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Purification and Properties of Trehalase from Larvae of *Pieris rapae* L.

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배추흰나비의 幼蟲期에서의 trehalase의 精製와 特性

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摘 要

배추흰나비의 變態過程에 따른 中腸內 trehalase의 變化와 分布 및 特性을 測定, 比較하였으며, 5令末幼蟲의 中腸內 trehalase를 精製하여 다른 器官과의 相互關係를 免疫學的 方法으로 究明하여 다음과 같은 結果를 얻었다.

中腸組織의 trehalase 活性은 5令初보다 5令末에서 높았으며 trehalase는 암, 수 모두 中腸 後部에 많이 존재하였다. 前蛹初부터 羽化直前까지는 中腸內容物에서 活性이 나타났는데 蛹直後에 가장 높았으며 中腸의 trehalase는 soluble form 이었다. 혈림프에서도 trehalase가 確認되었는데 蛹化直前에 높게 나타났다가 蛹化直後에 급진적으로 減少하였다. 中腸 trehalase의 최적 pH는 6.0으로 全變態段階에 걸쳐 일정하게 나타났으며, Km값은 2.86×10^{-3}M이었다. 最終精製된 酵素의 純度는 약 9.2이며, Rm값은 0.3이었다.

INTRODUCTION

Trehalase brings about the hydrolysis of trehalose, a non-reducing disaccharide, to glucose which participates in various metabolic activities. This enzyme was first demonstrated by Frerejacque (1941) in insects that feed on fungi and has also been found in microorganisms, mammalian kidney, higher plant, and plant pollen (Birch, 1963; Sacktor, 1968; Roberts and Tovey, 1969; Gussin *et al.*, 1969). This enzyme exists not only as a soluble form but also as particle-bound forms that are bound to cell membranes or other insoluble particles (Gussin and Wyatt, 1965; Gilby *et al.*, 1967; Sumida and Yamashita, 1977). The soluble and particle-bound forms of the enzyme have quite different biochemical properties

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(Gussin and Wyatt, 1965; Yamashita *et al.*, 1974). During larval-pupal development, the enzyme activity increased in the soluble form with a concomitant decrease in the particle-bound form. This change occurs via an intermediate form which is recovered from the particle-bound form but exhibits kinetic properties of the soluble form (Sumida and Yamashita, 1977). These developmental changes in trehalase forms are closely correlated with the physiologcal changes in midgut functions such as digestion, absorption, and excretion. Also trehalase is widely distributed in insect tissues such as muscle and gut, and even in haemolymph containing trehalose (Friedman, 1960, 1961; Matthews *et al.*, 1976). Matthews *et al.* (1976) have tried to separate cockroach haemolymph into serum and haemocytes and have found trehalase activity in both fractions.

Since the first partial purification of insect trehalase by Kalf and Rieder (1958), this enzyme has attracted the attention of a number of research workers. Purified and partially purified trehalase from various insects have been studied (Gussin and Wyatt, 1965; Gilby *et al.*, 1967; Marzluf. 1969; Yanagawa, 1971; Retief and Hewitt, 1973; Giebel and Domnas, 1976; Ogawa and Ariyoshi. 1981; Sumida and Yamashita, 1983; Han and Kim, 1987), Gussin and Wyattt (1965) found two distinct types of trehalase, with different properties, in *Cecropia* silkmoth, while Gilby *et al.* (1967) obtained the same results with cockroach, Marzluf (1969) found three trehalase bands during electrophoresis of *Drosophila melanogaster* trehalase. Yanagawa (1971) investigated silkworm muscle and midgut, and found three trehalases in each tissue, and their characteristics from muscle were identical to those from midgut. However, Retief and Hewitt (1973) isolated only one form of trehalase from whole body homogenate of the harvester termite by DEAE-Sephadex column chromatography and polyacrylamide gel electrophoresis. The differences between soluble and membrane-bound forms of this enzyme were found only in their kinetic parameters including pH and temperature optima and Michaelis constant.

In recent years(Sumida and Yamashita, 1983), one soluble trehalase was purified from whole midgut of pharate adults of silkworm. This enzyme was also shown to consist of a single polypeptide as analyzed by polyacrylamide disc gel electrophoresis.

In this paper, extraction and purification of midgut trehalase from *Pieris rapae* during larval-pupal development are conducted to determine the properties and distribution of this enzyme.

