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Antheraea mylitta cocoonase: An explorable protease

A. Rani[1,2], J.P. Pandey[1]*, K. Jena[1], K. Sathyanarayana[1] and D.M. Pandey[2]

[1]Central Tasar Research and Training Institute (Central Silk Board, Ministry of Textiles), Ranchi-835 303, India
[2]Birla Institute of Technology, Ranchi-835 215, India

***Corresponding Author Email :** pandeyjayprakash46@gmail.com ***ORCiD:** https://orcid.org/0000-0002-3378-8880

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Abstract

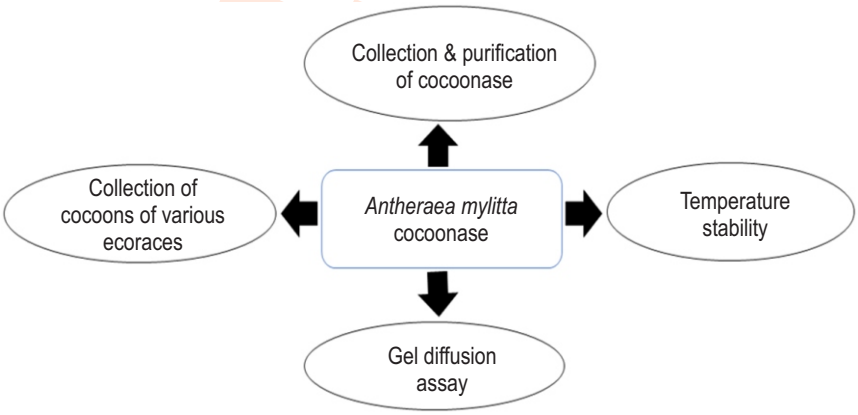
Aim: The study was conducted to explore the insect secreted protease *Antheraea mylitta* cocoonase.

Methodology: The cocoons of eleven ecoraces were collected followed by observation of cocoonase secretion behaviour, cocoonase collection, purification, and its quantification. In addition, the thermal stability of cocoonase and gel diffusion assay were investigated.

Results: *Antheraea mylitta* cocoons of eleven ecoraces were collected from different eco-pockets of India. Subsequently, the cocoonase secretion behaviour of different ecoraces during emergence was studied. It was observed that the quantity and concentration of cocoonase secreted depends upon various factors such as cocoon size, cocoon stiffness, and the habitat in which silkworm is reared. To observe the proteolytic specificity of *A. mylitta* cocoonase, a gel diffusion assay was conducted using casein and gelatin as probable substrates. The presence of hydrolytic ring confirmed protease activity. In addition, the thermal stability of cocoonase was also observed by protease activity.

Interpretation: The present study concludes that *Antheraea mylitta* cocoonase is an explorable protease that needs further exploration to unveil the unspecified properties of this insect-secreted protease, which can find beneficial applications in silk and other industries.

Key words: *Antheraea mylitta*, Cocoonase, Ecoraces, Gel diffusion assay, Temperature stability



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Introduction

The tropical tasar silkworm *Antheraea mylitta* species is indigenous to India (Anand *et al.,* 2021). The silk produced by *A. mylitta* in the form of silk cocoons is a complex fabric formed of fibroin protein bounded by sericin protein. The silk fiber comprises 22-25% sericin protein and 67-75% fibroin protein (Mahmoodi *et al.,* 2010; Srihanam *et al.,* 2009; Prasong *et al.,* 2009). The erection of a cocoon is the most potent approach for some holometabolous insects to shelter the dormant pupa from harmful natural predators, parasites and other adverse aspects before adult emergence. The emergence of moths generally depends upon the rearing season, their voltinism and favorable weather conditions. The emergence of adult moths is generally governed by a circadian rhythm (Horodyski,1996; Matthews and Matthews, 1988). This behavior is common among lepidopterans. Cocoonase is an active enzyme that helps in softening the anterior part of the cocoon to allow escape for the moth by making an opening near the peduncle region (Unajak *et al.,* 2015, Pandey *et al.,* 2018,Gai *et al.,* 2020, Phoeurk *et al.,* 2021).

