Total Proteolytic Activity on The Pupal Rectal Sac of *Bombyx mori* L. (Lepidoptera: Bombycidae)

Bombyx mori L. (Lepidoptera: Bombycidae)'ye Ait Pupa Rektal Kesesindeki Toplam Proteolitik Aktivite

Research Article

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ABSTRACT

This study is aimed to demonstrate histologically secretory materials of the rectal sac and also determine total proteolytic enzyme activity of sac's fluid of silkworm, *Bombyx mori* considered to be responsible for adult eclosion from its cocoon. The rectal sacs of pupae were fixed with Bouin's solution. The sections taken from the sac were stained by Paraldehyde Fuchsin and Periodic acid-Schiff dyes. Total protein concentrations and proteolytic activities of sac contents were determined by Bradford method at 595nm and azocasein substrate at 420nm, respectively. The secretions -proteinic and lipoid- were determined by different stains. As the sac grew up gradually, these two secretory materials were separated into darker and lighter phases. Protein concentration existed in the darker phase of secretion was higher than that in the lighter one. The total proteolytic activity measurements of the lighter phase were higher than the darker one, in both sexes. It was found that the activity of lighter phase in male is higher than that of female on the 10th day. This result supports that male moths escape earlier from cocoon than the female moths. Consequently, proteinic secretion synthesized by some cells of rectal sac could play a role in emerging from their cocoons of moths.

Key Words
Bombyx mori pupa, rectal sac, proteolytic activity, azocasein.

ÖZET

Bu çalışmada ipekböceği, *Bombyx mori* ergininin kozasından çıkışında etkin olduğu düşünülen rektal kese salgısının hem histolojik olarak gösterilmesi hem de toplam proteolitik enzim aktivitesinin belirlenmesi amaçlanmıştır. Pupaya ait rektal keseler Bouin solüsyonu ile tespit edildi. Kesesinin bölümleri Paraldehit fuksin ve Periyodik asit-Schiff boyası ile boyandı. Kese içeriklerinin toplam protein derişimi ve proteolitik aktivitesi sırasıyla Bradford metodu ile 595 nm'de ve azokazein substratı ile 420 nm'de belirlendi. Sekresyonlar -proteinik ve lipoid- farklı suşlar kullanılarak belirlendi. Kese giderek büyürken, bu iki salgı malzemesi koyu ve açık fazlara ayrıldı. Salgının koyu fazı içerisindeki mevcut protein derişimi, açık fazın sahip olduğu miktardan daha yüksektir. Açık fazın toplam proteolitik aktivite ölçümleri her iki cinste de, koyu fazdakine gore bir daha yüksektir. 10. günde, erkekteki açık faz aktivitesinin dişidekine göre daha yüksek olduğu tespit edildi. Bu sonuç, erkek kelebeklerin dişi kelebeklerden daha once kozasından ayrıldığı bilgisini desteklemektedir. Sonuç olarak, rektal keseye ait bazı hücreler tarafından sentezlenen proteinik salgı kelebeklerin kendi kozasından çıkmasında önemli bir rol oynayabilmektedir.

Anahtar Kelimeler
Bombyx mori pupa, rektal kese, proteolitik aktivite, azokazein.

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INTRODUCTION

In insects, especially in Lepidoptera species, the rectal sac which is common part of the digestive and excretory systems is formed during development [1]. The walls of the rectal sacs are observed to be dotted with hundreds of rectal pads which are responsible for reabsorbing the water and solutes [2]. The rectal pads are thought to have a mechanism involving a membrane system allowing transport of fluid across epithelia by osmosis [1, 3-7]. In different insect groups, these rectal pads are described with different number of cell layers [8], but they are indicated absent in some Lepidoptera species [9]. In *Bombyx mori* L. (Lepidoptera: Bombycidae), rectal sac consists of epithelium, loose connective tissue and a lot of rectal pads. These rectal pads possess an outer cortex and an inner medulla. The cortex is formed by squamous epithelium with spindle-shaped nuclei. The medulla has two to five big pyramidal epithelial cells with rather large nuclei [10].

