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Studies on Esterase of *Pieris rapae* L. I. Changes of Esterase Activity and Zymogram Pattern During Development and Purification

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Changes in esterase activity and zymogram pattern during development in *Pieirs rapae* L. were investigated and three esterases (E2, E6 and E11) from the late 5th instar larvae were purified. Esterase activity in whole body increased rapidly during 5th instar larval stages and reached a peak at the late 5th instar larval stage. The number and intensity of esterase band from whole body and midgut also showed a peak at the late 5th instar larval stage. Purification of esterase was performed using gel filtration on Sephadex G-100, ion-exchange chromatography on DEAE-trisacryl and preparative electrophoresis. The final purities of these enzymes were about 30 to 60-fold.

KEY WORDS: Cabbage worm, Esterase, Column chromatography

With the introduction of zone-electrophoresis on starch gels in mid 1950s, a successful method to separate various enzymes was realized and the application of histochemical reagents to such gels by Markert and Hunter (1957) has revealed the presence of numerous forms of esterase in a number of insects since 1960s (Menzel *et al.*, 1964; Cook and Forgash, 1965; Cook *et al.*, 1969; Briegel, 1972; Guss and Krysan, 1972; Turunen and Chippendale, 1977; Nunamaker and Wilson, 1982). Esterases show a drastic changes in activity and zymogram pattern during development (Afsharpour and O'Brien, 1963; Eguchi and Sugimoto, 1965; Clements, 1967) and particularly in connection with digestion of foods, they maintain high activity during the last instar larval stage showing active feeding ability (Eguchi and Iwamoto, 1975; Kapin and Ahmad, 1980).

Most of the studies on insect esterases, however, have been confined to infer the physiological function of esterases only on the basis of the changes in zymogram pattern during development, or to classify the esterases according to their sensitivities to specific inhibitors. Recently, it has been tried to purify esterases by a few researchers and the biochemical properties of the purified esterases and their physiological role in insects have been inferred (Devonshire, 1977; Danford and Beardmore, 1979; Mane *et al.*, 1983; Willadsen *et al.*, 1987; Kai *et al.*, 1987).

The present study was to elucidate the changes in esterase activity and zymogram pattern during development and then to purify some esterases which were physiologically meaningful as a part of investigating the physiological role of the esterases of *P. rapae L.*

Materials and Methods

Insects

Cabbage worms, *Pieris rapae* L., used in the present study were reared on kail in vinyl house and were observed at the intervals of late 4th instar, early 5th instar, mid 5th instar, late 5th instar, early prepupal, late prepupal, newly ecdysed pupal and 3 day-old pupal stages.

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Measurement of Esterase Activity

Esterase activity was measured by the method of Kapin and Ahmad (1980) with some modifications. Five hundred μl of enzyme solution was mixed with 2.5ml of the substrate solution (3.0×10^{-4}M α-naphthylacetate (Sigma) in 0.1M phosphate buffer (pH 7.0) containing 1%(V/V) acetone). After 20min of incubation at 30°C with shaking, 0.5ml of a freshly prepared dye solution, containing two parts of 1% (W/V) Fast Blue B salt (Merk) and five parts of sodium dodecyl sulfate (SDS, Sigma), was added. The esterase action on the substrate produced α-naphthol which was coupled to Fast Blue B salt. The coupling produced a strong blue color, which was measured at 590nm by Shimadzu UV-240 Spectrophotometer.

Protein concentration was determined by the method of Lowry *et al.* (1951) and on column chromatorgraphy monitored at 280nm.

Electrophoresis

Electrophoresis was carried out in 6% polyacrylamide gel with tris-glycine buffer (pH 8.3) at the current of 3 mA/tube according to the method of Davis (1964). After electrophoresis, the gel was equilibrated in 0.1M phosphate buffer (pH 6.6) for 10 min and transferred to a solution containing 0.04% (W/V) α-naphthylacetate (Sigma) and 0.07% (W/V) Fast Blue RR salt (Sigma) in 0.1 M phosphate buffer (pH 6.6). The esterase band appeared as the dark brown color.