MATERIALS AND METHODS

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

Assay of enzyme

Trehalase activity was determined by the method of Ishaaya and Swirski (1976) with

some modification. 0.15 ml of enzyme solution and 0.15 ml of 50mM phosphate buffer (pH 6.0) were added to 0.3ml of 0.6% trehalose solution (Sigma) and then incubated at 37°C for 90 min. After incubation, 1.2 ml dinitrosalicylic acid reagent was added and boiled in water bath for 5 min and diluted with 1.6 ml of distilled water. The enzyme activity was measured by Shimazu UV-360 Spectrophotometer at 550 nm and expressed as mg of released glucose per min per individual midgut. Proteins were assayed according to Lowry *et al.* (1951) and on colum chromatography monitored at 280 nm.

Preparation of crude extract

Crude extracts of midgut tissues centrifuged at 1,000 g for 20 min were recentrifuged at 67,000 g for 30 min. Supernatants were used for assay of soluble trehalase activity and precipitates were dissolved in the same saline solution and then centrifuged at 105,000 g for 120 min. Precipitates were used for assay of particulate trehalase activity.

Purification of enzyme

The abdominal part of late fifth instar larva was homogenized in 25 mM phosphate buffer (pH 6.0) containing 5 mM phenylthiourea using the freezing and thawing method. The homogenate was centrifuged at 10,000 g for 30 min and then the supernatant was precipitated between 50% and 80% ammonium sulfate saturations. The precipitate was dissolved in the same buffer and the resulting solution was dialyzed against a large volume of the same buffer for 24 hr and then the solution was applied to a column of DEAE Sephadex A-50. The column (1.6×40 cm) was equilibrated with 25 mM phosphate buffer (pH 6.0) and eluted with a linear gradient from 0 to 0.5 M NaCl in the same buffer. 4 ml fractions each were collected at a flow rate of 0.3 ml per min. Fractions of the main trehalase peak were pooled and dialyzed in the same buffer for 24 hr and then concentrated to 3 ml. Also, trehalase in the midgut tissue of late fifth instar was purified by a procedure similar to that for trehalase in abdominal part.

Preparation of antitrehalase

0.5 ml of purified trehalase solution and an equal volume of Freund's complete adjuvant were thoroughly mixed. Preparation of antiserum and immunodiffusion test were carried out as described previously (Yoe and Kim, 1987).

Analytical disc gel electrophoresis and specific trehalase stain

Polyacrylamide disc gel electrophoresis was carried out in 7.5% gel using Tris-glycine buffer (pH 8.9). The gels were run at 3 mA per column. Gels with thehalase activity were specifically stained as described by Talbot and Huber (1975). Gels were rinsed in 5 mM phosphate buffer(pH 6.5) after the electrophoretic runs and immersed in a solution of 0.6 % trehalose in the same buffer for 1hr. Gels were rinsed with distilled water and placed in 0.1 M iodoacetamide solution for 8 min at room temperature. Finally they were rinsed again with distilled water and reacted with a 2% solution of tetrazolium red dissolved in 0.5 M NaOH in a boiling water bath for about 4 min. Red bands appeared at the positions of enzymic activity. The stained gels were stored in 7.5% acetic acid at 4°C.

RESULTS

Change in trehalase activity during larval-pupal development

Changes of trehalase activities in the midgut and haemolymph were determined from fifth instar larva to just before emergence. As shown in Fig. 1, trehalase activity in the midgut

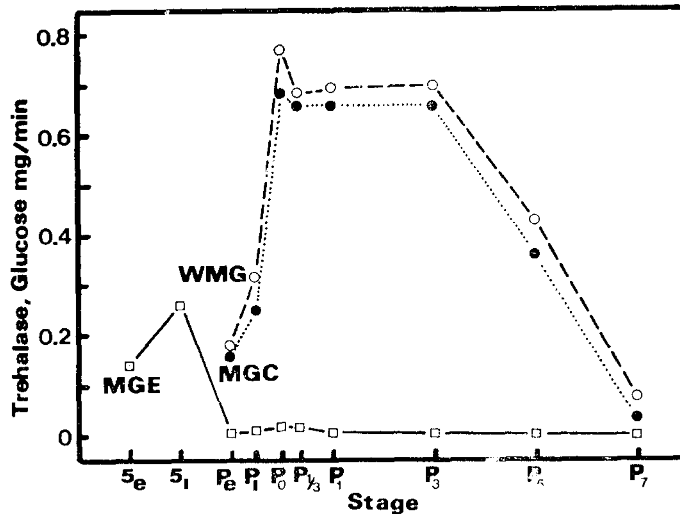

Fig. 1. The change of trehalase activity in the midgut during metamorphosis. MGE, midgut epithelium; WMG. whole midgut; MGC, midgut contents.

