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**INTERNATIONAL JOURNAL OF
 ADVANCED RESEARCH (IJAR)**

Article DOI: 10.21474/IJAR01/7305
 DOI URL: <http://dx.doi.org/10.21474/IJAR01/7305>



RESEARCH ARTICLE

PROSPECTIVE UTILIZATION OF *ANTHRAEA MYLITTA* COCOONASE AND ITS MOLECULAR HARMONY WITH NATURE.

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Manuscript Info

Manuscript History

Received: 17 April 2018
 Final Accepted: 19 May 2018
 Published: June 2018

Keywords:-

Cocoonase, harmony, natural, molecular sculpt, *Antheraea mylitta*, silk surface.

Abstract

Cocoonase is a proteolytic enzyme released by *Antheraea mylitta* during pupal-adult emergence. Interestingly, as a natural-molecular-mechanism, around 600-900µl enzyme get sprayed by emerging insect with the concentration of 221µg/ml which specifically target insect glue protein sericin without affecting the fibroin protein. This natural-reaction makes anterior portion of cocoons soft to facilitate the moth (adult) emergence. Moreover, cocoonase is the un-utilized crucial by-product of tasar silk industry. Therefore, in the present study molecular sculpt of cocoonase has been prepared to understand its molecular harmony with nature in order to utilize this by-product in future. Native cocoonase secretion process, place of secretion, secretion volume, sequential changes in cocoonase concentration, chronological secretion profile, quantification, purification by sephadexG100 column, characterization, proper storage conditions, enzymatic action and enzyme activity has been worked out successfully. SDS-PAGE analysis of purified cocoonase showed molecular weight 25-26 kDa. Elemental-mapping/profiling of secreted cocoonase, suitable condition for specific activity of cocoonase, utilization of purified native cocoonase for cocoon softening has been done. Around 2150 ml of cocoonase collection from 3000 emerging tasar silkworm has been done successfully. Tasar silk SEM study showed marked variation in silk fiber surface when cocoon softening was done using cocoonase. Maldi Tof-Tof (MS and MSMS) data of *A. mylitta* cocoonase MS 1320.477 showed similarities with cocoonase/proteolytic enzyme of other sericigenous insects. *Antheraea* cocoonase secretion showed it close harmony with nature. It is projected that, by using this enzyme, softening of tasar cocoon can be conducted to develop an enzyme based eco-friendly cocoon cooking and reeling technology in order to produce organic tasar silk with natural beautiful color.

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Introduction:-

Tasar silkworm, *Antheraea mylitta* is an economically important sericigenous insect produced silk at the end of larval stage in the form of cocoons shell. Silk cocoons mainly consist of two main proteins namely sericin and

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fibroin. It is observed that during pupa-adult metamorphosis moth secretes proteolytic enzyme cocoonase. This proteolytic enzyme is one of the un-utilized by-products of tasar silk industry. Cocoonase is having efficacy to soften anterior portion of cocoons and adult moths comes out from silk cocoons. Therefore, a study towards utilization of cocoonase in cocoon cooking has been conducted. It is reported that, several sericigenous insects including *A. mylitta* exude a proteolytic enzyme cocoonase as they near the final stages of their metamorphosis. This proteolytic enzyme makes anterior portion of cocoon soft which facilitates the moth exit from cocoon (Kafatos and Williams, 1964; Felsted *et al.*, 1973; Pandey *et al.*, 2011; Unajak *et al.*, 2015). Studies on cocoonase of different sericigenous insects have been made by number of researchers (Felsted *et al.*, 1973, Liu and Li 2002; Wang *et al.*, 2005; Wang *et al.*, 2005a; Yu-Dan, 2008; Wu *et al.*, 2008; Wang, 2008; Yang *et al.*, 2009; Rodbumer *et al.*, 2012; Fukumori *et al.*, 2014; Geng *et al.*, 2014; Unajak *et al.*, 2015) without much correlation with its possible-efficacy in cocoon cooking. It is proven that cocoonase directly acts on the sericin protein without affecting the fibroin protein. The SDS-PAGE analysis of freshly collected cocoonase showed molecular weight around 25-26 kDa. Although, our initial result indicates cocoon processing/cooking in cocoonase retains the tasar silk yarn natural beautiful unique tasar silk color, softness and luster (Pandey *et al.*, 2011) but utilization of *A. mylitta* cocoonase and its molecular harmony with nature has not been studied much. Therefore, present study was conducted to study the prospective utilization of *Antheraea mylitta* cocoonase and its molecular harmony with nature.

