from the culturing field.

Silkworms rearing in the culturing field at Kakatiya University, Warangal. Tasar silkworm, A. mylitta, Andhra Local, and Daba BV ecoraces were reared at an average temperature of 27- 31°C and relative humidity 75- 81% in 2018. A. mylitta Daba BV ecorace reared by treating with different polyamines Putrescine (Put), Spermidine (Spd), and Spermine (spm) at various concentrations (50 µM, 100 µM, and 150 µM) during the V instar of Tasar silkworm, in the year 2019 for two crops i.e., the first crop in July to September and the second crop in September to October. After the consumption of food from three successive treatments on day 4, day 7, day 10, and day 13, the silkworm larvae were collected randomly on day 16. Eight silkworms were sacrificed and collected various tissues i.e. Haemolymph, Fat body, Digestive tract, silk glands, and silk fibers estimated the protein content by Lowry method.

2.2 Collection, Homogenisation, Dialysis, and Preservation of tissues during V instar larval body and Fibrous extracts from Silk fibre in Tasar silkworm A. mylitta D.

Silkworms, A. mylitta, Andhra Local, Daba BV, and Daba TV ecoraces reared in the culturing field. Eight matured larvae of V instar, on day 16 were sacrificed in the collection of tissues i.e., haemolymph, fat body, gut, silk gland, and 8 pupae from the harvested cocoons sacrificed to collect the Hemolymph and fat body. Fibrous were collected from the cocoon pieces in laboratory methods (Figure-1 & 2) All the collected materials were preserved at laboratory conditions and used for biochemical estimations.

2.3 Collection of Haemolymph:

Larvae were brought to the laboratory and exposed to 4°C for 4- 5 minutes to increase the flow of blood in the coelom cavities. Forelegs of the worms selected worms of polyamines Put, Spd and spm each with 50 µM, 100 µM, and 150 µM, were cut with disinfected scissors and the haemolymph was collected in the 5 ml pre-chilled Eppendorf containing a pinch of thiourea (thiourea prevent the haemolymph from melanization) and stored at -80°C till use.

2.4 Collection of Digestive Tract epithelial tissue:

After the collection of Haemolymph, the silkworms were dissected via the central axis of the ventral surface with a fine disinfected blade. The undigested food material and other wastes were washed out, gut epithelial tissue of the digestive tract was collected into the pre-chilled 10 ml falcon tubes and stored at -20°C till use. To estimate the protein content, 100 mg tissue was taken and left over for 5 minutes at the RT to bring it to room temperature. 100 mg. tissue was weighed on a digital balance and placed in a pre-chilled porcelain mortar and added 1ml of pre-cooled ddHH2O and 9 ml of 10% TCA. The mixture was homogenized for 4-5 minutes thoroughly with the pistil. The solution was dialyzed with a 10K MWCO cellulose membrane against the ddHH2O and 2M Urea solution for two days. The dialysate solution was centrifuged at 10000 rpm (Eppendorf, Germany) at 4°C for 30 minutes, stored at -80°C (Eppendorf, Germany) for biochemical estimation of proteins by Lowry Method (1951).

2.5 Collection of Fat body:

After the removal of the digestive tract, a light yellowish fat body appeared. Disinfected forceps and spatulas were used and collected the tissue into the pre-chilled 10 ml falcon tubes and stored at -20°C till use. To estimate the proteins 100 mg. Wet tissue was weighed on a digital balance and brought to the RT for 5 minutes and taken into a pre-chilled porcelain mortar, added with 1ml of H2O and 9 ml of 10% TCA, and homogenized gently with pistil for 4-5 minutes. The solution was dialyzed with 10K MWCO cellulose membrane against the ddH_2O and 2M Urea solution for two days. The dialysate solution was centrifuged at 10000 rpm (Eppendorf, Germany) at 4°C for 30 minutes, stored at -80°C (Eppendorf, Germany) for biochemical estimation of proteins by Lowry Method (1951).

