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Efficient CDNA Preparation and Amplification of Laccase 2 Gene from Various Tissues of Tasar Silkworm for Genetic Studies

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Abstract

Tasar silkworms, scientifically known as *Antheraea mylitta*, a species of wild silkworms native to India. The tasar silkworm undergoes a complete metamorphosis, including the egg, larva (caterpillar), pupa (cocoon), and adult (moth) stages. This complex life cycle, coupled with their outdoor rearing environment, makes their cultivation distinct and labour-intensive compared to other silkworm species. To improve the quality of silk being produced, it is of utmost importance that genetic analysis with preference to gene expression is well understood. Transcriptome analysis is one of the most crucial studies performed to understand the level of gene expression in a living organism. With respect to *Antheraea mylitta*, currently there are very limited genetic data describing the gene expressions. Transcriptome analysis at different stages of larvae development can give useful insight. The data can also be used to study the response of the insect's immune system in case of pathogen attack. The study focused on optimization of cDNA synthesis from RNA isolated from *Antheraea mylitta*. Our study used *Laccase2* gene for PCR amplification to check the quality of isolated RNA. The study concluded that good quality RNA can be successfully isolated from *Antheraea mylitta*. Successful amplification of *Laccase2* gene reveals that Laccase2 gene is being expressed at different levels of different larval stages as it helps in cuticle tanning and sclerotization. This research focuses on synthesizing cDNA for the Laccase 2 gene from *Antheraea mylitta* and confirming its identity using PCR amplification and sequence analysis, contributing to a deeper understanding of its biological function.

Keywords: *Antheraea mylitta*, *Laccase 2* gene, tasar silk, RNA, Polymerase Chain Reaction, Transcriptome analysis, Haemolymph, Fat body, Pupae, etc.

Introduction

India is the second largest producer of silk in the world. All four kinds of silk, namely Mulberry, Tasar, Eri and Muga are produced in this country. Jharkhand, Orissa and Chhattisgarh are major tasar producing states. The tribal of Chotanagpur are known as inventors of tropical tasar silk, out of reported forty four Ecoraces of the country, ten of them are identified from Jharkhand. The tropical Tasar silkworm, *Antheraea mylitta Drury* (Lepidoptera: Saturniidae) is a sericigenous insect that produces silk (tasar silk) of high commercial importance. Tasar silkworm is holometabolous insect and undergoes a complete metamorphosis. In insects, the cuticle layer is made up of protein and chitin that covers most

of the body, becomes stronger through a process called sclerotization, where the proteins link together tightly. Insects utilize laccase as a catalyst in the development of their cuticle. Laccase plays a multifunctional role in insect sclerotization, driving both the biochemical hardening of the cuticle and contributing to processes like pigmentation, immunity, and morphology.

Its precise regulation and interaction with other enzymes are critical for insect survival, making it a potential target for biotechnological and pest management applications. Laccase is an enzyme containing multiple copper ions found in various organisms, including plants, fungi, and bacteria (Johnstone, 1994; Nawrath, 2006; Janel et al., 2005). In a follow-up study on the red flour beetle, *Tribolium castaneum*, researchers found that it was not the knockdown of laccase1 but rather laccase2 that led to lethality. This lethality was linked to defects in both cuticle hardening and pigmentation (Arakane et al., 2005). Since then, the expression and functions of laccase2 genes have been explored in various insect species across multiple orders (Arakane *et al*., 2005; Yatsu and Asano, 2009; Dittmer *et al.*, 2009; Gorman *et al*., 2008; Elias-Neto *et al.*, 2010; Futahashi and Tanaka *et al*., 2011; Masuoka *et al.*, 2013.)

It is thought to be essential for cuticle sclerotization because it can catalyze the oxidation of phenolic compounds into their corresponding quinones, such as dopamine (DA) and dihydroxyphenylethanol and Pigmentation, These two processes are known as Cuticle Tanning. Laccase works alongside phenoloxidase and dopachrome tautomerase to regulate the production of quinones and ensure proper timing and extent of cuticle hardening. These enzymes form part of the oxidative cascade required for efficient cross-linking, two types of phenoloxidase, tyrosinase and laccase are critical in this process. (Andersen, *et al.* 1990; Ashida and Yamazaki, *et al.* 1990; Hopkins and Kramer, *et al.* 1992) during sclerotization, phenolic precursors like N-acetyl dopamine (NADA) and N-β-alanyl dopamine (NBAD) are oxidized by laccase into quinones. These quinones are highly reactive and act as cross-linking agents that bind to cuticular proteins and chitin, reinforcing the structure of the cuticle.