5_e, early fifth instar larva; 5_l, late fifth instar larva; P_e, early prepupa; P_l late prepupa; P_0, newly ecdyzed pupa; $P_{1/3}$, 8 hr pupa; P_1, 1 day old pupa; P_3, 3 day old pupa; P_5, 5 day old pupa; P_7, very late pupa (just before emergence).

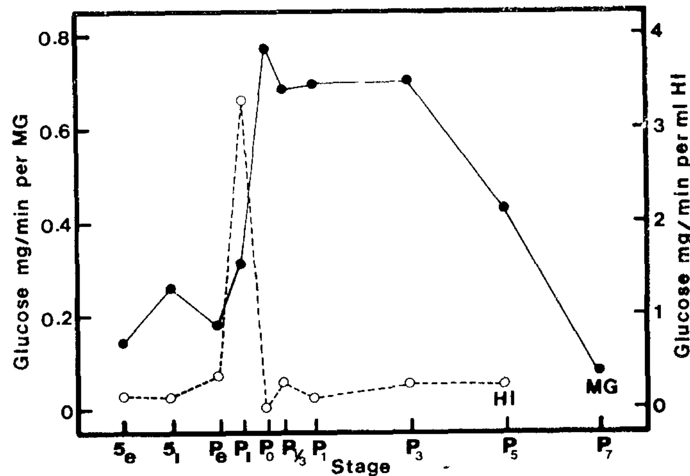

Fig. 2. The change of trehalase activity in the mid gut and haemolymph during metamorphosis. MG, midgut; Hl, haemolymph. Stage as in Fig. 1.

epithelia is low at early fifth instar stage and then increases during the first half of fifth instar stage but decreases during second half of fifth instar stage and maintains low level from early prepupal stage to just before emergence. However, trehalase activity in midgut contents could not be detected because of almost no digested foods in midgut of early and late fifth instar stages but found from early prepupal stage, and increases drastically until newly ecdysed pupal stage and drops suddenly from 3 day old pupae until just before emergence(Fig. 1) The change of trehalase activity in the whole midgut during metamorphosis shows almost similar pattern to that of midgut contents, suggesting that pupal midgut trehalase might be mainly accumulated in the midgut contents but scarcely in the epithelium.

The trehalase activity in the haemolymph is a high level at the late prepupal stage and drops suddenly until newly ecdysed pupal stage and thereafter maintains low level to just before emergence(Fig. 2).

Distribution of trehalase in midgut

The late fifth instar larvae were segregated into male and female, and as previously described (Yoe and Kim, 1987) trehalase activity was assayed in the anterior, middle, and posterior midguts respectively. Activities in the posterior portion are higher than in the anterior and middle portions, both in male and female (Table 1).

Optimal conditions for trehalase activity

Optimal conditions for pH, time course, and substrate concentration were first evaluated through a series of preliminary experiments. Thereafter, the optimal condition of each factor was determined for each factor separarely, all other factors being at the optimum.

1) Optimum pH: The effect of pH on hydrolysis of trehalose was determined with phosphate and glycine-NaOH buffers of suitable pH. Fig. 3 shows that the highest activity occurred at pH 6.0, this value in good agreement with that found by others with trehalase from ***M. sexta*** (Dahlman, 1971), ***C. erythrocephala*** (Duve, 1975), and ***C. pipiens*** (Giebel and Domnas, 1976). However, the value was slightly higher than the optimum pH of about 5.5 for trehalase from ***M. differentialis*** (Derr and Randall, 1966), ***S. oleae*** (Ishaaya and Swirski, 1976), and ***H. axyridis*** (Ogawa and Ariyoshi, 1981).