Purification of Esterase

Whole body of late 5th instar larvae was homogenized in 0.1M phosphate buffer (pH 7.0) and centrifuged at 15,000g for 30min and then the supernatant was precipitated with 40-70% ammonium sulfate. The pellet was taken up in a minimum volume of extraction buffer and dialysed against 0.05 M phosphate buffer (pH 6.6). The dialysate was applied to a Sephadex G-100 column (1.6 ×65cm) previously equilibrated with 0.05M phosphate buffer (pH 6.6). The flow rate was 16 ml/hr and 2 ml fractions were collected. Fractions with high esterase activity were pooled, concentrated, dialysed and applied to a DEAE-Trisacryl column (1.6 ×38 cm) previously equilibrated with 0.02 M phosphate buffer (pH 8.0). The column was eluted with a linear gradient of 0 to 0.5 M NaCl in the equilibration buffer. The flow rate was 20 ml/hr and 5ml fractions were collected. Three esterase peaks were obtained and fractions under the two esterase peaks which showed the separation of each isozyme were pooled separately, concentrated and applied to preparative electrophoresis with a 6% polyacrylamide slab gel. After electrophoresis, the gel was stained slightly for estrease activity and the bands were excised. The gel slices were put into dialysis tubes containing tris-glycine buffer (pH 8.3) and eluted at a constant voltage of 100 V for 3hrs. The eluted esterase was concentrated, dialysed and then used in subsequent experiments. All purification steps described above were performed at 4°C.

Results

Changes in Esterase Activity and Protein Concentration during Development

As shown in Fig. 1, esterase activity in whole body was low at early 5th instar larval stage but increased rapidly until late 5th instar larval stage, followed by a gradual decline during pupal stages.

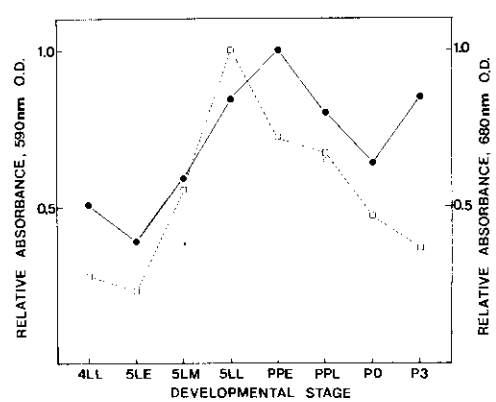

Fig. 1. The changes of the whole body esterase activity (| ····· |) and protein concentration (● —— ●) during development. 4LL, late 4th instar larvae; 5LE, early 5th instar larvae; 5LM, mid 5th instar larvae; 5LL, late 5th instar larvae; PPE, early prepupae, PPL, late prepupae; PO, newly ecdysed pupae; P3, 3 day-old pupae.

The change of protein concentration in whole body during development showed similar pattern to that of esterase activity except for the peak, which revealed at early prepupal and 3 day-old pupal stages.

Electrophoretic Patterns of Esterase during Development

The number and intensity of esterase band from whole body increased gradually during larval stages and then showed a maximum at late 5th instar larval stage but decreased during pupal stages. Sixteen esterase bands were identified at late 5th instar larval stage and these bands were numbered by 1 to 16 according to their mobility starting from the origin (Fig. 2).

The change in zymogram pattern of esterase from midgut during development was similar to that from whole body, showing a maximum at late 5th instar larval stage in both of the number and intensity of esterase band and 12 esterase bands were detected at this stage (Fig. 3).

Distribution of Esterase in Each Organ of Late 5th Instar Larvae

At late 5th instar larval stage, 3 to 12 esterase bands were indentified according to the organs tested. The esterase bands appeared in midgut (12), foregut (5), hindgut (4), silk gland (3) and haemolymph (8) but no esterase band was detected in the cuticle (Fig. 4).

Purification of Esterase

The results obtained in each purification step were summarized in Table 1. Crude extracts from whole body of late 5th instar larvae were saturated with 40-80% ammonium sulfate at 10% intervals and measured for their activities and protein concentrations. As shown in Fig. 5, 40-70% ammonium sulfate fraction revealed relatively high specific activity. These fractions were subjected to gel filtration and the elution profile is shown in Fig. 6. Only one peak appeared in fractions 12-17 and each fraction under the peak was electrophoresed to see if each isozyme was separated. Fig. 7 showed that E11 with high molecular weight appeared as the major band in the lane for fraction 10 and E6 with low molecular weight appeared as the major band in the lane for fractions 12-17 with high enzymic activity. The latter was pooled, concentrated and chromatographed on DEAE-Trisacryl column. As shown in Fig. 8, three esterase peaks were observed but fractions under the lst peak were put into no further purification steps because they showed several esterase bands on the gel after electrophoresis. Elec-