Laccase activity also contributes to the pigmentation of the cuticle, as many of the phenolic compounds involved in sclerotization are precursors to melanin. The same quinone-based chemistry involved in cross-linking can lead to the formation of melanin, giving the cuticle color. Laccase enzymes are expressed and activated at specific times and locations in the insect body. This ensures that sclerotization occurs only where and when needed (e.g., after molting or in particular body parts like mandibles and wings. Laccase also helps to reduc**e** water l**oss** and strengthens the insect's barrier against microbial pathogens.

In some insects, there are different isoforms of laccase (such as Laccase-1 and Laccase-2), each with distinct roles: Laccase-1 is mainly involved in immunity and wound repair. Laccase-2: Critical for cuticle sclerotization and pigmentation. The laccase-2 gene showed high expression in the epidermis prior to ecdysis. Cuticle laccase is synthesized as an inactive precursor, which is activated after the ecdysis stage.

Literature review suggests that there are scholarly articles on the commercial usages of this insect but only some about bimolecular changes and Transcriptome studies. Here we report the isolation of RNA from different samples of Instar larva and pupa of *A. mylitta.* By using an enzyme Reverse transcriptase, we convert this isolated RNA into cDNA and design a primer for the specific gene Laccase 2. The study demonstrated that high-quality RNA can be effectively isolated from *Antheraea mylitta*. The successful amplification of the *Laccase2* gene indicates its expression varies across different larval stages, supporting its role in cuticle tanning and sclerotization. Currently, no studies have explored the Laccase 2 gene in the tasar silkworm (*Antheraea mylitta*), leaving a gap in our understanding of its role in this

species. Since Laccase 2 is involved in important functions like cuticle formation and defense in other insects, studying it in *A. mylitta* could reveal unique insights into silkworm biology. This research could also help to improve practices in silk production and pest control, making it valuable for the sericulture field.

Material and Methods

Experimental material: The Tasar silk moth is the main insect for performing all the experiments and different samples were prepared by different larval stages were obtained from the Central Tasar Research and Training Institute (CTR &TI), piska Nagri Ranchi, Jharkhand, India.

Sample collection

A sufficient number of disease-free instar larvas and pupae of the Bivoltine Daba eco race of Tasar silkworm *(Antheraea mylitta*) were collected with labeled perforated paper boxes for the study. Silkworm rearing was conducted on *Terminalia arjuna (*Arjun*)* food plants in the field laboratory of Central Tasar Research and Training Institute, Piska Nagri Ranchi, India. Then the Sample was carried to Genomics Laboratory, Aakriti Biotechnology, Ranchi (Jharkhand) for conducting the genomics studies that is the expression profile of the Laccase 2 gene at the cDNA level of different samples of the larval stages of *Antheraea mylitta.*

Sample processing

Insect was dissected dorsally; the yellow color fat body was isolated from different larval stages, by dissecting the different instars larva (3rd, 5th) and pupae. Mechanical grinding of the sample in pestle and mortar was performed with pre-chilled Liquid Nitrogen. Samples were collected and stored at -20ºC.

RNA extraction and cDNA preparation

RNA isolation from the sample was performed by GT-based solvent extraction method, by using RNA isolation Kit for Insects by Aakriti Biotechnology, Ranchi (Jharkhand) Ramalho *at el.* (2004). Total RNA (1 µg) isolated from the fat body tissue of *A. mylitta* was used as the template for cDNA synthesis. To eliminate genomic DNA contamination, the RNA was treated with DNase I according to the manufacturer's instructions. cDNA synthesis was tried with various protocols as per previous reports (Wang *et al.* 2018 Harbers, *et al.* (2008). In the current study modified single nucleotide insertion oligo dT primer was used to obtain a good length and quality product.

PCR amplification Programme: 30ng of template was taken for PCR amplification, PCR programme was set for 94ºC for 5 min followed by three steps cycle of Denaturation at 94ºC for 1 min, Annealing at 55ºC for 40 sec, Extension at 72ºC for 1min and final extension at 72ºC for 5 min.

Result and Discussion

Total RNA was successfully isolated from different tissue sample of A. mylitta using the GT based solvent extraction method. The RNA yield was good, resulting approx in a total of 10 µg from 20 mg of tissue. Purity assessment showed an A260/A280 ratio of 2.0 and an A260/A230 ratio of 2.1, indicating minimal protein and phenol contamination. RNA integrity was verified by agarose gel electrophoresis. The RNA was of high quality and suitable for cDNA synthesis.