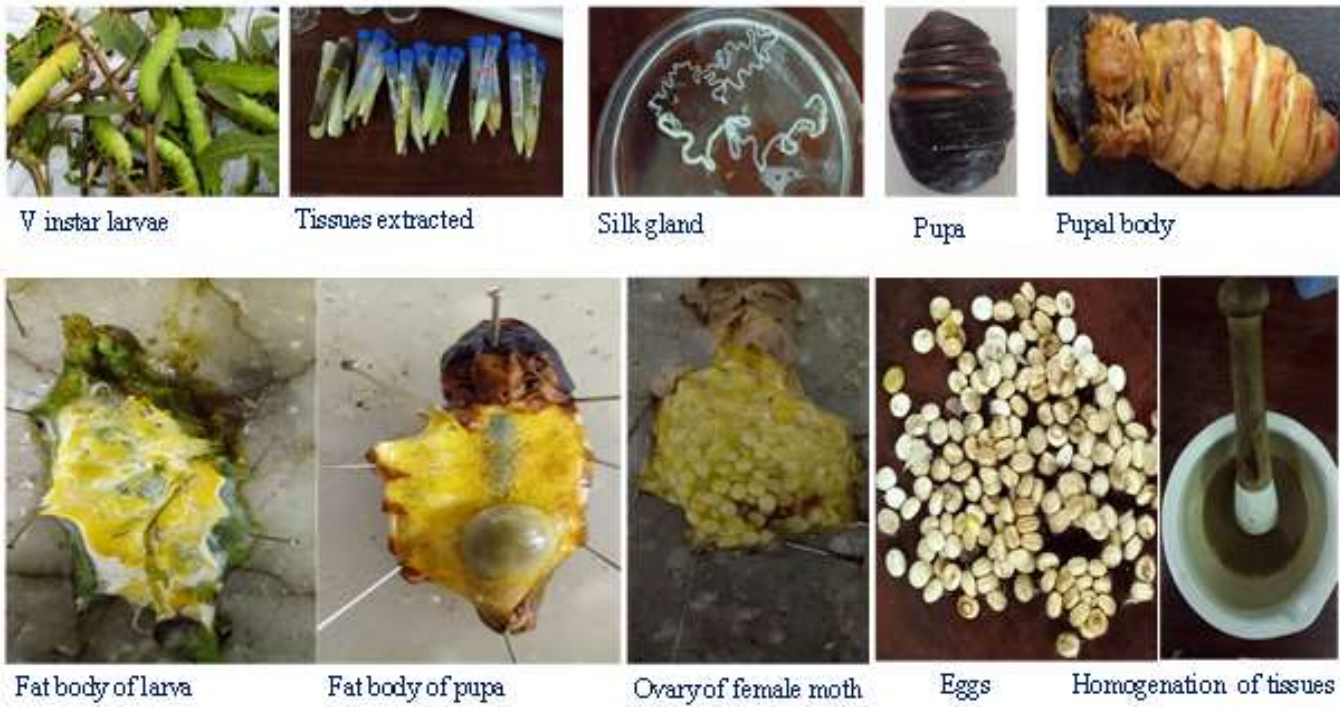


Figure-1. Extraction of various tissues from the Tasar silkworm A. mylitta Drury, Daba BV ecorace.