2) Substrate concentration: The rate of trehalase reaction was determined as a function of

Table 1. Distribution of trehalase activity in the midgut of late fifth instar larvae of ***Pieris rapae***

	Male		Female	
	*Activity	**%	Activity	%
Anterior midgut	0.018	8.2	0.042	14.6
Middle midgut	0.075	34.3	0.117	40.6
Posterior midgut	0.126	57.5	0.129	44.8
Total	0.219	100.0	0.288	100.0

* Trehalase activity are expressed as mg of glucose formed per min per animal.

** Values are percentage of total activity.

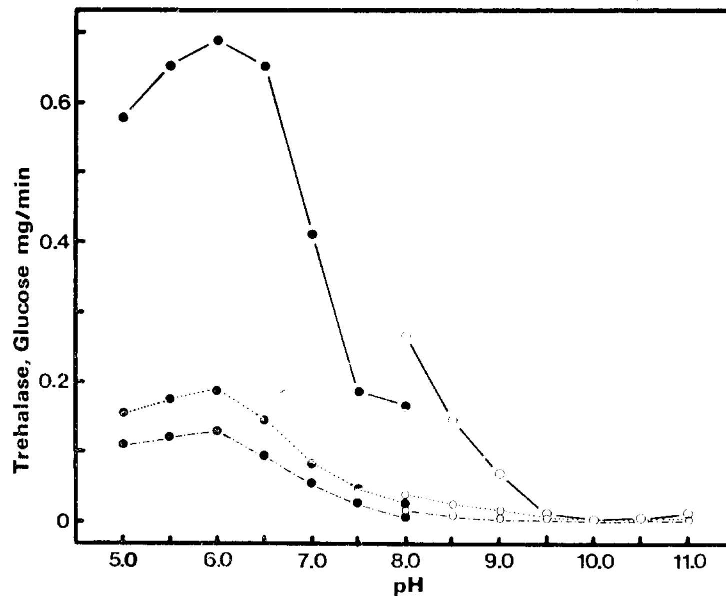

Fig. 3. The effect of pH on the trehalase activity in the midgut of different stages. ······, late fifth instar larva; -·-·-, early prepupa; ——, 1 day old pupa. Enzyme activity in the pH 5.0 to 8.0 range carried out in 50 mM phosphate buffe (•); in the pH 8.0 to 11.0 range in 50 mM glycine-NaOH buffer(○).

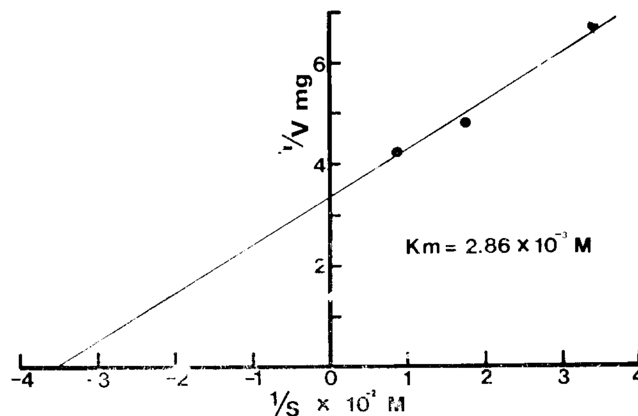

Fig. 4. Lineweaver-Burk plot to determine K_m of soluble trehalase in the fifth instar larval midgut at pH 6.0.

trehalose concentration. The Km value calculated from a Lineweaver-Burk (1934) plot was approximately 2.86×10^{-3} M at 37°C, this value in good agreement with that found by Shimada (1976) with trehalase from *Bombyx mori*. A representative plot is shown in Fig.4.

3) Time course: A catalytic period of up to 240 min at 37°C was found to be linear (Fig. 5).

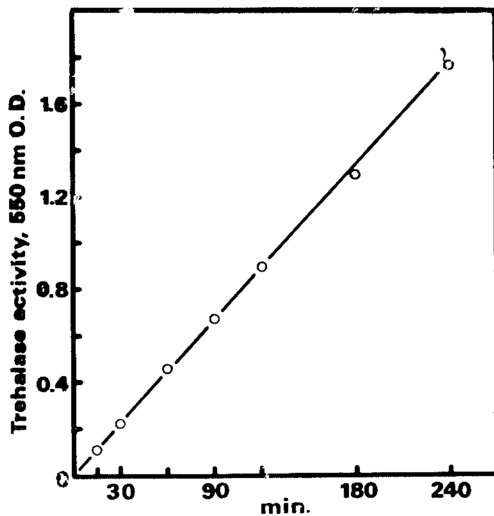

Fig. 5. Time course of the midgut trehalase activity in the late fifth instar larva.