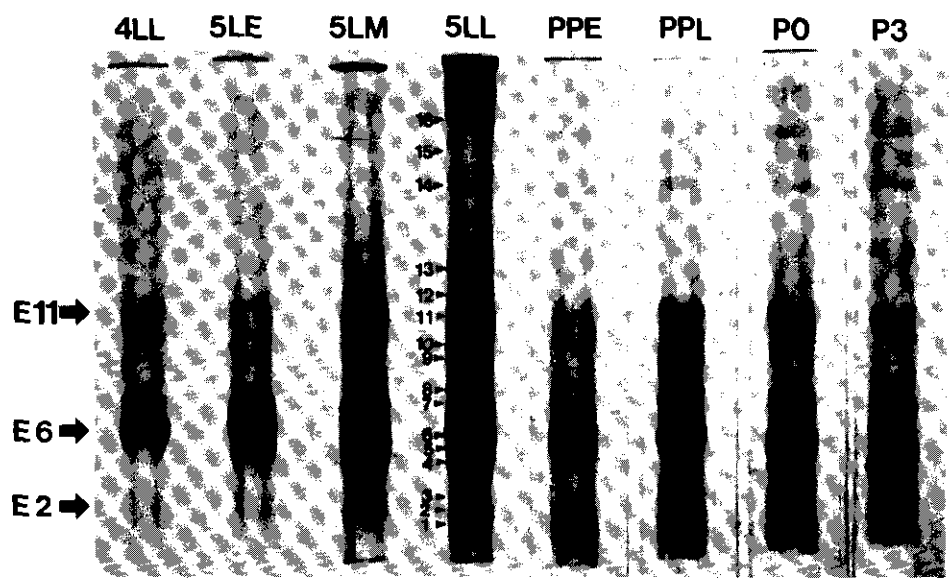

Fig. 2. Electrophoretic patterns of esterase from whole body during development. Stage as in Fig. 1.

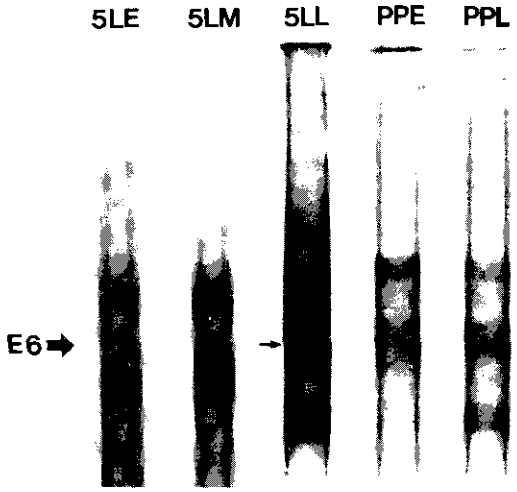

Fig. 3. Electrophoretic patterns of esterase from midgut during development. Stage as in Fig. 1.

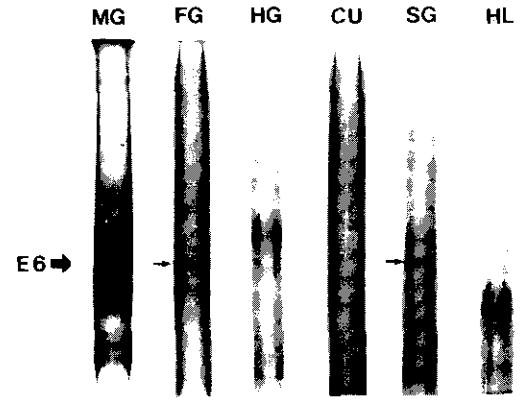

Fig. 4. Electrophoretic patterns of esterase in each organ of the late 5th instar larvae. MG, midgut; FG, foregut; HG, hindgut; CU, cuticle; SG, silk gland; HL, haemolymph.