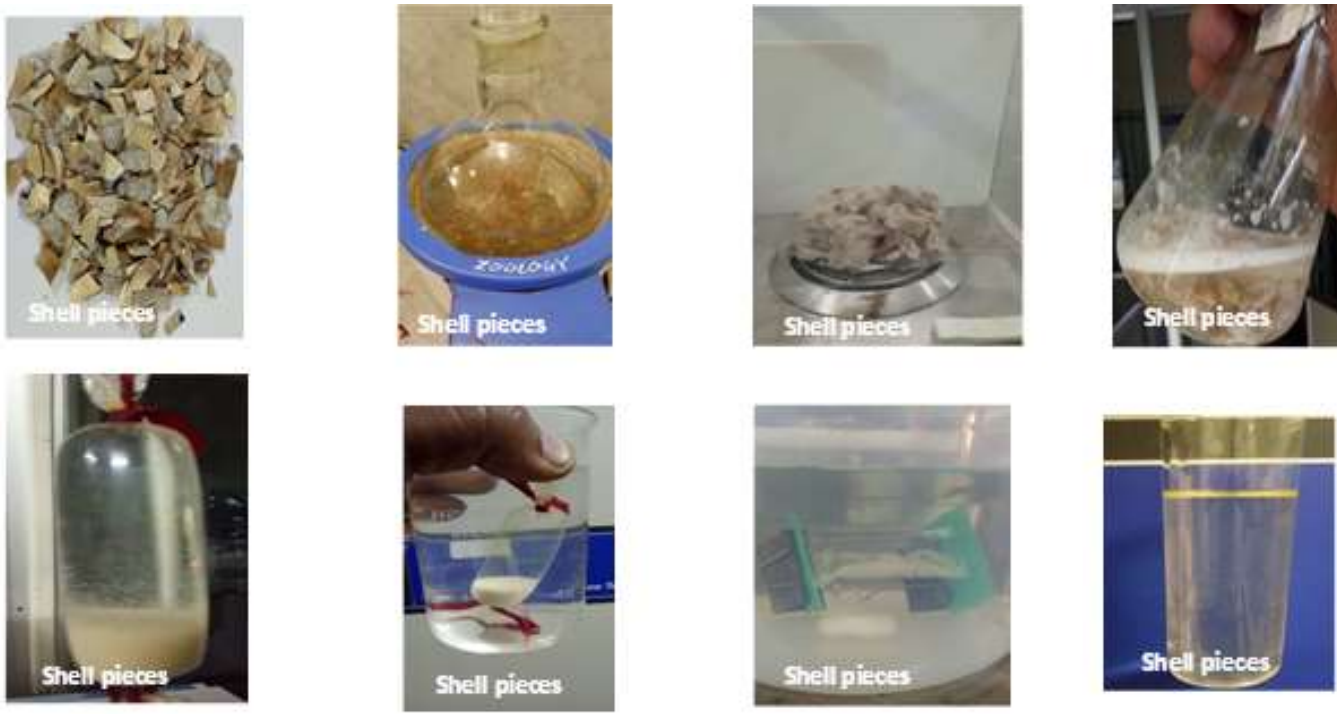


Figure-2. Dialysis of Sericin and Fibroin protein solutions from the Tasar silkworm *A. mylitta* Drury, Daba BV ecorace