Silkworms are protected in silken cocoons in the course of their pupal development. In *B. mori* and in other silkworms the cocoon wall is softened and dissolved with liquid containing a proteolytic enzyme secreted from the mouth of the pharate adults [11-14]. Despite reaching the more information about the digestive system, its enzymes and the excretory systems in silkworms, the data about the morphology and the role of the rectal sac especially in their pupal stage is very limited. It, therefore, seems to be important to investigate the nature of this proteolytic enzyme and to know the functional involvement of the sac in silkworm development. It is considered as the proteases responsible for the eclosion of silkworm from the cocoon which is known to be secreted by several organs. However, it has been pointed out that crop, maxillae and midgut are important in such secretions [11, 13-15]. The midgut protease in the pharate adult is one of the sources of the cocoon-digestion enzyme which changes its nature in the crop [13, 15, 16]. According to findings of Eguchi and Iwamoto [15], some reservoir organs concerned with the production and utilization of cocoon-digesting enzymes, work in a combined system with the midgut. The same researches have shown that the proteolytic activity in the midgut of pharate adults reaches a peak just before emergence and falls steeply afterwards and only a traceable amount of enzyme exists in the midgut. Although the existing literature has mainly been focused on the crop, the appearance of the rectal sac in the late pupae and pharate adult is found to coincide with the storage function related not only with the excretion but also with some other activities [14,17].

The contents of the rectal sac have two-phased fluids (light and dark) including cocoon-digesting enzymes. During the emergence from the cocoon, adult silkworms release a fluid which is the same colour as the dark fluid in the rectal sac of pupa. When moths are dissected, the rectal sac appears as an air-filled membranous structure [14].

Although being one of the key regulators of eclosion, the actual role of rectal sac is presently not well-known. The present study has been designed to undertake two goals: 1) to determine total proteolytic activity of the fluids of rectal sac in late pupa 2) to describe a correlation between released fluid and aforementioned role of the rectal sac in eclosion.

MATERIALS and METHODS

Animals

The silkworm larvae were reared on fresh mulberry leaves in laboratory conditions at 24 ± 1°C and 70% relative humidity [18,19]. For each insect, passing from larva to pupae (Day 0) was monitored 5-6 days after the onset of cocoon shell formation. Rectal sac began to appear on the 6th day pupae and was dissected on the 6th, 8th, and 10th days. Number of dissected insects on the 6th day was more than that of the 10th day for obtaining a sufficient amount of the sac content.

Preparation

For light microscopy, the rectal sacs were embedded in paraffin wax and sectioned at 5 μm, stained with Periodic Acid Schiff (PAS) and Paraldehyde Fuchsin (PAF) for showing the fluid of rectal sac [20-22]. Evaluation of preparations was made using an Olympus BX51T-32P01 type research system microscope and photographs were made using Olympus CX31-Altra 20 soft imaging system.

Lyophilization

Lyophilization (Edwards Freeze Dryer, UK) was performed for 2 days. This process was carried out due to density of lighter phase in sac content, dissolving and diluting procedures were accomplished easily.

Total Protein Concentration

Total protein concentration was determined according to the Bradford method [23]. Bovine serum albumin (BSA, Sigma, A-4503, USA) was used to create a standard reference. Absorbance was measured at 595 nm with a spectrophotometer (Jasco V-530 UV/VIS spectrophotometer).

Total Proteolytic Activity

Total proteolytic activity in rectal sac was determined by the method of Pereira et al. [24] in absorbonce at 420nm (ΔAbs) min^{-1} mg/protein (unit) (Jasco V-530 UV/VIS spectrophotometer).

RESULTS

Histological Results

Secretory deposits were observed to be localized in the medulla of the rectal pads, among the epithelial cells and the connective tissue (Figure 1). Two types of secretions were determined in proteinic and lipoid character by differential staining. These secretions were seen in the lumen of the sac. Proteinic secretory material was synthesized in medullary cells of the rectal pads. However, it was postulated that lipoid secretion was synthesized by the epithelial cells of midgut and delivered to the rectal sac via hindgut. Synthesis and accumulation of the both secretion types increased in developmental process.