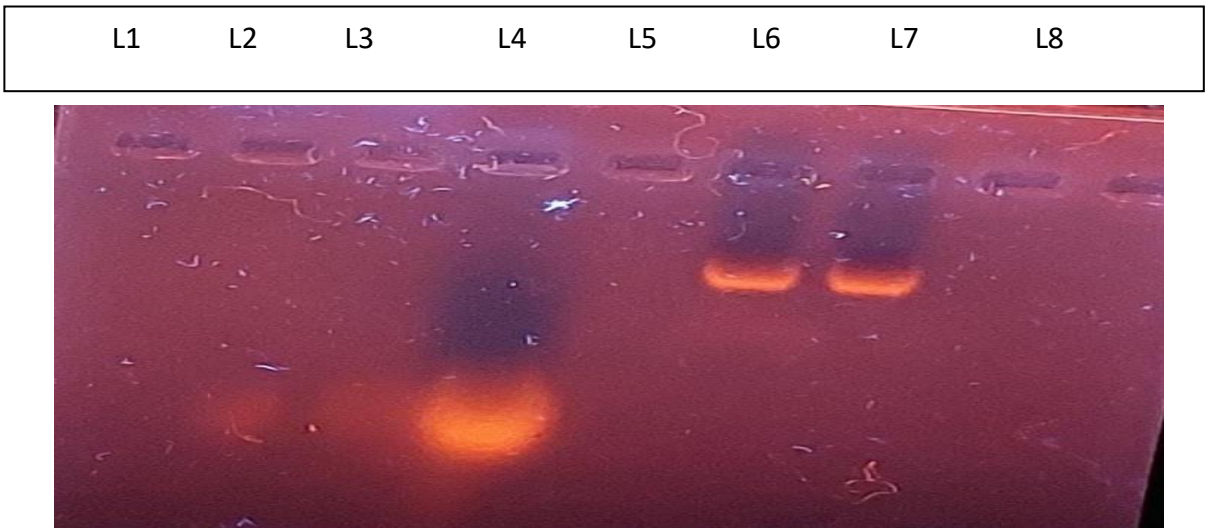

Fig- Gel picture of RNA isolation from different sample of instar larva and pupa of *A. mylitta* L4- fat body sample of 3rd instar larva, L6- fat body sample of 5th instar larva, L7-fat body sample of Pupa

Total RNA (1 µg) isolated from fat body tissue of *A. mylitta* was used as the template for DNA synthesis. To eliminate genomic DNA contamination, the RNA was treated with DNase I according to the manufacturer's instructions. cDNA based analysis is the most accepted when performing expression profile studies. This step is vital for converting the RNA template into a more stable form that can be amplified and analyzed. The choice of primers, such as oligo (dT) primers for mRNA-specific synthesis for a broader range of RNA species, influenced the cDNA yield and specificity. Transcriptome level analysis becomes crucial to perform the expression level studies, response of the larvae in case of stress and other related studies. cDNA synthesis from the sample using modified oligo dT primers and PCR amplification using Laccase 2 gene confirms that high quality cDNA having high molecular weight fragments have been synthesized.

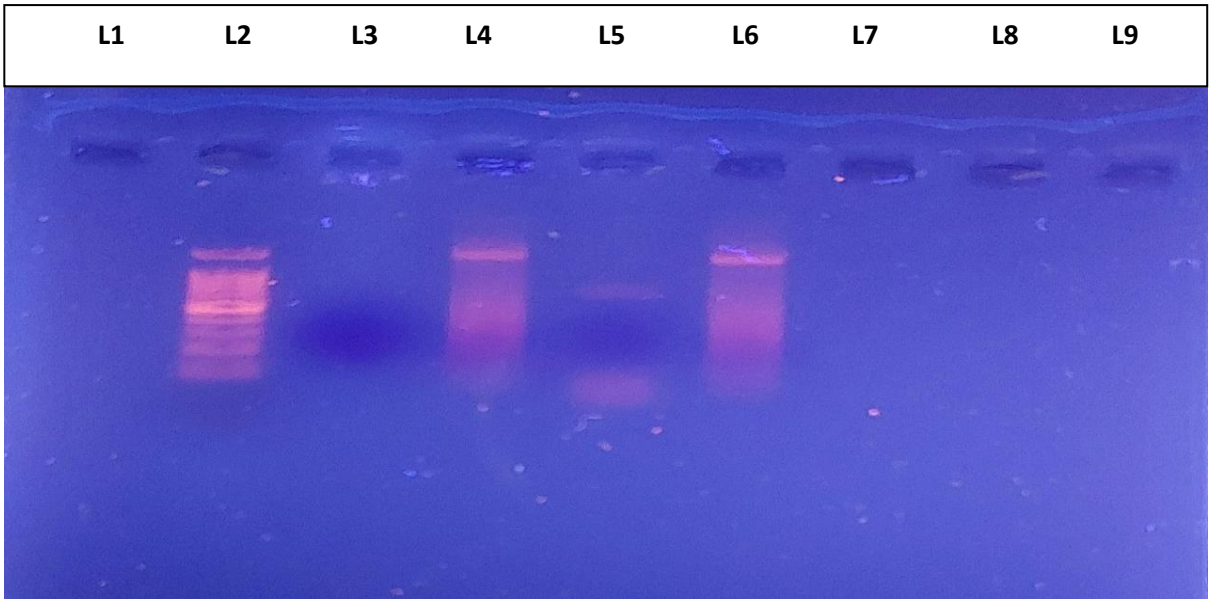

Figure: c-DNA PCR amplification of Different larval stages of *Antheraea mylitta* using Laccase 2 gene