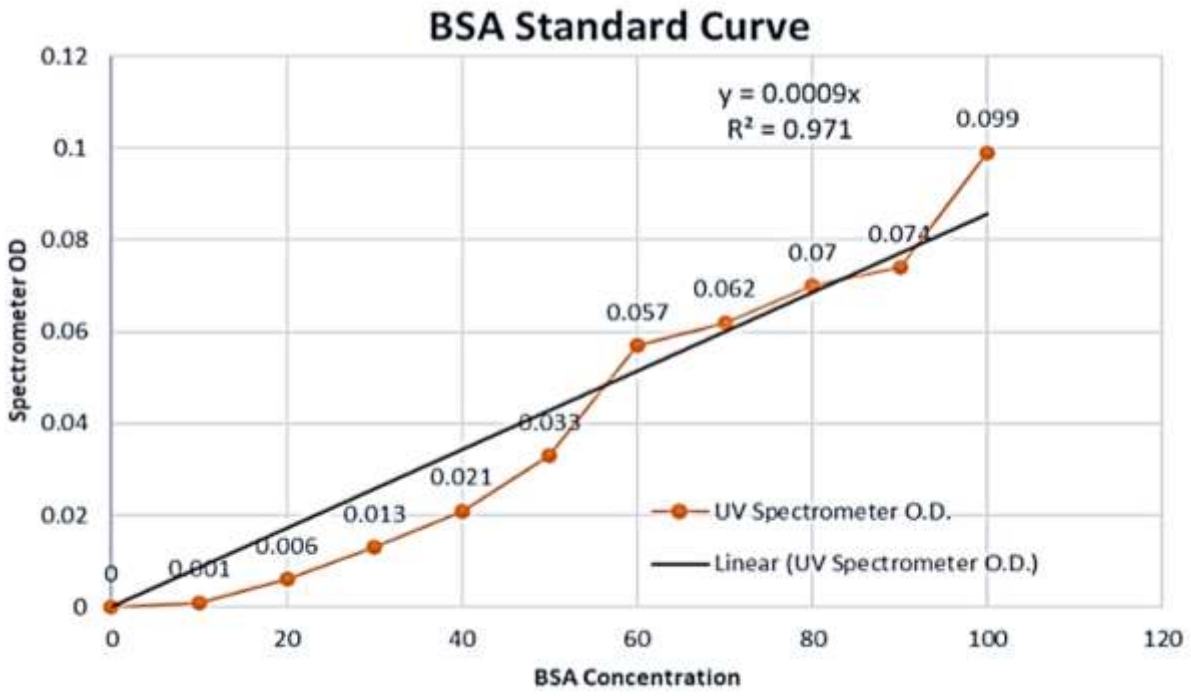


Figure-3. BSA standard values

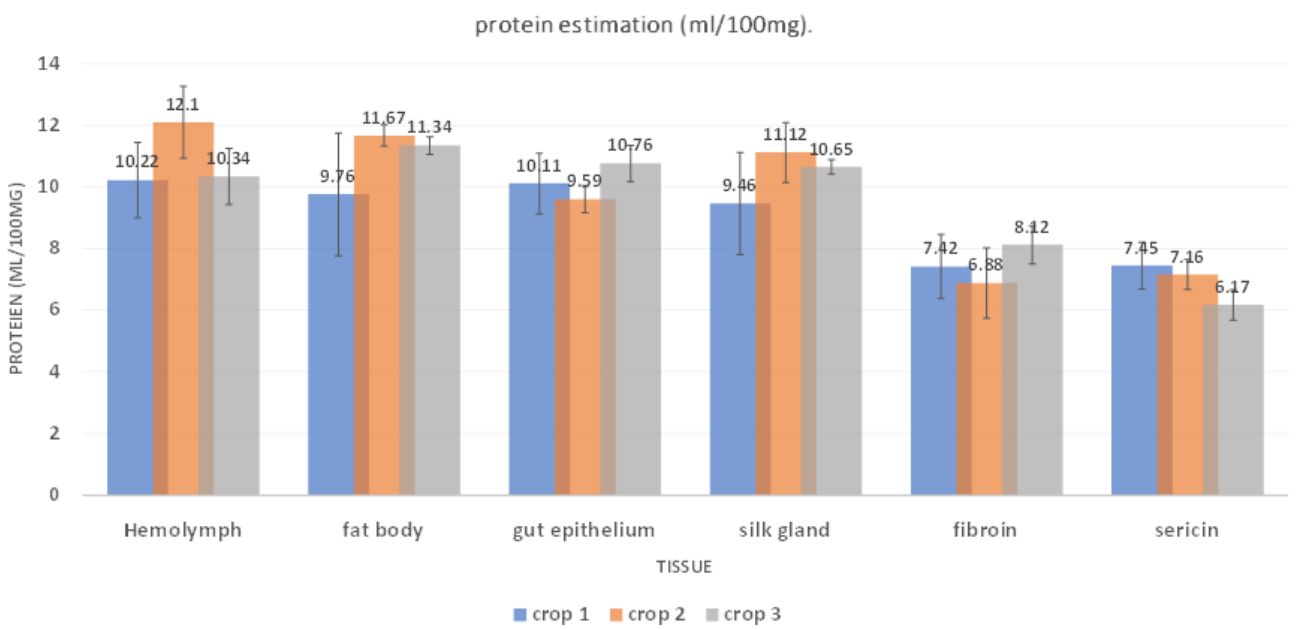


Figure-4. Proteins (mg/ml or 100 mg tissue) estimated in the different tissues of Tasar silkworm Anteraea mylitta D, Daba BV ecorace during Crop-1; July-August. Crop-2: August to October, Crop-3: October to November during 2018