Cocoonase is also documented as a trypsin-like serine protease (Fukumori *et al.,* 2014; Anand *et al.,* 2021; Geng *et al.,* 2014; Yamazaki *et al.,* 1995). This proteolytic enzyme is produced and stored within the specific secretory cells in the galeae of moth, in the form of an inactive zymogen (Berger *et al.,* 1971). Later, the zymogen releases to the surface, activates, and then reduces to a dry deposit of principally pure enzyme (Kafatos *et al.,* 1967 a,b). During emergence, the enzyme dissolves in a copious potassium bicarbonate buffer secreted by the labial glands (Kafatos, 1968), and the pH of cocoonase is naturally adjusted to around 8.3 (Kafatos *et al.,* 1967a; Berger *et al.,*1971), which breaks down the sericin protein and enables the moth to escape from the cocoon. The tropical tasar silkworm *A. mylitta* is widespread and distributed in diverse geographical regions of India. Numerous ethologically and morphologically distinct ecotypes are known for this species that diverge in their primary food plant specificity (Chakraborty *et al.,* 2015).

Till date 44 ecoraces are documented in this species, which forage primarily on *Shorea robusta* and *Terminalia* species and also on a number of secondary food plants (Jolly *et al.,* 1974). The ecoraces are uni, bi or trivoltine based on the geographical environment and vary from each other in several quantitative and qualitative traits (Srivastav *et al.,* 2002) such as the weight of cocoon and its color, color of larvae and many other prospects. Ecoraces were created as a result of distinct life cycle variances that arose from the foraging of silkworms on a variety of food plants, annual rainfall, day length, and plant succession, among other characteristics like latitude, longitude, altitude, and other factors. (Srivastava *et al.,* 2004).

In the present study, main focus was to collect *A. mylitta* cocoons of various ecoraces, which are geographically distributed across different parts of India, to observe the cocoon characteristics, and pattern of cocoonase secretion during emergence. In addition, the thermal stability of *A. mylitta* cocoonase and the correlation between quantity and the concentration of cocoonase secretion were observed.

Materials and Methods

Cocoons and cocoonase collection: Cocoons of eleven ecoraces of tasar silkworm *A. mylitta* were collected from their native eco-pockets of India. The rearing of Daba (B.V. and T.V.) ecoraces was performed at Central Tasar Research and Training Institute, Ranchi. Cocoonase collection during moth emergence was carried out following the standard laboratory protocol of Pandey *et al.* (2018). Before cocoonase collection late stage pupae were identified and selected for cocoonase collection and are kept in an upside down position over a funnel. During emergence the pupae produces a crackling sound due to breaking of the pupae exoskeleton, the cocoonase exudes out from the mouth part region and gets deposited in the test tube. It takes around 1.5 - 2 hr for oozing out approximately 800-1500µl of cocoonase. After the required volume of cocoonase is collected, it needs to be removed from the test tube in which it was being collected to avoid any excretory contamination from the emerged moth.

Protein purification, quantification and gel electrophoresis: The native cocoonase was collected and purified using Sephadex G-50 column (Himedia). After purification, the protein was concentrated using acetone precipitation. Thereafter, the cocoonase was quantified by the Bradford (1976) method with slight modifications. Followed by protein separation using 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis which was carried out according to the method of Laemmli (1970). The gel after electrophoresis was stained in Coomassie brilliant blue R-250 overnight and was then destained to visualize the cocoonase band.