A great comparable difference was found in the amount of secretion granules on that day and on the 6th and 8th days (Figure 1a-d). No lipoid secretion was found in connective tissue and a decrease in the amount of the proteinic secretory material was obvious when it was compared with the 6 and 8th days (Figure 1a-d). The decreased secretion was supported by diminishing number of rectal pads in epithelial cells surrounding to sac. The maximum size of rectal sac was reached on the 10th day with an increasing storage (Figure 1e, f).

Total Protein Concentration Results

As can be seen in the table values, a significant difference was not observed between female and male. But, differences were found between the phases. It was occured that total protein concentration of dark phase was higher than that of light phase (measurement of total protein concentration of dark phase at male on the 10th day of pupal stage was 0.3584 mg and that of light phase was 0.0055 mg) (Figure 2).

Total Proteolytic Activity Results

Rectal sac content of light and dark phases considering the results of the total proteolytic activity was observed in the differences between male and female individuals. Total proteolytic activity of light phase was higher than that of dark phase (measurement of total proteolytic activity of light phase at male on the 10th day of pupal stage was 0.2969 unit and that of dark phase was 0.1772 unit) (Figure 3).

There was a difference found between light and dark phases of female and male in total proteolytic activity in that while total proteolytic activity of dark phase in female was 0.1175 unit, light phase was 0.1772 unit. Total proteolytic activity of light phase in male was 0.1175 unit higher than that of dark phase in was 0.0548 unit on the 10th day of pupal stage (Figure 3).

DISCUSSION

During adult development in especially Lepidoptera species, in *Hyalophora cecropia* [2] that the rectal sac is determinated to continue its development as a small size tubular-shaped organ of the digestive system from the 2nd of pupal stage. However, it is also followed in *Bombyx mori* that a ball-shaped rectal sac is developed as a common part of the digestive and excretory systems from the 5th day of pupal stage and reached its maximum size in late pupae. This result agrees with previous studies [10, 17]. Its content is observed as proteinic and lipoid substances which were in accordance with inactivated ecdysteroids that accumulate in rectal sac as well as meconium [25].