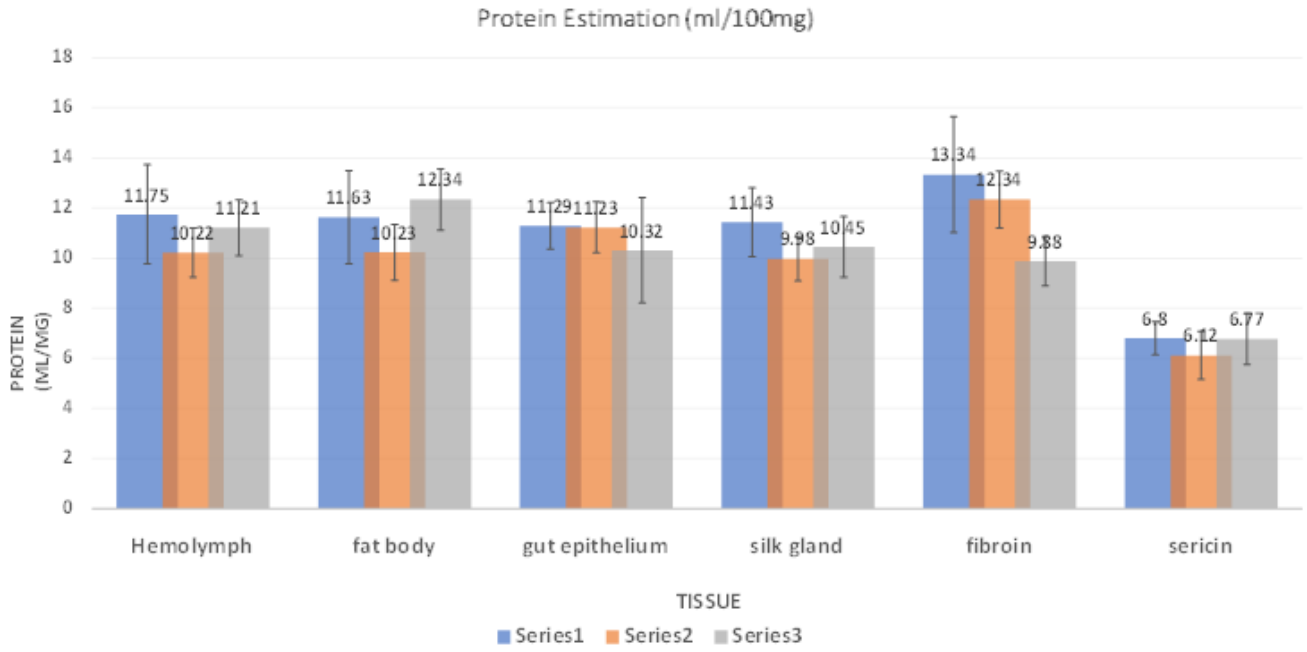
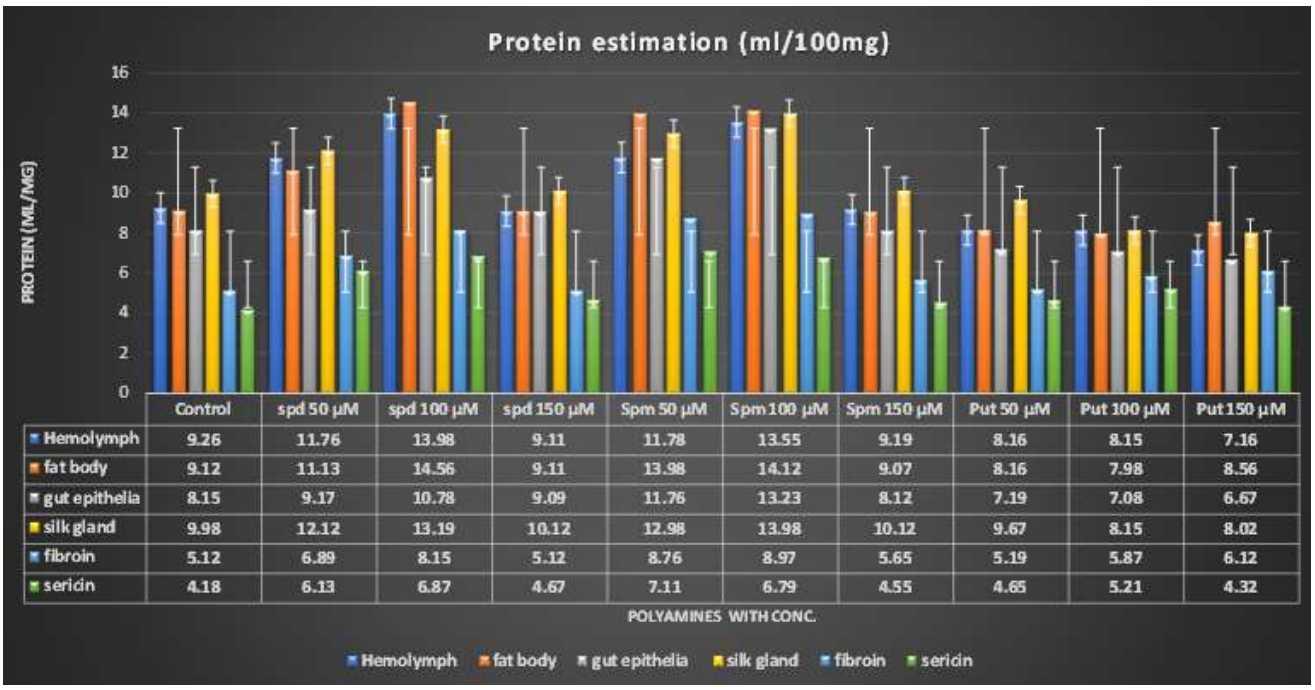
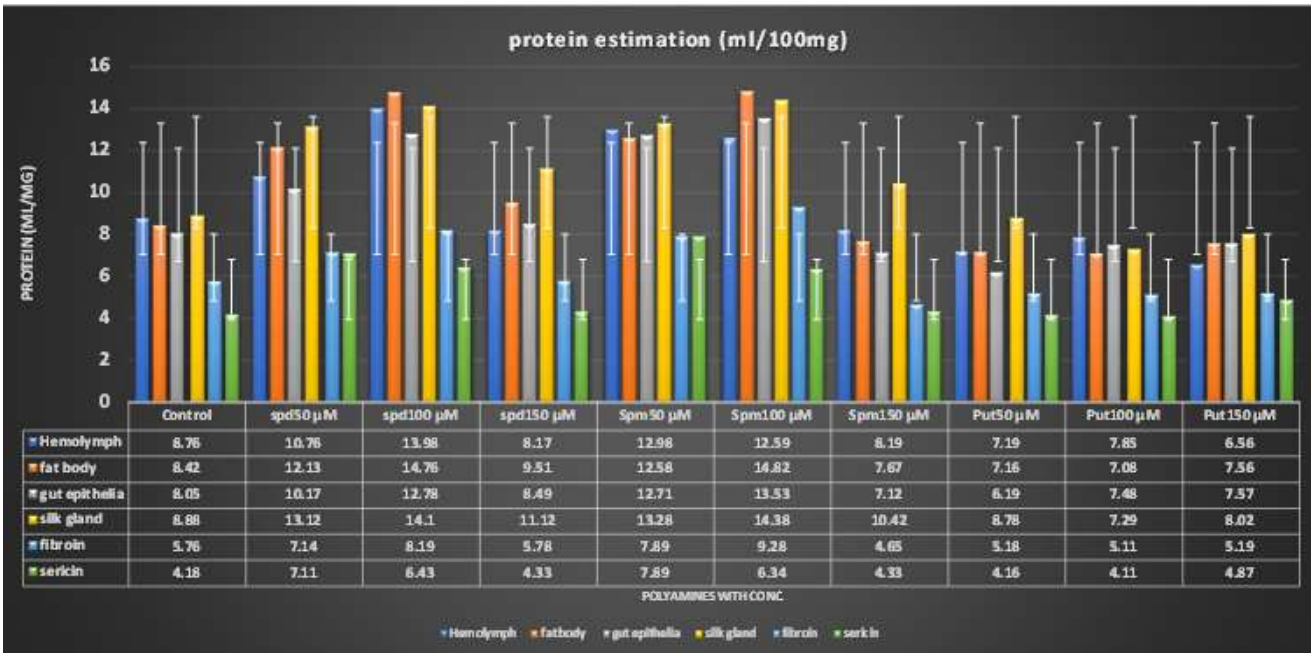