Gel diffusion assay for cocoonase: To check the specificity of cocoonase as a protease towards its substrate, a gel diffusion assay was performed. For this, the casein plate medium was prepared following the method of Zang *et al.* (2021). Thereafter, 10 ml of the medium-prepared mixture was poured into the plates in a laminar air flow chamber. The poured medium, containing casein as substrate was allowed to solidify for around 10 min, three holes were punctured once the medium was solidified. Three different temperature treatments were given to cocoonase before being poured into each of the holes. The cocoonase sample was divided into three aliquots, each of which was incubated in a water bath for 12 hrs at 37 °C, 55 °C and 60 °C, respectively.

Dual-substrate plate diffusion assay for cocoonase: The plates for protease assay were prepared containing 1% casein, 1% gelatin, and 1.5% agar (Montville,1983). The plates were punctured to make three holes. The cocoonase sample was divided into three aliquots, each of which was incubated in a water bath for 12 hrs at 37 °C, 55 °C and 60 °C, respectively. Ten

microlitre of each incubated cocoonase sample was poured into each hole and the plates were incubated at 37 °C for 5 hrs in the incubator. After incubation, the plates were observed for the appearance of hydrolysis ring.

Temperature stability test of cocoonase: To check the temperature stability, the cocoon strips were cut in uniform dimensions and were soaked in cocoonase followed by incubation at 35 ºC, 45 ºC, 50 ºC and 60 ºC. After every 1 hr, the cocoon strips were observed to check the proteolytic activity of each incubated cocoonase at respective incubation temperature.

Statistical analysis: Student's t-test was used to statistically analyse the data using Microsoft Excel 2007.

Results and Discussion

The silkworm *A. mylitta* is a polyphagous sericigenous insect also known as tropical tasar silkworm which is designated as a wild silk moth species spread across the forest belts in different states of India (Gattu *et al.,* 2021, Saha *et al.,* 2008, Renuka and Shamitha, 2016). The tender leaves of *Shorea robusta, Terminalia tomentosa* and *T. arjuna* are the major food plants for tasar silkworms (Jolly *et al.,* 1968). The diverse population of this silkworm present in various forest patches are known as ecorace which are genetically adapted to particular habitats (Renuka and Shamitha, 2016). In the present study, cocoons of eleven different ecoraces were collected from different geographical ecopockets of India (Fig.1). Moreover, the study revealed that different ecoraces of *A. mylitta* of same species showed varied differences in terms of food plants preference (Renuka and Shamitha, 2016), cocoon size (Srivastava *et al.,* 2002), thickness as well as in concentration and quantity of cocoonase secretion (Table1).

Moreover, cocoonase collected from various ecoraces (Table 1). is concerned, variations were observed in the volume of cocoonase secreted and their protein concentration in each sample of cocoonase collected from different ecoraces. Variations in the primary food plants were also observed among them which might be due to the availability of resources/ plantations at the particular ecopocket. Among the eleven ecoraces mentioned here, except for Daba (B.V and T.V ecorace), which is a semi-domesticated (Kar *et al.,* 2005) type whereas, all other nine discussed here were wild types that are generally reared in the forest area. These, wild-type silkworms have to face many challenges to survive in the forest environment conditions (Kumar *et al.,* 2020). In the forest belts, they have to deal with various pests and predators as well as the forest environmental conditions, that are most challenging for the silkworm to survive (Agrawal and Seth 1999). Interestingly, according to the present observation with respect to the geographical distribution of *A. mylitta* silkworm species, it can be inferred that the variations in cocoon size and stiffness or

Table 1: Details of various ecoraces collected from different ecopockets of India, their food plants and quantitative variations in cocoonase secreted by silkworm of respective ecoraces.