Methodology:-

Large scale rearing of *A. mylitta*:

Large scale rearing of tropical tasar silkworm, *A. mylitta* Drury (Daba ecorace) was conducted in outdoor conditions and larvae were fed on leaves of *Terminalia tomentosa*. After spinning, 3000 cocoons were collected and stored at room temperature in grainage house which was utilized for cocoonase collection.

Cocoonase collection

The specific stage for proteolytic enzyme cocoonase collection was identified. These pupae were allowed to get ready for adult emergence which was monitored based on changes in integument color. The pupae close to adult emergence alter their integument color from natural red-brownish to black with its loosening. Before emergence, pupae were transferred to cocoonase collection set-up for cocoonase secretion and collection.

Cocoonase quantification, purification and MALDI analysis

Purified cocoonase enzyme activity was assayed as per standard protocol with slight modification. (Pandey *et al.*, 2011). Cocoonase activity was assayed spectrophotometrically at 255 nm and subsequent kinetic comparisons were calculated using the k_{cat} values. Standardization of cocoonase activity was performed at different pH, temperature and substrate concentration. Protein estimation (Bradford, 1976) with slight modifications. SDS-PAGE analysis (Laemmli, 1970) with slight modifications was also done. Cocoonase was purified using Sephadex 50-column. Purified cocoonase MALDI analysis was carried out as per standard protocol.

Cocoon softening in cocoonase and scanning electron microscopy (SEM) of silk

Impact of native cocoonase on *A. mylitta* DABA cocoons softening was assessed as per post cocoons technology protocol (Addis *et al.*, 2011) with slight modifications. Silk surface modification was observed through SEM (Mondal *et al.*, 2007). Simultaneously, element analysis was done using element analyzer.

Recoup of cocoonase sequences and its molecular harmony with nature

The complete coding nucleotide and protein sequences of cocoonase were recovered from NCBI database and analysis was conducted by using bioinformatics tools. NCBI database available sericigenous insect cocoonase molecular similarity and harmony with other variants has been conducted.

Data Analysis:-

The data was subjected to statistical analysis using Student's t-test. Microsoft Excel 2007 software was used to analyze the data. Molecular data was analyzed by using bioinformatics tools.

Results And Discussion:-

Cocoonase is a proteolytic enzyme secreted by many sericigenous insect including tasar silkworm *A. mylitta* during pupal-adult-eclosion. This enzyme is by-products of tasar silk industry. It was observed that, emerging adults exude around 500-850 μ l cocoonase which soften the anterior portion of cocoon shell (peduncle region) and facilitates

emergence of moths (Fig 1). Interestingly, cocoonase was secreted by *A. mylitta* very slowly drop-by-drop and this secretion process continue 2-4 hrs. It is noticed that, cocoonase directly acts on the sericin protein without affecting the fibroin protein (silk fiber). It evidently indicates that, sericin is excellent natural-substrate for cocoonase. This natural observable fact generates an idea to use cocoonase for cocoon softening and silk surface modification in order to minimize chemical based cocoon-cooking/silk processing. Existing chemical methods influence the natural unique beautiful color and soft texture of tasar silk.