Figure-5. Estimation of Proteins (mg/ml or 100 mg tissue) estimated in the different tissues of Tasar silkworm Anthraea mylitta D, Daba BV ecorace treated by different concentrations of polyamines during crop-1: July-August in 2020.



	Control	spd 50 µM	spd 100 µM	spd 150 µM	Spm 50 µM	Spm 100 µM	Spm 150 µM	Put 50 µM	Put 100 µM	Put 150 µM
Hemolymph	9.26	11.76	13.98	9.11	11.78	13.55	9.19	8.16	8.15	7.16
fat body	9.12	11.13	14.56	9.11	13.98	14.12	9.07	8.16	7.98	8.56
gut epithelia	8.15	9.17	10.78	9.09	11.76	13.23	8.12	7.19	7.08	6.67
silk gland	9.98	12.12	13.19	10.12	12.98	13.98	10.12	9.67	8.15	8.02
fibroin	5.12	6.89	8.15	5.12	8.76	8.97	5.65	5.19	5.87	6.12
sericin	4.18	6.13	6.87	4.67	7.11	6.79	4.55	4.65	5.21	4.32

Figure-6. Estimation of Proteins (mg/ml or 100 mg tissue) estimated in the different tissues of Tasar silkworm Anthraea mylitta D, Daba BV ecorace treated by different concentrations of polyamines during crop-1: July-August in 2019.



	Control	spd50 µM	spd100 µM	spd150 µM	Spm50 µM	Spm100 µM	Spm150 µM	Put50 µM	Put100 µM	Put150 µM
Hemolymph	8.76	10.76	13.98	8.17	12.98	12.59	8.19	7.19	7.85	6.56
fat body	8.42	12.13	14.76	9.51	12.58	14.82	7.67	7.16	7.08	7.56
gut epithelia	8.05	10.17	12.78	8.49	12.71	13.53	7.12	6.19	7.48	7.57
silk gland	8.88	13.12	14.1	11.12	13.28	14.38	10.42	8.78	7.29	8.02
fibroin	5.76	7.14	8.19	5.78	7.89	9.28	4.65	5.18	5.11	5.19
sericin	4.18	7.11	6.43	4.33	7.89	6.34	4.33	4.16	4.11	4.87

Figure-7. Estimation of Proteins (mg/ml or 100 mg tissue) estimated in the different tissues of Tasar silkworm Anthraea mylitta D, Daba BV ecorace treated by different concentrations of polyamines during crop-1: July-August in 2020.

2.6 Collection of silk gland tissues

Silk glands were extracted from two lateral sides of dissected silkworms into pre-chilled 10 ml falcon tubes and washed with 0.5 % NaCl and stored at -80°C till use. 100 mg of tissue was taken into a pre-chilled porcelain mortar and added with 1 ml of ddH2O and 9 ml of 10% TCA, homogenized thoroughly with pistil for 4-5 minutes. The solution was dialyzed with 10K MWCO cellulose membrane against the ddH_2O and 2M Urea solution for two days. The dialysate solution was centrifuged at 10000 rpm (Eppendorf, Germany) at 4°C for 30 minutes, stored at -80°C (Eppendorf, Germany) for biochemical estimation of proteins by Lowry Method (1951).