Antheraea mylitta ecoraces	Places of cocoons collection	Primary food plant	Volume of cocoonase secretion (µl)	Protein in secreted cocoonase (mg ml^{-1})
Modal	Mayurbhanj, Odisha	*S. robusta*	1800-2000	5.00 ±0.53
Raily	Bastar, Chhattisgarh	*S. robusta*	1700 -2200	5.50±0.57
Jhanjhi	Mayurbhanj, Odisha	*S. robusta*	1500 -1800	5.48±0.69
Barf	Korba, Chhattisgarh	*S. robusta*	800 -1500	4.15±0 .25
Bhandara	Bhandara, Maharashtra	*T. tomentosa*	800 -1500	4.20 ±0.30
Jata	Keonjhar, Odisha	*T. tomentosa*	800 - 1400	4.25 ±0 .27
Sarihan	Dumka, Jharkhand	*T. arjuna*	800 -1500	4.18 ± 0.75
Daba (TV)	Ranchi, Jharkhand	*T. arjuna*	900 -1400	3.06± 0.50
Nalia	Mayurbhanj, Odisha	*S. robusta*	800 - 1100	5.20 ± 0.25
Daba (BV)	Ranchi, Jharkhand	*T. arjuna*	1000 -1500	3.19 ±0.50
Andhra local	Adilabad, Telangana	*T. arjuna*	900-1000	3.0 ±0.47

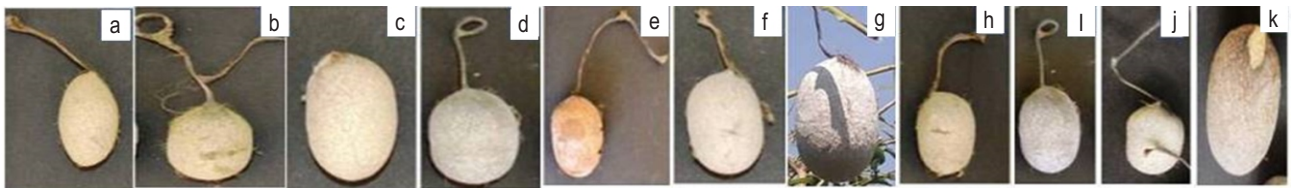

Fig.1: Cocoons of various ecoraces collected from different ecopockets of India (a) Andhra local, (b) Jata, (c) Bhandara, (d) Modal, (e) Nalia, (f) Sarihan, (g) Daba, (TV), (h) Jhanjhi, (i) Daba, (BV), (j) Barf and (k) Raily.

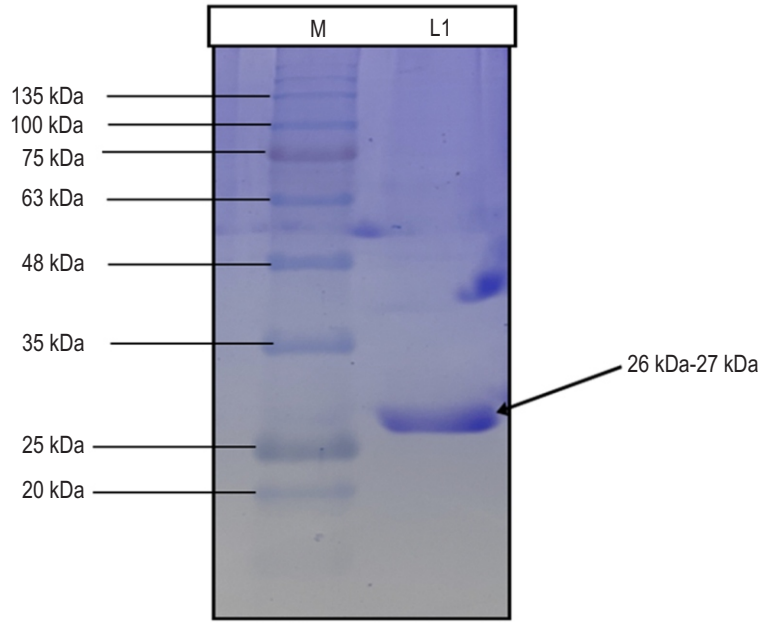

Fig. 2: Separation of purified *A. mylitta* cocoonase on 12% SDS-PAGE. Lane M: shows the prestained protein marker of Himedia. Lane L1: shows a purified cocoonase band of 26-27kDa.