Table 1. Purification steps of esterase from whole body of late 5th instar larvae of *P. rapae*

Step	Total volume (ml)	Total activity (α-NA mM)	Total protein (mg)	Specific activity	Yield (%)	Purification factor (-fold)
Crude extract	83	604	1853	0.33	100	1
Ammonium sulfate (40-70%) fractionation	19	427	667	0.64	71	2
Sephadex G-100 filtration	12	77	28.6	2.7	13	8
DEAE-Trisacryl column chromatography i)*	3.5	24	2.6	9.2	4	28
ii)**	10.0	17	3.3	5.2	3	16
Preparative electrophoresis						
Esterase 2)	8.5	4.6	0.5	11.5	0.7	35
Esterase 6)	5.0	12.5	0.6	20.6	2.0	62
Esterase 11)	10.5	3.2	0.3	10.0	0.5	30

*Fractions 30-35 from DEAE-Trisacryl column containing esterase 6.
**Fractions 40-49 from DEAE-Trisacryl column containing esterase 2 and 11.

tropherogram (Fig. 9) revealed that fractions under the 2nd peak contained only single esterase band (E6) and those under the 3rd peak showed two distinctly separated esterase bands (E2 and E11). These fractions were further purified separately to eliminate proteins other than esterase by preparative electrophoresis and electro-elution. The homogeneity of each purified isozyme was checked by electrophoresis and the result showed that each isozyme was detected as single band on the gel (Fig. 10).

Discussion

In general, *P. rapae* used in the present study ingest foods mostly during their larval stages especially from early 5th instar larval to late 5th instar larval stage. During this period, therefore, the alimentary canal is composed mostly of midgut and filled with undigested foods. As shown in Fig. 1, esterase activity during development increased

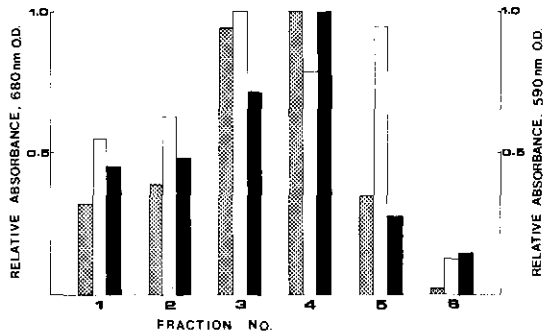

Fig. 5. Esterase activity (▨), protein concentration (□), and specific activity (■) after treatment with ammonium sulfate on crude enzyme extract. Fraction No. 1-5, precipitate after treatment with 40, 50, 60, 70, 80% ammonium sulfate, respectively; No. 6, supernatant after treatment with 80% ammonium sulfate.

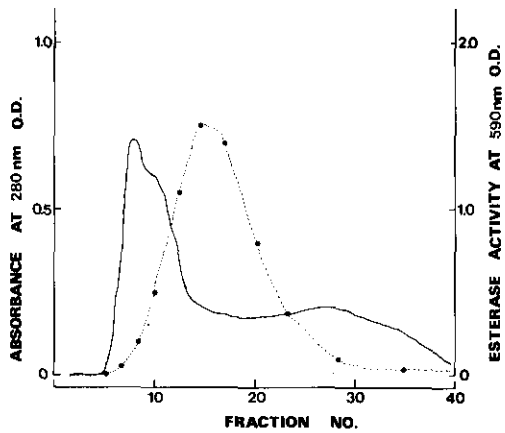

Fig. 6. Gel filtration of the 40-70% ammonium sulfate fraction on Sephadex G-100 column. —— protein concentration; • ····· •, esterase activity.

continuously from early 5th instar larval stage to late 5th instar larval stage, followed by a gradual decline during pupal stage. Thus, it is considered that the increasing rate of food consumption is correlated with the increase of esterase activity. Eguchi and Iwamoto (1975) reported that esterase activity in the midgut tissue of *B. mori* reached a peak just before spinning. Turunen and Chippendale (1977) deteted 7 esterase bands from midgut of *D. grandiosella* after electrophoresis and examined the effect of feeding activity on the midgut esterase pattern. When the larvae were starved, the titre of midgut esterases was found to fall to a low level, suggesting a good agreement between

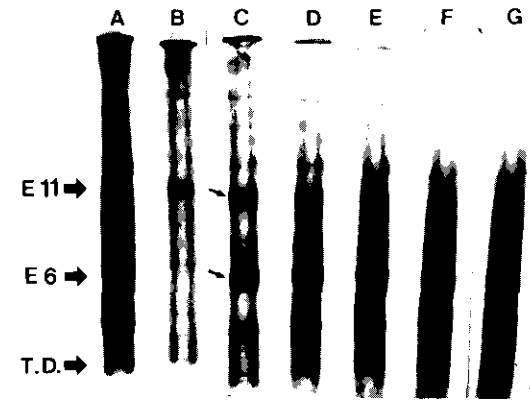

Fig. 7. Electrophoretic patterns of esterase from Sephadex G-100 fraction. A, 40-70% ammonium sulfate fraction; B, fraction 10; C, fraction 12; D-G, fraction 14-17.