2.7 Homogenization of Eggs extract

The diapause completed eggs were weighed on digital balance in 1g (approximately ten in number), washed 2-3 times with ddH2O till free from glue material, the meconium, followed by 0.5% NaCl treatment for 2-3 minutes with slow shaking. The eggs were placed in clean, disinfected, pre-chilled porcelain mortar added 5 ml Millipore water, and homogenized with the pistil, gently. The mixture was centrifuged at 8000 rpm (Eppendorf, Germany) at 4°C for 10 minutes. The supernatant was extracted into a 3.5 K MWCO cellulose membrane and dialyzed against ddH2O for 24 hours. The purified protein solution was centrifuged at 12000 rpm (Eppendorf, Germany) at 4°C for 30 minutes, stored at -80°C (Eppendorf, Germany) for biochemical estimation of proteins by Lowry Method (1951).

2.8 Preparation of Sericin solution:

The cocoon shell was cut into pieces (1x1 cm), washed with ddH2O two times followed by boiled in ddH2O containing 0.02M Na2CO3 for 60 minutes. Fibers were cooled to room temperature and filtered through 0.7µm (Minisart®- sartorius, Germany). The solution was concentrated to 300 ml by heating on a rotary evaporator (Aditya Scientific, India). The Solution was dialyzed through a cellulose membrane of MWCO-3500K (3.5kDa) against ddH2O in a magnetic stirrer containing a rotating bit at 1000 rpm. Water was being changed after an hour, at the 3rd hour, 6th hour, 9th hour after a day. The Final solution was centrifuged at 10000 rpm (Eppendorf, Germany) at 4°C for 30 minutes and the solution was stored at -80°C (Eppendorf, Germany) for biochemical estimation of proteins by Lowry Method (1951).

2.9 Preparation of fibroin solution:

The cleaned and dried cocoon shell was cut into pieces (1x1 cm) and washed two times with dd H2O. 5g of pieces were weighed on a digital balance and boiled in dd H2O containing 0.33M Na2CO3 for 1 hour. Fibers were removed and soaked in dd H2O for 10 minutes followed by repeated heating in ddH2O as mentioned earlier for 5 times. The Fibrous material was kept in the incubator (WiseCube®WIS-20, India) for 1 hour at 60°C left in the fume hood for a day. The fibrous mesh was weighed on a digital balance. The fibrous network was soaked in Ajisawa's Reagent (CaCl2: H20:C2H5OH in 1:8;2 ratios) for 1 hour followed by boiled in a water bath for 1 hour at 100°C with occasional stirring and cooled to room temperature. This was repeated 4 times and the fibers were squeezed and the fibroin solution was dialyzed with 10K MWCO cellulose membrane against the ddH_2O and 2M Urea solution for two days. The dialysate solution was centrifuged at 10000 rpm (Eppendorf, Germany) at 4°C for 30 minutes, stored at -80°C (Eppendorf, Germany) for biochemical estimation of proteins by Lowry Method (1951).

2.10 Proteins estimation- Lowry Method (1951):

In this method, the development of blue colors due to the reaction of cupric copper ions with the peptide bond of proteins.

Peptides under alkaline conditions and reduction of Phosphomolybdic acid by tryptophan and tyrosine, the aromatic amino acid residues of proteins. The wavelength is calculated in the spectrophotometer absorbance at calculated the number of proteins by taking the BSA as standard (Figure-3). A 0.5 ml of Haemolymph was taken into the pre-cooled test tube placed on dry ice and 1 ml of ddH2O and 8.5 ml of TCA was added (total10 ml solution) to precipitate the proteins. In the case of a fat body, digestive tract, and silk gland tissue 0.5 ml of homogenate supernatant prepared from 100 mg. tissue was taken. The homogenate solution was mixed for 10 minutes thoroughly and centrifuged at 1000 rpm, at 4°C in a microcentrifuge (Eppendorf). The supernatant was discarded and the pellet was added with 4.5 ml of NaOH (0.1N) and dissolved protein mixture for 10 minutes. Centrifuge the solution for 5 minutes at 4°C in a microcentrifuge (Eppendorf) and 0.5ml of supernatant was taken into a cleaned and dried test tube and added 4.5 ml of freshly prepared reagent- D (alkaline CuSo4 reagent) (appendix) and leave the test tube on the stand without disturbing for 10 minutes. Add 0.4 ml of Folin Ciocalteu (diluted commercial reagent at 1N strength) reagent to the solution. A blank (for reference) was prepared by adding4.5 ml of NaOH (0.1N), 4.5 ml of freshly prepared reagent- D, and 0.4 ml of Folin Ciocalteu reagent. All the test tubes were left over for 30 minutes on the stand without disturbing incubation at RT. The blue color developed in solution and O.D. was read on a UV Spectrophotometer at 720 nm. Bovine Serum Albumin (BSA) was used as standard. Total protein content was calculated and interpreted in micrograms /1 ml or 100mg. wet weight of tissue.