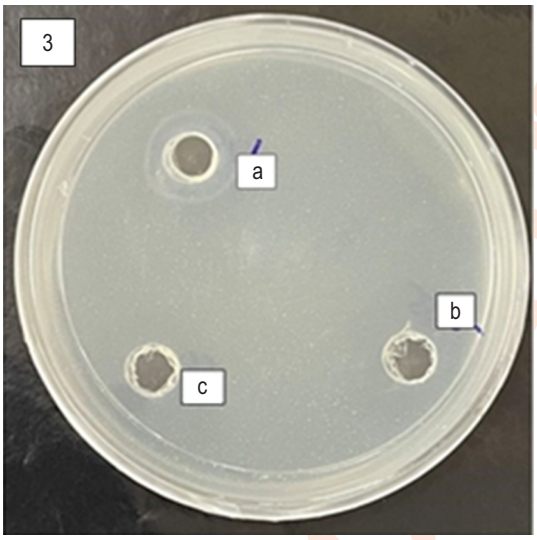

Fig. 3: Gel diffusion assay: (a) 10µl cocoonase incubated at 37 °C, (b) 10 µl cocoonase incubated at 55°C and (c) 10 µl cocoonase incubated at 60°C.

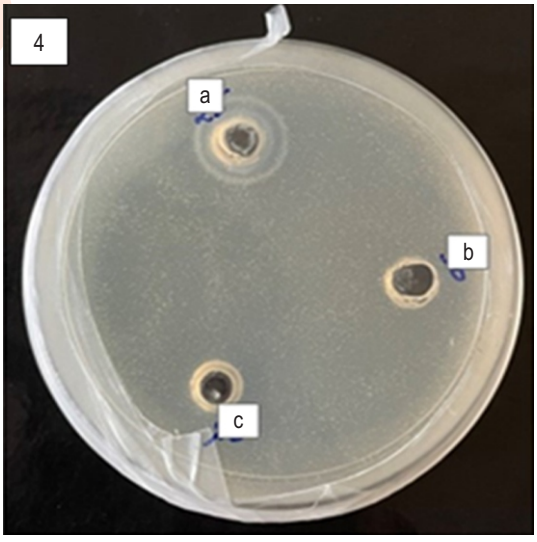

Fig. 4: Dual-substrate plate diffusion assay: (a) 10 µl cocoonase at 37 °C, (b) 10 µl cocoonase incubated at 55°C and (c) 10 µl cocoonase incubated at 60°C.

hardness might be due to their adaptation to different ecopockets (Acharya *et al.,* 2017; Deka and Kumari, 2013) and the variations in concentration and volume of cocoonase can be correlated to the quantity and concentration of enzyme sufficiently essential for softening the anterior position of the cocoon to let an easy exit of the moth out of cocoon during emergence. Hence, it can be assumed that the quantity and quality of cocoonase secreted directly depends upon the thickness of the cocoon.

The thickness of cocoon, which is one of the quantitative traits of cocoon phenotype (Deka and Kumari, 2013; Nayak and Guru, 1998), is directly related to food plants (Jena *et al.,* 2021)