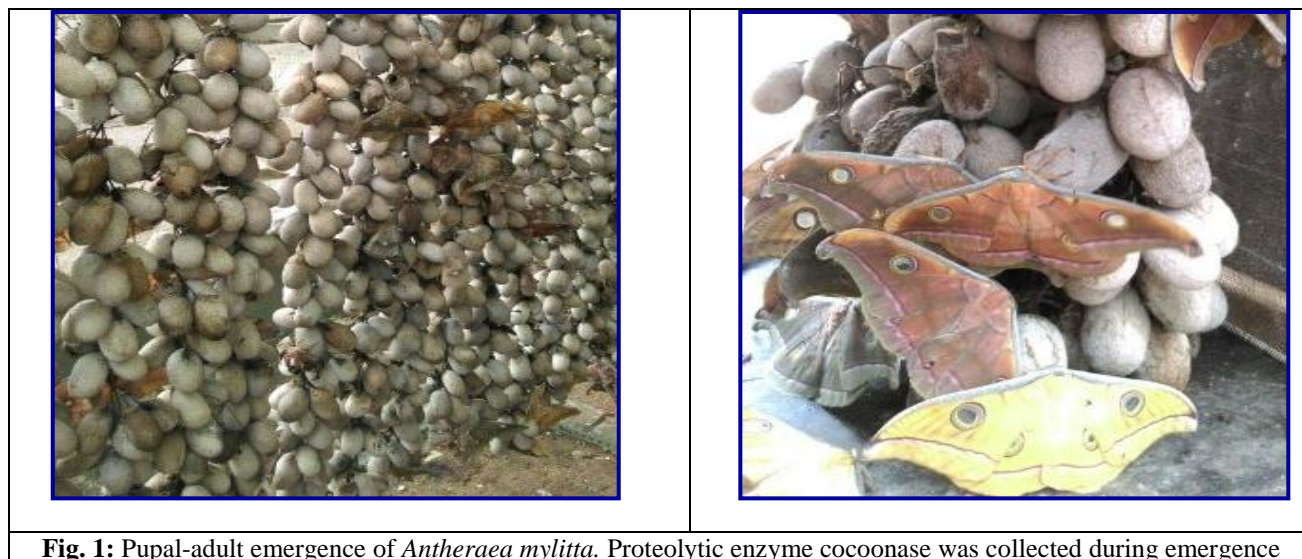


Fig. 1: Pupal-adult emergence of *Antheraea mylitta*. Proteolytic enzyme cocoonase was collected during emergence

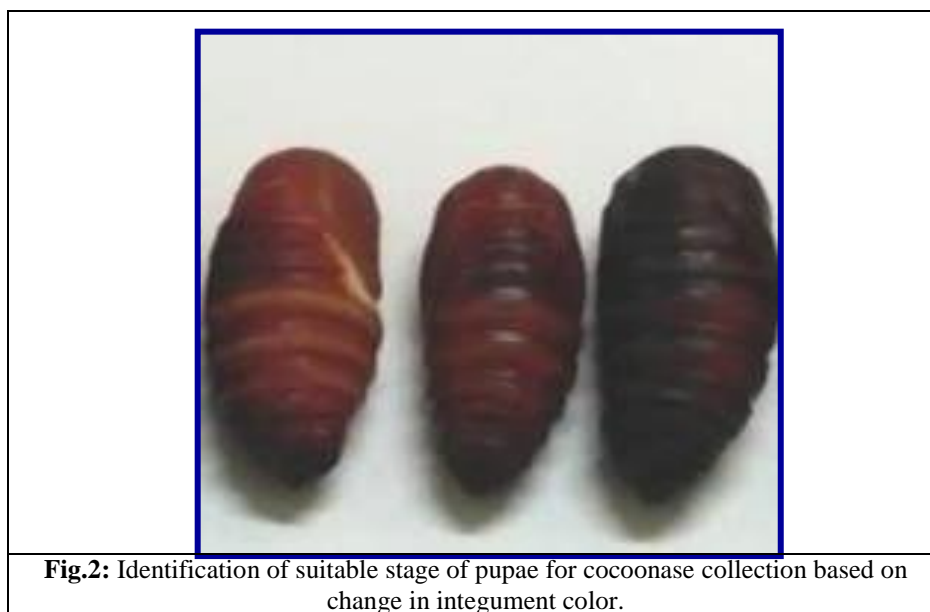


Fig.2: Identification of suitable stage of pupae for cocoonase collection based on change in integument color.

Identification of suitable stage of pupae for cocoonase collection based on change in integument color. Pupae having dark black color integument is most suitable stage for cocoonase collection (Fig 2). Our experiments suggest cocoonase rational relevance and molecular harmony in silk surface modification. Using native enzyme, softening of tasar cocoon can be conducted which showed prospective relevance to develop an enzyme based eco-friendly cocoon cooking and reeling technique in order to produce organic tasar silk with natural beautiful color. Around 2150 ml of cocoonase collection from 3000 emerging tasar silkworm has been done successfully. Identification of crucial stage for cocoonase collection was done based on temporal changes in color of pupae integument. In addition, native cocoonase secretion process, place of secretion, secretion volume, sequential changes in cocoonase concentration, chronological secretion profile, quantification, purification by sephadexG100 column, initial

