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Proteolytic Enzyme in the Midgut during Metamorphosis of *Pieris rapae* L.

Kim, Hak Ryul and Sung Moon Yoe
(Department of Biology, Korea University, Seoul 132, Korea)

배추흰나비의 變態에 따른 中腸內 蛋白質分解酵素

金 學 烈·余 聖 文
(高麗大學校 理科大學 生物學科)

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摘 要

배추흰나비의 變態에 따른 中腸內 蛋白質分解酵素의 變化, 分布 및 性質을 調査 分析하였다.

中腸內 蛋白質分解酵素는 5齡 幼蟲과 前蛹期에서 變態直前에 높은 活性이 나타났으나 蛹時期에는 變態後 1日에 가장 높았다. 또한 中腸內의 optimum pH는 5齡 幼蟲에서 pH 8, 前蛹期에서 pH 6.5, 羽化直前에 pH 8.5로 각각 相異하게 나타났다.

細胞內 酵素 分布는 5齡에서는 주로 中腸의 組織에서 發見되었으나 이후 羽化直前까지는 주로 中腸 內容物에서 發見되었고 中腸 組織에서는 거의 식별할 수 없었다. 이러한 結果로 보아 蛋白質分解酵素는 幼蟲期동안 中腸組織에서 合成되었다가 蛹化期에 中腸 內腔으로 放出되는 것 같다.

INTRODUCTION

Proteolytic enzymes have been demonstrated in the midgut of many insects, and most insect proteinases have been proved to be active at neutral or alkaline pH (Eguchi *et al.*, 1972; Baker, 1976; Eguchi and Iwamoto, 1976; Ahmad *et al.*, 1976; Kunz, 1978a; Engelmann and Geraerts, 1980).

In general, digestive proteases of insects are described as trypsin-like or chymotrypsin-like on the basis of their pH optima in the alkaline range and the inhibition by specific enzyme inhibitors. It was also found that in cockroach species proteases are induced upon feeding on protein such as casein, fibrin, or glutenin (Engelmann, 1969).

Present work describes the change of properties, distribution, and electrophoretic pattern of proteases from midgut tisse and digestive fluid during the development of *Pieris rapae* L.

MATERALS AND METHODS

Cabbageworms used in present study were reared in vinyl house and these were observed at the intervals of early 5th instar, late 5th instar, early prepupal, late prepupal, newly ecdysed pupal, 8 hr pupal, 1 day pupal, 3 day pupal, 5 day pupal and very late pupal (just before emergence) stages.

Preparation of enzyme solution

Midgut of 5th instar larvae, prepupae and was dissected out under the dissecting microscope and midgut epithelial tissue was homogenized in cold Ringer's solution (4°C) after removing its contents, and whole midgut of prepupae and pupae also homogenized in the same solution as above but including contents since the tissue was fragile and the separation of tissue and contents was not easy. The supernatant of the centrifugation at 4000 RPM for 10 min was stored at -20°C until assayed.