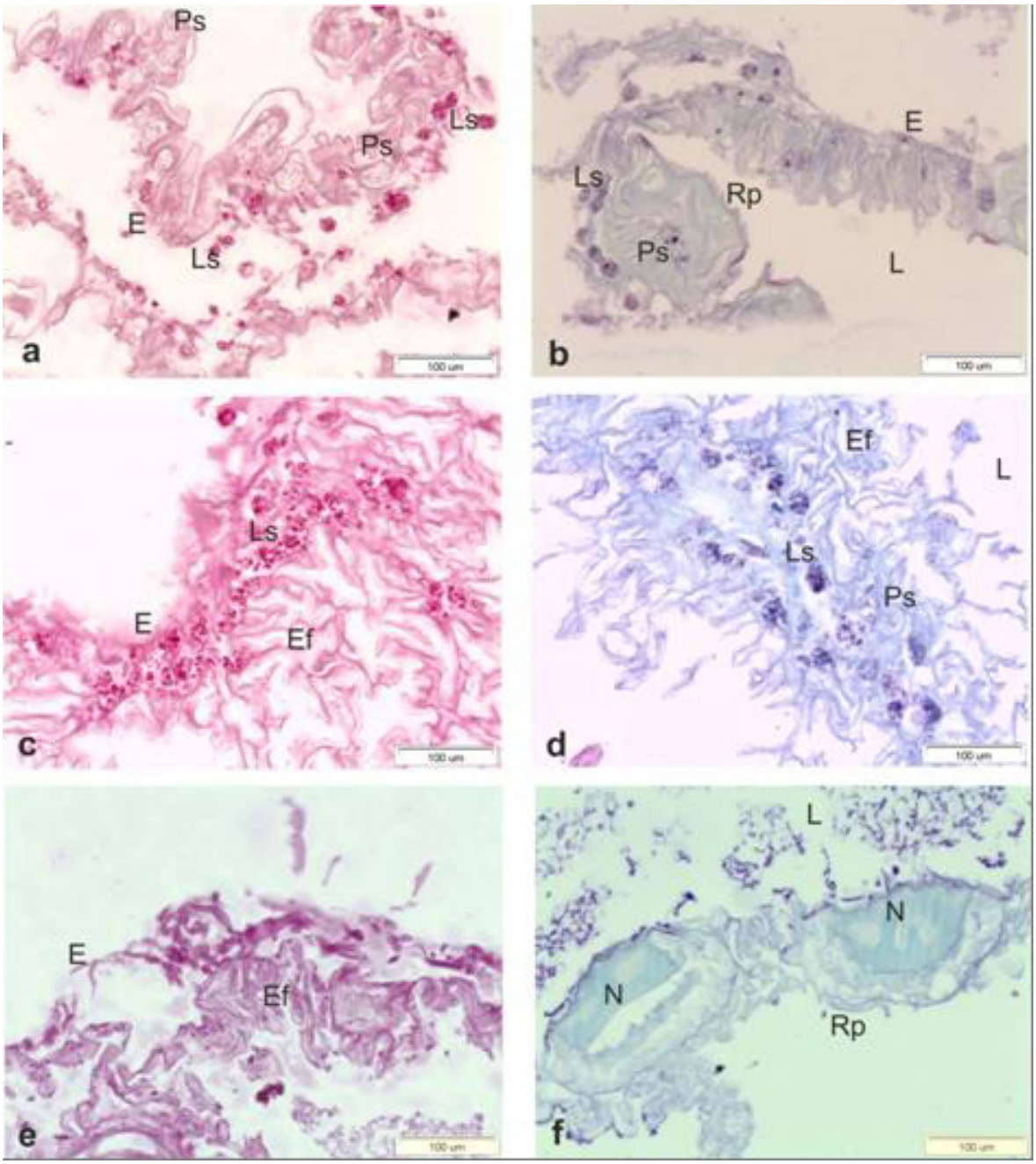

Figure 1. Demonstration of rectal sac secretions by different stains in pupae. a-b. 6th day, c-d. 8th day, e-f. 10th day. a-c-e. section/PAS, b-d-f. section/PAF. E; Epithelium, Ef; Epithelial folding, L; Lumen, Ls; Lipoid secretion, N; Nucleus of medullary cell, Ps; Proteinic secretion, Rp; Rectal pad.

Wild and domestic silkworms are protected in cocoon during pupal development. It has been determined for years that the emergence of pupa carried out by dissolving the cocoon by secreting a fluid of proteolytic nature [11, 13, 14]. The midgut and other organs, such as the crop and maxillary gland, were studied together as the production and usage of cocoon-digesting enzyme. Particularly, the crop is responsible for reservoiring and pumping. The proteases which the midgut synthesizes have reservoired in the crop [13-16]. The same investigators have indicated that the midgut proteases of the pharate adults hydrolyse the cocoon forming proteins, fibroin and sericin. Also, they have demonstrated that the protease activity in the midgut of the silkworm, *B. mori*, increased in the pharate adult, reached a peak just before emergence of the moth, decreased markedly