characterization, proper storage conditions, enzymatic action and enzyme activity has been worked out successfully (Fig 1-4). SDS-PAGE analysis of purified cocoonase showed molecular weight 25-26 kDa (Fig.3). Per insect cocoonase collection volume is 500 to 800 μ l volume with 221 μ g/ml concentration. Recognition of sericin as natural substrate of cocoonase and it is established that cocoonase directly acts on the sericin without affecting the fibroin protein. Elemental-mapping/profiling of secreted cocoonase, suitable condition for specific activity of cocoonase, utilization of purified native cocoonase for cocoon softening has been done. Results indicate that cocoonase reutilization (one time) is also possible. SEM study showed marked variation in silk fiber surface when cocoon softening was done using cocoonase. Maldi Tof-Tof (MS and MSMS) data A& B of *A. mylitta* cocoonase MS 1320.477 showed similarities with cocoonase/proteolytic enzyme of other sericigenous insects. Molecular matching of *Antheraea* spp. cocoonase was also performed in order to identify the possible variants available in nature. Silk cocoons mainly consist of two main proteins, sericin and fibroin, with a glue-like layer of sericin coating two singular filaments of fibroin. Cocoon shell contains around 68-74% fibroin and 21-26% sericin. It is noticed that, cocoonase directly acts on the sericin protein without affecting the fibroin protein (silk fiber). It obviously indicates that, sericin is excellent natural-substrate for cocoonase. Based on sequential change in secreted cocoonase concentration specific time for cocoonase expression has been identified. Interestingly, the chronological changes in native cocoonase concentration were observed. Maximum concentration was observed in early collection followed by mid and late. The cocoonase secretion was more during first 30 minutes. Around 260-300 μ l of cocoonase was secreted during this period, 150-200 μ l cocoonase was secreted during 30-60 minutes and 200-300 μ l in last 60-120 minutes. Concentration of various elements present in naturally secreted liquid cocoonase was done. The carbon nitrogen (C/N ratio) ratio of cocoonase was 17.04 by using element analyzer. The NCBI database available sericigenous insects cocoonase sequences were analyzed by various web based online bioinformatics tools. For prediction of protein structure from the sequence procured was constructed from the newly constructed sequence. Molecular matching of cocoonase was also performed and predicted possible variants available in nature.

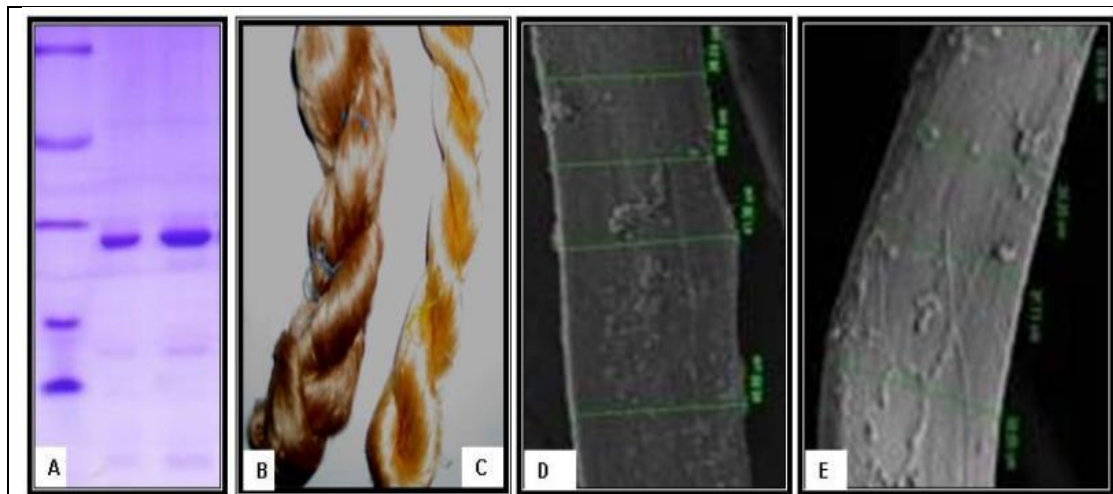


Fig.3: Impact of *Antheraea mylitta* native cocoonase on silk surface. **A.** 12.5% SDS-PAGE analysis showing 25-26kDa cocoonase purified using Sephadex G100 column. **B.** Morphology of silk yarn obtained after cocoon softening in cocoonase **C.** Morphology of silk yarn obtained after cocoon softening in soap & soda. **D.** Scanning Electron Microscopy (SEM) showing surface morphology of silk yarn obtained after cocoon softening in cocoonase and **E.** existing method (using soap & soda).

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