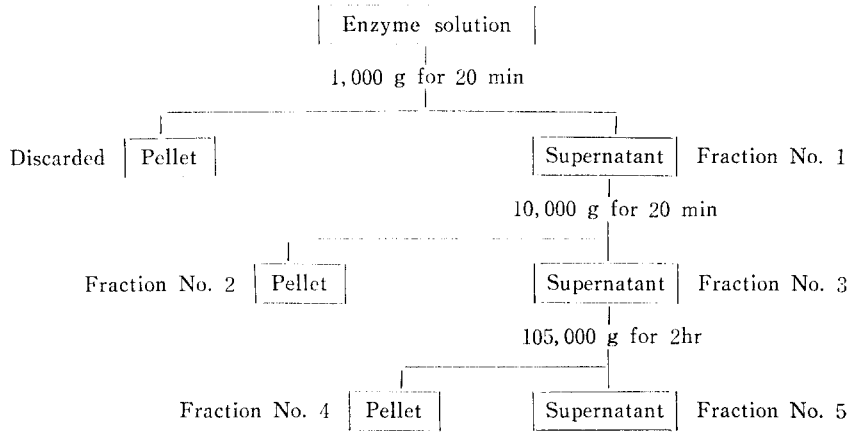
Assay of protease activity

Protease activity was determined by the method of Kunitz (1947) with some modification. 0.3 ml of enzyme solution and 0.3 ml of 0.1 M phosphate buffer (pH 8.0) were added to 0.6 ml of 1% casein solution (Merck, Hammarsten) and then incubated at 37°C for 90 min. After digestion, intact casein was precipitated with 1.8 ml of 5% trichloroacetic acid and centrifuged at 4000 RPM for 10 min. The enzyme activity of the supernatant was measured by Shimazu UV-360 spectrophotometer at 280 nm. The enzyme activity was expressed as μg of tyrosin formed per min and protein concentration was determined by the method of Warburg and Christian (1941).

Fractionation of enzyme solution

Fractionation of enzyme solution was performed using Hitachi 65P-7 Automatic Ultra-

Table 1. Fraction of enzyme solution



centrifuge as described in Table 1.

Electrophoresis

Electrophoresis was carried out by the method of Eguchi (1972) with some modification. Samples was electrophoresed on glass plate covered with agar gel (0.7% w/v in calcium lactate buffer, containing 1% casein) in Tris-glycin buffer at 13 v/cm for 1.5 to 2 hr and incubated at 37°C for 1 hr. Agar gel plate was stained with amido black 10 B for 15 min and then washed with 7% glacial acetic acid. The protease activity was observed as colourless band on the coloured background produced by the protein.

RESULTS

The change of protease activity in midgut during metamorphosis

As shown in Fig. 1, the change in protease activity of midgut during metamorphosis is as follows. The activity is low at early 5th instar stage and increases during 5th instar stage and then falls during terminal period of this stage. The activity during prepupal stage also represents the same pattern as that of 5th instar stage, falling to the minimum value at pupation period. The activity of protease during pupal stage shows almost similar pattern to those of previous two stages rising up to maximum at 1 day pupal stage and then falling until just before emergence.

The general pattern of protein concentration change is almost similar to that of protease

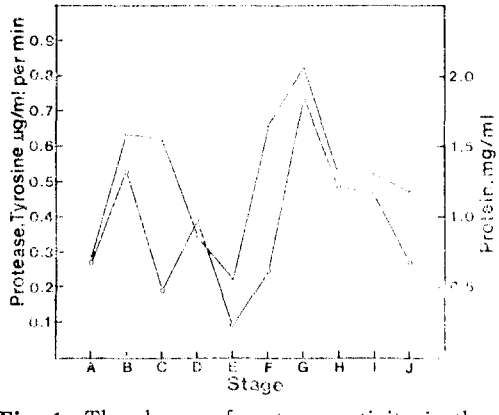


Fig. 1. The change of protease activity in the midgut during metamorphosis. ○——○, protease activity: ●——●, protein concentration. A, early 5th instar lavae: B, late 5th instar larvae: C, early prepupae: D, late prepupae: E, newly ecdysed pupae: F, 8 hr pupae: G, 1 day pupae: H, 3 day pupae: I, 5 day pupae: J, very late pupae (just before emergence)

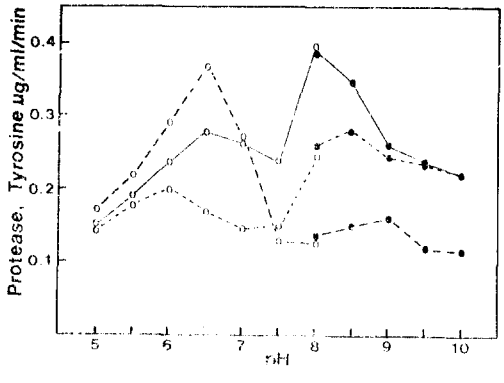


Fig. 2. The effect of pH on the protease activity in the midgut of different stages. ○——○, 5th instar larvae: ○-·-·-○, prepupae: ○·····○, very late pupae. Enzyme activity in the pH 5.0 to 8.0 range carried out in 0.1 M phosphate buffer (○); in the pH 8.0 to 10.0 range, in 0.1 M Tris-HCl buffer (●).

activity during metamorphosis, but the only opposite pattern takes place during early prepupal stage. In general, in the late 5th instar, early prepupal, 5 day pupal and very late pupal stages, protease activity is markedly low compared with protein concentration and during early pupal stage up to 8 hr after pupation protease activity shows rather slight increase relative to that of protein concentration.