Changes in particulate and soluble trehalase activities of midgut during larval-pupal development

To elucidate the mechanism of translocalization of midgut trehalase during metamorphosis, we examined the subcellular localization of midgut trehalase by differenrial centrifugation techniques. Crude homogenates of fifth instar larval midgut epithelia and whole midguts after larval maturation were centrifuged at 67,000 g for 30 min. From early fifth instar to just before emergence, the trehalase activity is mainly present in the soluble fraction but not in the precipitate fraction of midgut (Fig. 6). Therefore, midgut trehalase of *Pieris rapae* is a soluble form.

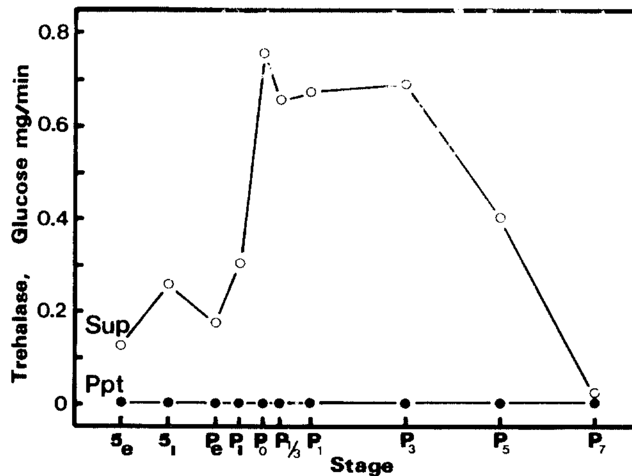

Fig. 6. The change of the particulate and soluble trehalase activity in the midgut during metamorphosis. Stage as in Fig. 1.

Purification of trehalase

1) Trehalase of midgut: 10 ml enzyme solutions of fifth instar midgut tissue precipitated at ammonium sulfate saturations between 30% and 80% were chromatographed on DEAE Sephadex a-50 column (Fig. 7). Two peaks of trehalase activity are eluted at 0.23 to 0.33 M of NaCl concentration (fractions 33 to 45). Of these fractions, fractions 35 to 41 were pooled and concentrated to 1 ml and then used as sample for analytical electroporesis

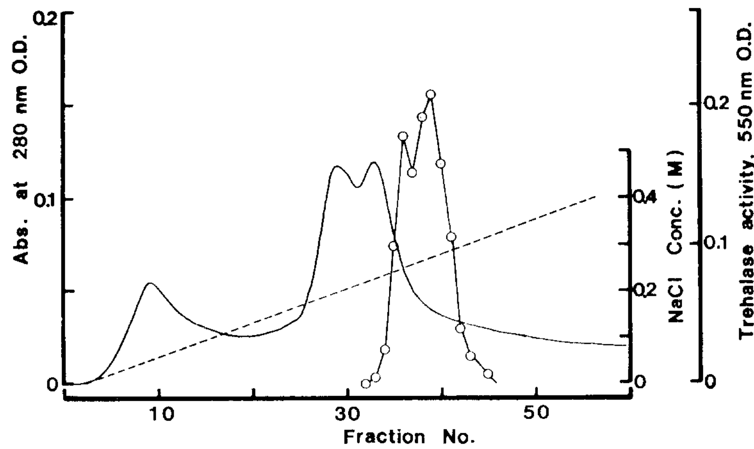

Fig. 7. Column chromatography of the soluble trehalase in the midgut epithelia of the last fifth instar larvae on a DEAE-Sephadex A-50 column. The column (1.0×25 cm) was equilibrated with 50 mM phosphate buffer (pH 6.0). 3 ml fractions were collected at a flow rate of 0.2 ml per min. —, protein concentration; ○—○, soluble trehalase activity; ···, NaCl linear gradient.