3. Results

3.1 Results of quantitative protein estimation in various tissues were depicted as µg/ 1 ml. or 100 mg. tissue.

3.1.1 Andhra Local ecorace:

In Andhra Local ecorace, the average protein content in the hemolymph, fat body, epithelium, silk gland and fibroin, and sericin was8.54µg, 9.18µg, 8.03µg, 9.06µg, 8.09µg, and 6.31µg per unit of tissue (1ml/100mg), respectively in 3 successive seasons. The ratio of total protein content in larval tissues to fibroin and sericin was approximately 1:1 and 3:2.3, respectively.

3.1.2 Daba BV:

In Daba BV ecorace, the mean protein content in the hemolymph, fat body, epithelium, silk gland, and fibroin and sericin were 11.1µg, 11.4µg, 10.9µg, 10.6µg, 11.9µg, and 6.6µg per unit of tissue (1ml/100mg), respectively in 3 successive seasons. The ratio of the sum of protein content in larval tissues to fibroin and sericin was approximately 1:1 and 3:2.2., respectively.

3.1.3 Daba TV:

In Daba TV ecorace, the mean protein content in the hemolymph, fat body, epithelium, silk gland, and fibroin and sericin were 10.89µg, 10.92µg, 10.15µg, 10.41µg, 09.47µg, and 6.93µg per unit of tissue (1ml/100mg), respectively in 3 successive seasons. The ratio of the sum of protein content in larval tissues to fibroin and sericin was approximately1:1.2 and 3:2.4., respectively.

3.2 Quantitative Estimation of Proteins in tissues of Tasar Silkworm, A. mylitta D, Daba BV ecorace treated by Polyamines.

Polyamine promotes the synthesis of proteins and contributes to growth and development in Escherichia coli (Igarashi and Kashiwagi 2018). PAs are essential for protein synthesis and the growth of eukaryotic cells **(Heby** and Persson 1990). Polyamine binding studies in B. mori reveal that Spermidine and Spermine increased the potentiality to bind the DNA for proper expression, especially the spermine in testicular development (Mysarla, Yerra, et al. 2016). Sex-dependent proteins were synthesized during the metamorphosing pupae (Mine, Izumi, et al. 1983). Silk gland proteins Fibroin and Sericin levels were increased during the mid-larval period of V instar (Uppula And Gangupanthula 2021).

During the larval stage the storage proteins such as SP1 and SP2 concentration was increased (Tojo, Nagata et al. 1980). In the present estimations, Haemolymph and fat body showed a rise in protein levels during V instar in crop-2 than in crop-1 and crop-3, gut epithelium whereas fibroin protein content was high in crop1 and crop-3. Sericin protein content was noticed high during crop-1 and crop-2 (Figure-4,5). In insects, the larval stage is the only feeding period. The development of the total stages depends on larval growth. A huge number of proteins are synthesized during this period viz., storage proteins (Tojo, Kiguchi, et al. 1981), transporter proteins, defense proteins, membrane-associated proteins, etc. A wide number of proteins were synthesized during the larval period of the Greater wax moth, Galleria mellonella (Godlewski, Kłudkiewicz, et al. 2001). During the larval period, especially in V instar haemolymph proteins were synthesis was at a peak, in Bombyx mori (Shigematsu 1958) and also in the fat body (Tian, Xue et al. 2017). Larval and pupal haemolymph protein levels were increased in the silkworm treated with the Spd with 100 and 50, Spm with 100 and 50 concentrations. Silk gland proteins Fibroin and Sericin were increased elevated especially by Spm50 and 100 than even spermidine. Gut epithelial protein content was high in Spd 100, Spm 50, and Spm 100 than all. Putrescine and PAs with 150 concentrations were declined the protein levels even than the control. These PAs might be function antagonistically to the protein synthesis in the Tasar silkworm. The PAs Spd 50, 100, and Spm 50, 100 were showed a predominantly positive effect on all the growth and development parameters in Tasar silkworm, Antheraea mylitta D., Daba BV ecorace (Figure-6,7).

4. Conclusion

Spermidine with 50 and 100, as well as Spermine with 50 and 100 concentrations showed enhanced development in protein level in all the tissues throughout all stages of the life cycle and fibrous proteins.

Conflicting Interests

The authors have declared that no conflicting interests exist.

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