Properties of midgut protease

pH optimum: Fig. 2 shows the effect of pH on the proteolytic activity in 5th instar, prepupal and very late pupal (just before emergence) stages. Two peaks were obtained in each stage; at pH 6.5 and pH 8.0 in the 5th instar stage and at pH 6.5 and pH 9.0 in the prepupal stage, and at 6.0 and 8.5 in the stage just before emergence.

Time course: Time course of protease activity is shown in Fig. 3. Protease activity increases linearly until 90 min, and then shows rather slower rate of increase.

Substrate concentration: The substrate concentration-activity curve is shown in Fig. 4. Casein is rapidly hydrolyzed in proportion to concentration at comparatively low concentrations (up to 1%) of substrate, but hydrolyzed slowly at higher concentrations.

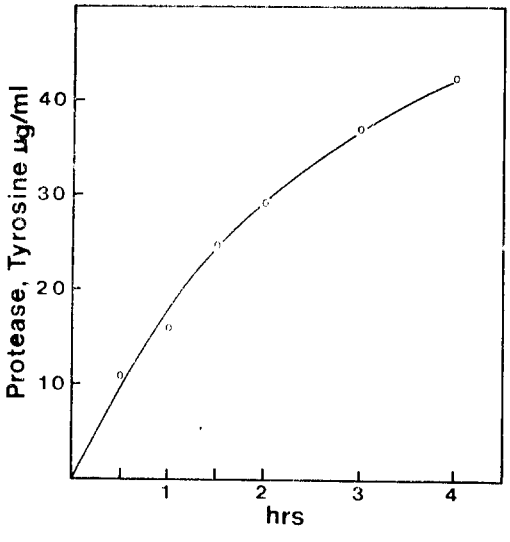


Fig. 3. Time course of the midgut protease activity in the 5th instar larvae.

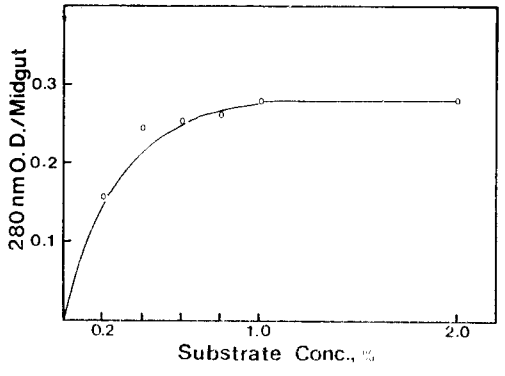


Fig. 4. Relation between substrate concentration and protease activity in the midgut of 5th instar larvae. The incubation mixture contained 0.6 ml of indicated concentration of casein dissolved in 0.1 M phosphate buffer (pH 8.0) and 0.6 ml of enzyme solution in 0.1 M phosphate buffer (pH 8.0).

Distribution of protease during metamorphosis

Distribution of protease in midgut during metamorphosis given in Fig. 5. In the late 5th instar stage the proteolytic activities are higher in fraction No. 2 and 4 (precipitates of centrifugation at 10,000 g and 105,000 g) than in fraction No. 1, 3 and 5 (supernatant of centrifugation at 1,000 g, 10,000 g and 105,000 g) (Fig. 5A). In the late prepupal stage,

however, most of enzymes are located in the supernatant and very few in precipitates (Fig. 5B). Thereafter there is a gradual decrease of protease activity in precipitates during the metamorphosis and almost no enzymes in precipitate just before emergence (Fig. 5C, D).