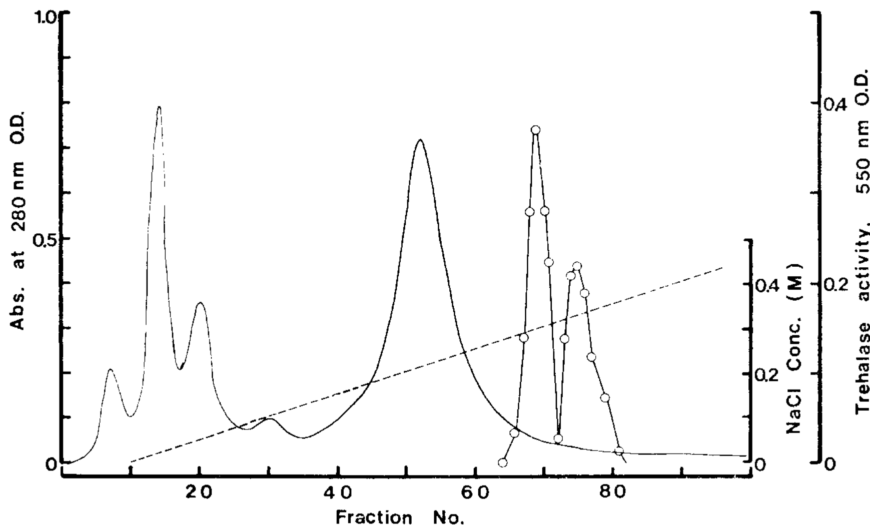

Fig. 8. Column chromatography of the trehalase in the abdomen of the late fifth instar larvae on a DEAE-Sephadex A-50 column. The column (1.6×40 cm) was equilibrated with 50 mM phosphate buffer (pH 6.0). 4 ml fractions were collected at a flow rate of 0.3 ml per min. —, protein concentration; ○—○, trehalase activity; ···, NaCl linear gradient.

Table 2. Purification steps of trehalase from abdomen of late fifth instar larvae of *Pieris rapae*

Step	Total volume (ml)	Total activity (glucose mg)	Total protein (mg)	*Specific activity	Yield (%)	Purification factor (fold)
Crude extract (supernatant at 27,000g)	40	21.287	435.17	0.049	100	1
$(NH_4)_2SO_4$ fractionation (50~80%) and dialysis	24	14.671	58.34	0.251	68.9	2.57
DEAE-Sephadex A-50 column chromatography	40	2.395	5.33	0.449	11.3	9.18

* Specific activity=Total activity divided by total protein (mg)

and immunological studies.

2) Trehalase of abdomen: Trehalase in the abdominal part of late fifth instar larvae was purified by a procedure similar to that for trehalase in midgut tissue (Fig. 8). The final purity of trehalase was increased about 9.2 fold. The yield and degree of trehalase at each step of purification is summarized in Table 2.

Purity of trehalase

Active fractions of two peaks from column chromatography were pooled, and concentrated