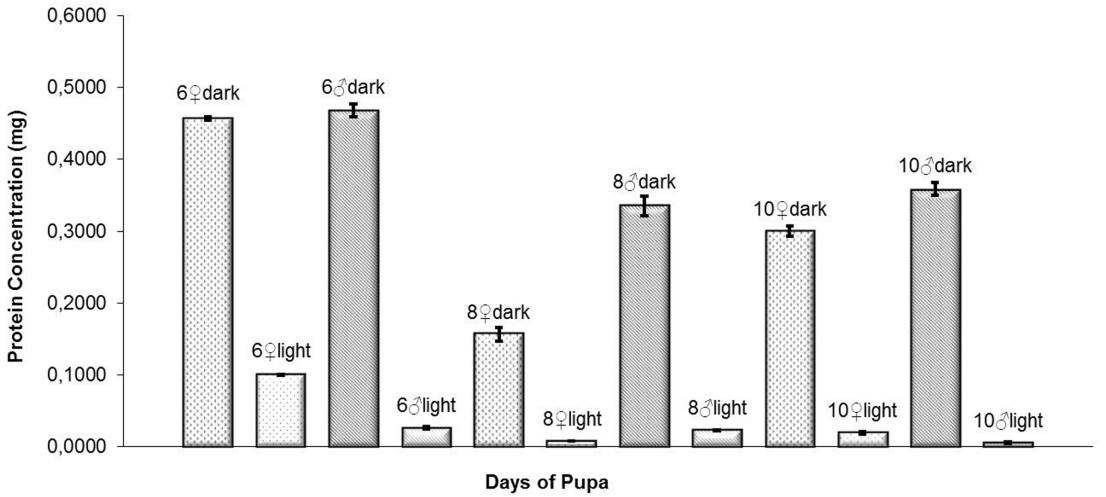

Figure 2. Total protein concentration of rectal sac secretions in pupae. The results were averages ±SD from three independent expriments.

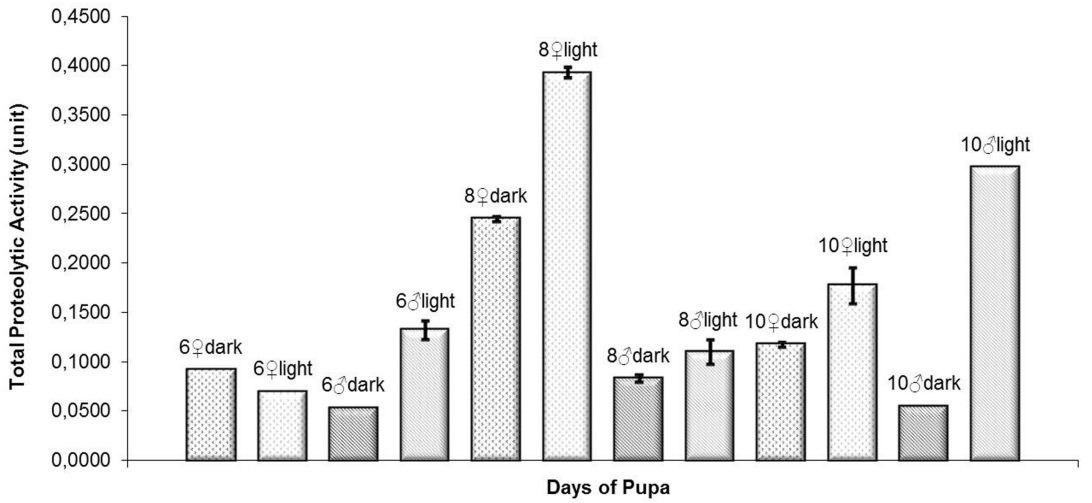

Figure 3. Total proteolytic activity of rectal sac secretions in pupae. The results were averages ±SD from three independent expriments.

thereafter. For this reason, it is pointed out that the midgut proteases of the pharate adult could be a source of the cocoon-digesting enzyme [13,14,16, 26]. Because the rectal sac is a reservoir organ closely related with the midgut, the discharge of the midgut content, in relation to cocoon-digestion, is achieved as an anal emission through the rectal sac while the crop leads to an oral emission [14].

In this study, it appeared that the proteinic secretion localized in the rectal pads, under the connective tissue and among the epithelial folding, the rectal sac in *B. mori* by staining with different ways. Lipoid secretion appeared around the pads and among the epithelial folding, as the sac increased in size. It was determined that secretion was gradually decreased around and inside of pads depending on developmental process and then disappeared completely.

The total proteolytic activity of light fluid phase of rectal sac in male pupae was shown higher than in females that is supported earlier emergence of male moths from pupal case than the females. This idea was supported by the results of total proteolytic activity of light phase being higher than

that of darker phase for both sexes on the 10th day of pupal stage.

In conclusion, histology of the rectal sac is primarily determined through its content with two different phases that might have a critical role for emergence of silkworms. Proteinic secretory material showing proteolytic enzyme activity, synthesized in medullary cells of the rectal pads, is in agreement with the proposition to be responsible for solving the cocoon.

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