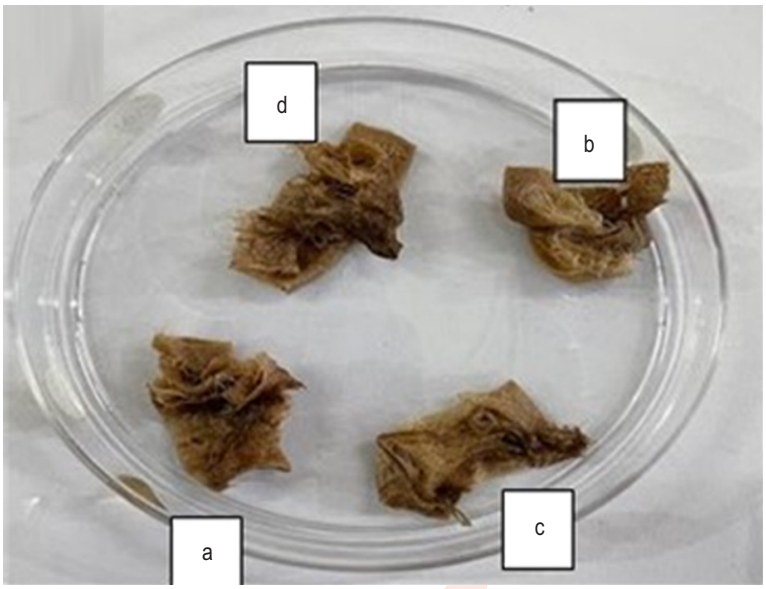

Fig. 5: Cocoonase activity on cocoon strips incubated in cocoonase at various temperatures (a) 35°C; (b) 45°C; © 50°C and (d) 60°C.

that silkworm consumes, which in turn is directly related to the ecopockets the *A. mylitta* silkworm species are adapted to dwell. Although, further study is needed to establish a concrete conclusion for this observation and also to find out the most active cocoonase among the naturally available cocoonase of various ecoraces. In this study, the proteolytic specificity of purified cocoonase (Fig. 2) was confirmed through gel diffusion assay using casein (Unajak *et al.,* 2015; Kafatos *et al.,* 1967b) and gelatin (Kafatos *et al.,* 1967b; Kafatos and Williams,1964) as probable substrates. In addition to the substrate specificity of cocoonase, the gel diffusion assay also evidenced the thermal stability of *A. mylitta* cocoonase. Within 4 hr of incubation, a hydrolytic ring was observed in the hole in which cocoonase incubated at 37°C was poured confirming the protease activity with both types of substrate plates (Fig. 3-Fig. 4). Whereas, no hydrolytic ring was observed in the hole in which cocoonase incubated at 55°C and 60°C were poured. From this study, it is inferred that heat treatment at 55°C and 60°C might have affected the proteolytic activity of cocoonase due to which hydrolytic ring could not appear even after 4 hr of incubation which might be due to denaturation of the cocoonase enzyme because of high-temperature treatment.

To further, confirm the effect of temperature treatment on the activity of cocoonase attempts have been made to observe the enzyme-substrate activity using sericin, which is a natural substrate (Pandey *et al.,* 2011) of cocoonase. It was observed that the strips incubated in cocoonase at 35°C and 45°C showed fast loosening of strips in comparison to those incubated at 50°C and 60°C, and the strip incubated at 60°C showed very less loosening of fiber and was stiff from inside (Fig. 5). This observation infers that cocoonase activity towards the breakdown of its substrate is temperature dependent. Cocoonase specifically acts upon sericin (Sakata *et al.,* 2022) to loosen the silk fiber at suitable pH and temperature (Unajak *et al.,* 2015).

Thus, the cocoonase actively digested the sericin of the cocoon strips incubated in cocoonase at 35°C and 45°C, however, cocoonase activity was decreased at higher temperatures due to denaturation. This observation, preliminarily concludes that cocoonase remains proteolytically stable around 45°C, which is an indication for cocoonase to be one of the stable proteases. However, to further confirm the temperature stability other quantitative thermal analysis such as Circular Dichroism spectroscopy, Differential Scanning Calorimetry, and other methods need to be carried out in future to confirm the thermal stability of cocoonase. Moreover, the present study concludes that *Antheraea mylitta* cocoonase is an explorable protease that needs further detailed exploration to unveil the unspecified properties of this insect-secreted protease, including the cocoonase of various ecoraces, which can find beneficial applications in silk and other industries.

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Authors' contribution: J.P. Pandey: Suggested the study, carried out its design, interpreted the results and approved the final manuscript; **A. Rani:** Carried out the experiments, recorded the observations and wrote the MS; **K. Jena, K. Sathyanarayana and D.M. Pandey:** Language editing, interpretation of the results and data analyses.

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