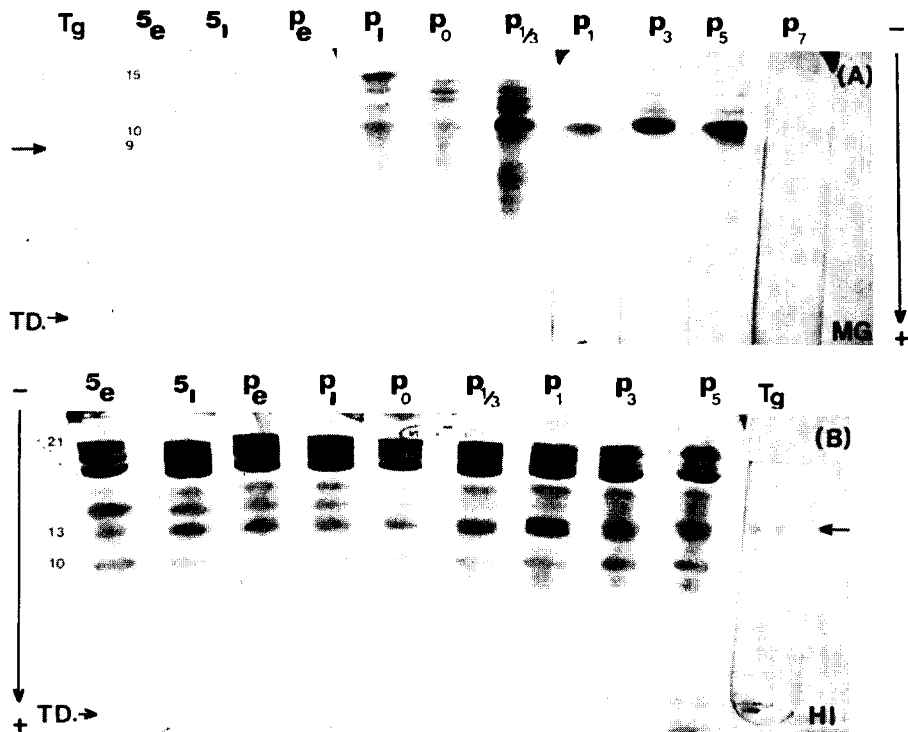

Fig. 9. Electrophoretic patterns of midgut (A) and haemolymph (B) proteins durng metamorphosis and midgut soluble trehalase of the late fifth instar larvae. T_g, purified midgut trehalase. Stage as in Fig. 1.

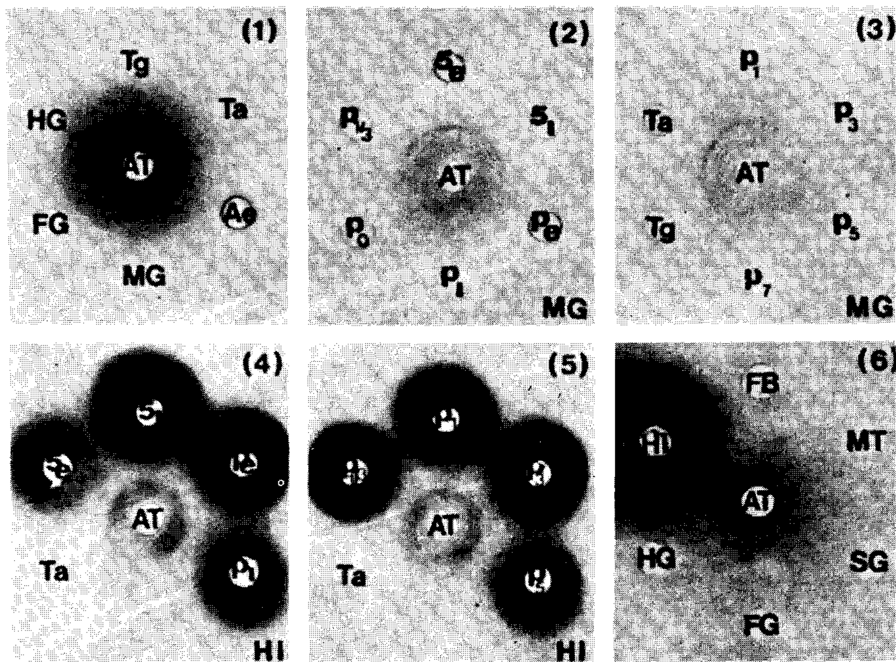

Fig. 10. Immunodiffusion patterns given by purified soluble trehalase and other organs at different stages. The centre well contained antiserum against purified abdomen trehalase. (1), (6): the late 5th intar larvae. T_g, purified midgut trehalase; T_a, purified abdomen trehalase; Ae, crude extracts of abdomen; MG, midgut; FG, foregut; HG, hindgut; FB, fat body; MT, Malpighian tubule. SG, silk gland; AT, Antitrehalase. (2), (3): midgut. Stages as in Fig. 1. (4), (5): haemolymph. Stages as in Fig. 1.

in a freeze dryer. Purity of trehalase preparation was examined by polyacrylamide gel electrophoresis at pH 8.9 (Fig. 9). A single band of trenalase activity observed on gel corresponds to protein band 9 (Rm 0.3) of the midgut (Fig. 9A) and band 13 of the haemolymph (Fig. 9B). This result suggests that trehalase preparation is completly homogeneous.

Immunological study

An immunological study was carried out to confirm the presence of trehalase in other organs. Purified abdominal trehalase of late fifth instar was used as an antigen. As shown in Fig. 10, one continuous precipitin line appears in purified midgut and abdominal trehalases (Fig. 10(1)) but not in fat body, Malpighian tuble, salivary gland, foregut, and hindgut (Fig. 10(6)). In the haemolymph, however, a single line was observed but was not fused with line against the purified abdominal trehalase (Fig. 10(4)).