Lane 2-100bp of DNA ladder, Lane 3- Negative control, Lane4 - 5th Instar larva, Lane 5 – positive control, Lane 6 - 3rd Instar larva

Discussion

Previous research has predominantly explored microbial laccases and their potential for various industrial applications (Cao et al., 2004). However, with the progress in molecular biology techniques, our understanding of insect laccase has significantly improved, highlighting their potential for practical applications. (LIU Zhen-gang et al 2018) The amplification of cDNA from various larval stages of the tasar silkworm (*Antheraea mylitta*) offers critical insights into its developmental biology and gene expression patterns. This approach facilitates the identification of stage-specific genes and their regulatory mechanisms, which are pivotal for understanding growth, silk production, and environmental adaptability. Complementary DNA (cDNA) synthesis from mRNA is a fundamental technique for studying gene expression. By targeting mRNA from different larval stages, researchers can identify and quantify the expression levels of genes involved in processes such as silk protein synthesis, immunity, and metabolic regulation. (Harbers, M *et al*. 2008) .This provides a foundation for exploring stage-specific molecular mechanisms, contributing to advancements in sericulture and pest management.

The process of RNA isolation and cDNA synthesis is a critical step in molecular biology research, particularly in gene expression studies. The quality and integrity of the isolated RNA directly impact the success of downstream applications, such as reverse transcription and quantitative PCR (qPCR). However, RNA is inherently unstable and highly susceptible to degradation by RNases. Careful handling and the use of RNase-free reagents and equipment were crucial for preserving RNA integrity. The use of DNase I treatment to eliminate genomic DNA contamination was particularly important, as any residual DNA could interfere with downstream reverse transcription and qPCR assays, leading to inaccurate results.

In this study, we obtained the amplified full-length cDNA band of Laccase2 using PCR and are now uncovering the properties of insect laccases, broadening their potential for practical use. Observation and Result of cDNA level of laccase 2 gene amplifications is similar in different larval stages. PCR based amplification of laccase 2 gene confirms the level of cDNA present in different larval stages. The isolation of high-quality RNA and its efficient conversion into cDNA are critical steps for conducting precise gene expression analyses. Ensuring RNA purity and integrity through stringent quality control measures is essential to maintain the accuracy and reliability of downstream processes. The synthesized cDNA will be utilized as a template for qPCR, enabling the quantification of specific gene laccase 2 expression levels and offering valuable insights into the field of genomic study under immunisation and melanisation of A. *mylitta.* In this study, Lac2 was expressed in all developmental stages, although expression levels were same as 1.5kb band is appear in all the different larval stages of *A. mylitta,* as Laccase, are a multi-copper oxidases, plays a crucial role in insect molting, a key developmental process that allows insects to grow by shedding their old exoskeleton and forming a new one. This enzyme is primarily involved in the hardening (sclerotization) and pigmentation of the newly formed exoskeleton, processes that are critical for the structural integrity and functionality of the cuticle. In *Anopheles* species an experiment during the dominant expression period, silencing AsLac2 *(Anopheles* Laccase 2) in pupae resulted in unpigmented cuticles that were thin and extremely soft. This condition significantly reduced the eclosion rate of adults and negatively affected their overall fitness. Additionally, the pupae exhibited a marked decline in melanisation-based immune responses, leading to weakened resistance against

microbial infections. (MH Du *et al*. 2017) Laccase is also involved in cuticle pigmentation, which is essential for camouflage, thermoregulation, and species-specific coloration. The enzyme oxidizes phenolic compounds into pigmented melanin or other derivatives, contributing to the final color of the exoskeleton. Sclerotization is the process of hardening the new cuticle through cross-linking of cuticular proteins. Laccase facilitates this by oxidizing catecholic and phenolic compounds (e.g., catechols and N-acetyldopamine), leading to the formation of quinones. These quinones then cross-link chitin and cuticular proteins, resulting in a rigid and durable exoskeleton. (SO Andersen *et al* 2012)The cross-linked, sclerotized exoskeleton acts as a protective barrier against physical damage, dehydration, and microbial invasion. Laccase's role in ensuring the robustness of this barrier highlights its importance in insect survival. Laccase enzymes are indispensable for successful insect molting, ensuring that the newly formed exoskeleton is both functional and protective. Disruptions in laccase activity can lead to incomplete or defective molting, affecting an insect's survival. (Cerenius, L *et al.* 2021).These are some major role of laccase enzyme that play important role in survival of the insect. This work is the new approach regarding the activity of laccase 2 gene towards *A.mylitta.*

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