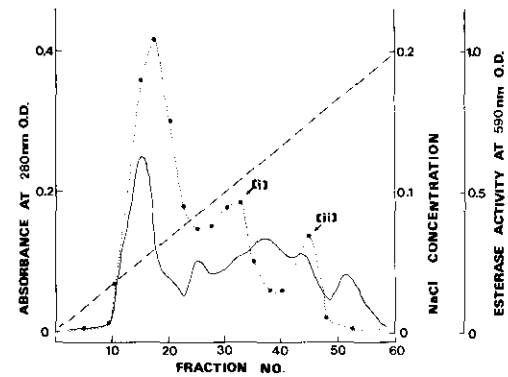

Fig. 8. Column chromatography of 14-21 fractions after gel filtration on DEAE-Trisacryl column. —— protein concentraton; • ····· •, esterase activity.

Fig. 9. Electrophoretic patterns of esterase from DEAE-Trisacryl fraction. A, crude enzyme extract; B, 40-70% ammonium sulfate fraction; C-J, fraction 30, 31, 33, 36, 40, 43, 47, 49, respectively.

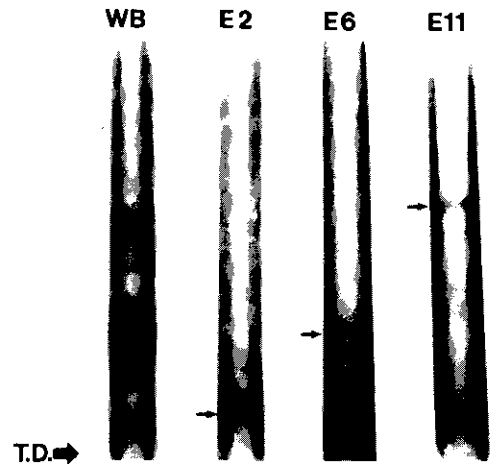

Fig. 10. Electrophoretic patterns of purified esterase. WB, whole body; E2, esterase 2; E6, esterase 6; E11, esterase 11.

feeding ability and esterase activity. Also, Wigglesworth (1958) noted that the esterase reaction of oenocytes in the newly fed *R. prolixus* was weak. But as growth and feeding proceeded, the oenocytes became charged with lipoprotein and the esterase activity of these cells also intensified.

It is customary to give a common name and classification to every esterase visualized on a gel after electrophoresis. However, it is essential that purification of an enzyme should be preceded to reveal the more precise classification, biochemical properties and physiological role of the enzyme. For this reason, the present study was focused on the two major esterases of late 5th instar larval stage during which the insects show great feeding activity and these enzymes were purified using column chromatography. The purities of these enzymes increased by 30 to 60-fold compared to crude extracts but the yields are extremely low (0.5 2%) largely because of the separation of the esterase of interest from a number of other esterases. In this connection, characterization and biochemical properties of these esterases will be studied in the subsequent experiments and also their physiological role will be elucidated using immunological methods.

Acknowledgements

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배추흰나비(*Pieris rapae* L.)의 esterase에 관한 연구 I. 변태에 따른 esterase의 활성변화 및 zymogram pattern의 변화와 정제

박천호 · 김학열* · 여성문(단국대학교 자연과학대학 생물학과 및 고려대학교 이과대학 생물학과*)

배추흰나비(*Pieris rapae* L.)의 변태에 따른 esterase의 활성변화 및 zymogram pattern의 변화를 조사하였으며 5령말 유충으로부터 3개의 esterase (E2, E6, E11)를 순수 분리하였다. Esterase의 활성은 5령초에서 5령말에 이르는 동안 급격히 증가하여 5령말에서 최대의 활성이 나타났으며 전기영동의 결과 whole body와 중장내 esterase band의 수나 강도는 5령말에서 최대로 나타났다. Gel filtration과 ion-exchange chromatography 및 preparative electrophoresis를 통해 정제된 각 esterese의 최종순도는 약 30-60배였다.