DISCUSSION

The control of the activity of typical digestive enzymes in insect intestine takes place by

nervous, hormonal or secretagogue mechanisms. In ***Pieris rapae***, trehalase activity in the midgut tissue was low at early fifth instar stage and decreases during late fifth instar stage (Fig. 1). These results are suggesting that the secretion of trehalase is probably regulated by secretagogue mechanism. A similar secretagogue control mechanism of trehalase activity in the intestine was reported by Rosiński *et al.* (1979) in *Tenebrio molitor*.

As shown in Table 1, in *P. rapae* male and female high trehalase activity is found in the posterior midgut. Terra *et al.* (1979) reported that α-amylase of *R. americana* is more secreted in anterior than posterior midguts, whereas trehalase and protease are more secreted in posterior than anterior midguts. These results, therefore, are supported by our finding that high trehalase activity is found in the posterior midgut. In comparision with the change of midgut trehalase, on the other hand, trehalase in the haemolymph drastically increases at late prepupal stage and then decreases at just after pupation (Fig. 2). This result is suggesting that a portion of trehalase in the haemolymph is directly released into midgut. Pant and Morris (1974) reported that intestinal trehalase in ***Philosamia ricini*** sharply increased after pupation and then maintained a regular activity during early pupal stage, while in the haemolymph decreased just before pupation. They also suggested that intestinal tissue trehalase probably plays an important role in regulating the blood sugar level.

It is generally accepted that properties of digestive enzyme in insect differ from differences of food, habitat, or interspecies. Fig. 3 shows that the highest activity of midgut trehalase occurred at pH 6.0 from fifth instar to prepupal stages, this value in good agreement with that found by others with trehalase from *Menduca sexta* (Dahlman, 1971), mosquito (Giebel and Dimnas, 1976) and honey bees (Talbot and Huber, 1975). According to Friedman (1975), however, midgut trehalase in adult *Phormia regina* has an optimum pH 4.5 whereas muscle trehalase has an optimum pH 5.0 to 5.5. Also, Talbot and Huber (1975) reported different properties between soluble trehalase in the cytosol of midgut cells and particulate trehalase bound to membrane of cell organells of flight muscle. It is well known that enzyme activity usually increases in proportion to substrate concentration up to a certain point, from which the rate of hydrolysis shows scarcely any increase. In the present study, trehalose is rapidly hydrolyzed in proportion to concentration from comparativeyl low concentration until 0.6% but scarcely at concentration above 0.6%. Km value for midgut trehalase was calcuated to be 2.86×10^{-3} M (Fig. 4). This value corresponds well to those to those of *Sarcophaga barbata* (Clements *et al.*, 1970), *Menduca sexta* (Dahlman, 1971), *Apis mellifera* (Talbot and Huber, 1975), and *Phormia regina* (Friedman, 1975).

The soluble trehalase was purified from the midgut tissue of fifth instar larvae of *Pieris rapae*. This preparation was homogeneous as assessed by analytical polyacrylamide gel electrophoresis at pH 8.9. Only single band Rm 0.3 exhibited the trehalase activity (Fig. 9). Same result was obtained by Sumida and Yamashita (1983) in midgut of *Bombyx mori*. They reported that a faint but distinct band is observed corresponding to the protein band with trehalase activity, and a PAS positive reaction.

In the present work trehalase activity was found in the midgut and haemolymph but not in other organs. A single line observed in haemolymph was not fused with line formed against midgut (Fig. 10). This is similar to result reported in silkworm (Sumuda and Yamashita, 1983). They indicated that different pattern of precipitin lines was a monomer-dimer relationship between the particulate and soluble trehalases from the midgut of silkworms. Although the physiological role of trehalase in haemolymph remains unknown, it functions most probably as a regulated of trehalase level in haemolymph.

SUMMARY

The change and properties of trehalase in the midgut of *Pieris rapae* L. were determined during metamorphosis and also trehalase in the midgut of late fifth instar larvae was purified and their relationship with other organs were determined using immunological method.

Trehalase of the midgut exists as a soluble form and its activity in the midgut is higher in late fifth istar than in early fifth instar. The level of trehalase is higher in the posterior than in the anterior and middle portions of midgut both in male and female. The activity presents in midgut contents from prepupa to just before emergence with highest peak at newly ecdysed pupae. Trehalase in the haemolymph drastically increases just before pupation and then decreases just after pupation. Optimum pH of trehalase in the midgut is 6.0 throughout the whole stage and Km value is 0.86×10^{-3} M. The final purity of enzyme is about 9.2 and Rm is 0.3.

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