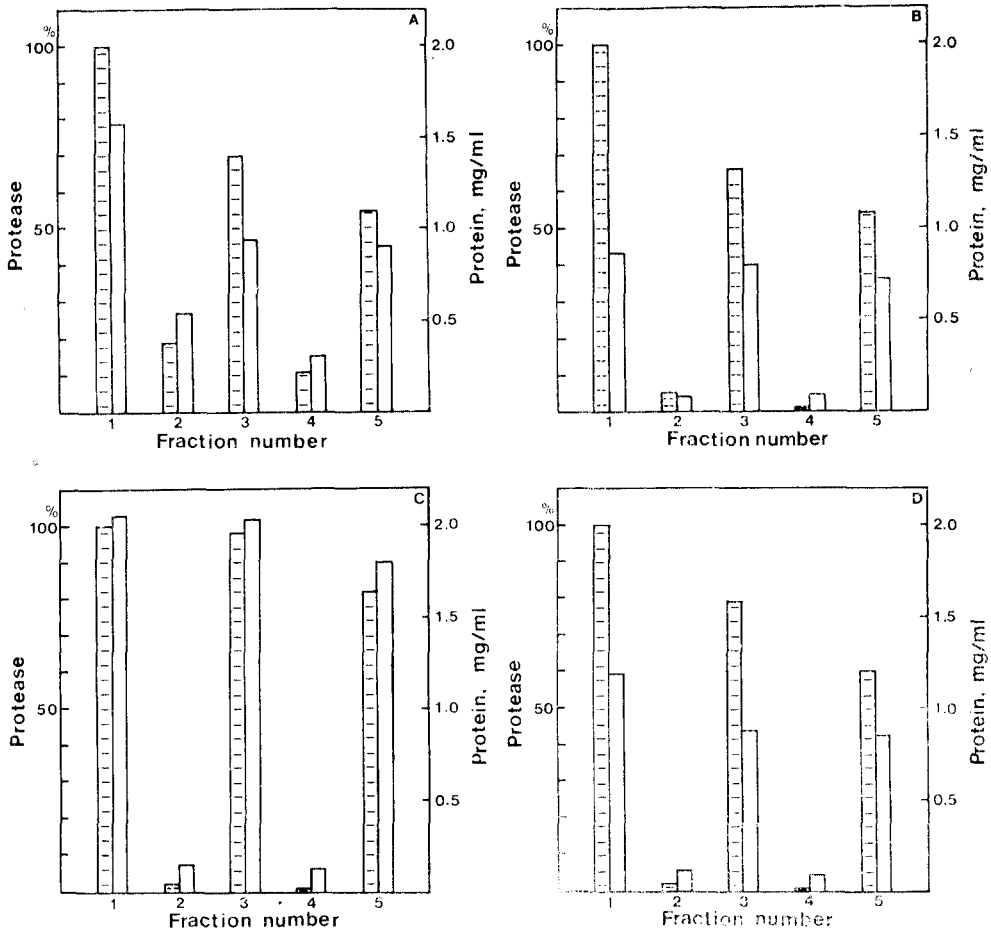


Fig. 5. Distribution of protease in the midgut of late 5th instar lavae (A), late prepupae (B), 1 day pupae (C) and very late pupae (D). Fraction No. 1, supernatant of centrifugation at 1,000 g for 20 min; No. 2 and 3, precipitate (mitochondria) and supernatant at 10,000 g for 20 min, No. 4 and 5, precipitate (microsome) and supernatant at 105,000 g for 2hr. Transversal lines, protease; white column, protein.

Electrophoresis

The midgut of prepupal and 1 day pupal stages were separated into midgut tissue and lumen contents, and these samples were used for agar gel electrophoresis. As given in Fig. 6, in the prepupal stage protease band appears faintly both in midgut epithelial tissue and lumen contents (Fig. 6A, B), but in 1 day pupal stage there is strong protease band in lumen contents (Fig. 6C) but none in midgut tissue (Fig. 6D).

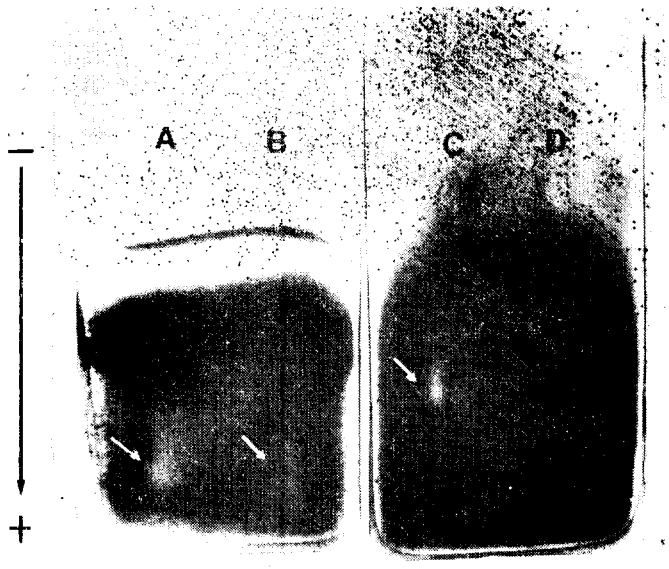


Fig. 6. Photograph showing electrophoretic patterns of midgut protease in the prepupal (A, B) and 1 day pupal stages (C, D). A, C, midgut lumen contents; B, D, midgut epithelial tissue.

Thus, from the results of the electrophoresis and enzyme assay, it was found that in the 5th instar stage the enzyme activity appears mostly in midgut tissue but thereafter until just before emergence the activity appears not in midgut tissue but in lumen contents.

DISCUSSION

The proteolytic activity of the alimentary canal in relation to feeding or proteins was studied in many insects (Engelmann, 1969; Hamano and Mukalyama, 1970; Persaud and Davey, 1971; Briegel and Lea, 1975). As shown in Fig. 1, it is evident from the similar changes between protein concentration and protease activity that the protein concentration appears to influence the protease activity. Such a similar pattern has also been demonstrated in larvae of *Spodoptera litura* (Ahmad *et al.*, 1976) and *Bombyx mori* (Eguchi and Iwamoto, 1976). Moreover, the observation that proteolytic activity of several insects decreases during starvation and increases again on refeeding (House, 1965; Engelmann, 1966, 1969; Janda and Krieg, 1969) also suggests the influence of the protein on gut proteolytic activity.

It is generally accepted that insect midgut proteases usually have pH optima in the neutral and alkaline region (House, 1974). Rather high pH optima, often above pH 9, have been reported, for example, in *Tenebrio* (Pfleiderer and Zwilling, 1966), *Pieris* (Lecadet and Dedonder, 1966), *Aedes* (Yang and Davies, 1971; Kunz, 1978a, b) or

Bombyx (Eguchi and Iwamoto, 1976). On the other hand, an acid pH optimum for midgut proteases of insects is rarely found and thus *Musca* or *Stomoxys* larvae (Lambremont *et al.*, 1959) and *Rhodnius* (Okasha, 1968; Garcia *et al.*, 1978) appear to be the exception to what is known for the majority of insect species. In *Pieris rapae*, protease activity has two peaks at pH 6.0 to 6.5 and pH 8.0 to 9.0 each throughout larval to pupal stages, and is maximum at pH 8.0 in 5th instar stage and at pH 6.5 in prepupal stage and pH 8.5 in pupal stage each. Therefore, *Pieris rapae* is considered to deviate a little from an alkaline property of protease of many other insects and also suggests at least the presence of more than one protease. Moreover, it is worth noticing that the protease activity of prepupal stage is maximum at pH 6.5.

It is also generally assumed that midgut enzymes are produced by the midgut epithelium and secreted into gut lumen (Dadd, 1970). Based on the results of fractionation and electrophoresis, it is apparent that in *Pieris rapae* protease is synthesized in the midgut tissue before prepupal stage and then released into the lumen during pupation period.

SUMMARY

The activity, properties, and distribution of midgut protease during metamorphosis in *Pieris rapae* L. are determined using spectrophotometer, ultracentrifuge and agar gel electrophoresis.

Proteolytic activity of midgut reaches the peak just before ecdysis in 5th instar and prepupal stages each but 1 day after ecdysis in pupal stage. Also, optimum pH of midgut protease is pH 8.0 in 5th instar stage, pH 6.5 in prepupal stage, and pH 8.5 immediately before emergence respectively. Protease is found mostly in midgut tissue in 5th instar stage but thereafter until just before emergence the enzyme only in lumen contents, suggesting that protease is synthesized in midgut tissue during larval stage and then released into lumen during pupation period.

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