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Studies on Esterase of *Pieris rapae* L. II. Biochemical Properties and Immunological Studies

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The properties of three esterases (E2, E6 and E11) which were previously purified from *Pieris rapae* L. were determined and physiological role of E6 was inferred using immunological methods. Based on inhibitor study, all of the purified esterases were found to be carboxylesterases (EC 3.1.1.1). The Km values for E2, E6 and E11 were determined to be 6.89×10^{-4}M, 3.19×10^{-4}M and 3.69×10^{-4}M, respectively. The molecular weights of E2, E6 and E11 were estimated to be 42 KD, 81 KD and 174 KD, respectively. The isoelectric points of E2, E6 and E11 were estimated to be pH 5.54, pH 5.89 and pH 6.50, respectively. The concentration of E6 during development was highest at the late 5th instar larval stage and that according to organs at the same stage was highest in midgut. These results suggest that E6 might be a hydrolase involved in the digestion of dietary lipids.

KEY WORDS: Cabbage worm, Esterase. Midgut

Esterases are a heterogeneous group of enzyme, widely distributed among animal and plant tissues, that mainly hydrolyze esters of short-chained fatty acids (C_2 – C_4) and are different from lipases, which preferentially hydrolyze esters of long-chained fatty acids (C_8 and upwards). At the present time, esterases are classified into carboxylesterases (EC 3.1.1.1), arylesterases (EC 3.1.1.2), acetylesterases (EC 3.1.1.6) and cholinesterases (EC 3.1.1.7 and EC 3.1.1.8), based on their reactions with different substrates and on their sensitivities to specific inhibitors (Augustinsson, 1961; Holmes and Masters, 1967, 1968; Masters and Holmes, 1972; Pearse, 1972; Hayat, 1973).

Insect esterases have attracted many researchers since a few decades ago, because they exhibit a variety of molecular forms among the species and they appear to participate in digestion (Kapin and Ahmad, 1980), regulation of juvenile hormone titre (Whitmore *et al.*, 1975), reproduction (Richmond *et al.*, 1980) and insecticide resistance (Motoyama and Dauterman, 1974; Devonshire, 1977). Most of the researches, however, have been confined to the study at the biochemical properites of esterase using crude extracts and to classification of the esterase bands appeared on the gel using various inhibitors.

The present study reports on the kinds and biochemical properties of the esterases which were previously purified and also on the physiological role of the enzymes in the cabbage worm, *Piers rapae* L.

Materials and Methods

Insects

Cabbage worms, *Pieris rapae* L., used in the present study were reared on kail in vinyl house.

Assay and Characterization of Esterase

Esterase assays were carried out according to

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the method of Kapin and Ahmad (1980) with some modifications.

Characterization of esterase was accomplished by the use of three generally known specific inhibitors, paraoxon (inhibitor of carboxylesterase and cholinesterase), *p*-hydroxymecuribenzoic acid (inhibitor of arylesterase) and eserine (inhibitor of cholinesterase). Final molar concentrations of these inhibitors used were 1×10^{-5}M (paraoxon), 1×10^{-4}M (PHMB) and 1×10^{-5}M (eserine). Five hundred μl of enzyme solution was preincubated with a specific inhibitor for 10 min at 30°C and assayed according to the procedure mentioned above.

Determinatin of Molecular Weights

The molecular weight of native esterase was determined by polyacrylamide gel electrophoresis as described by Hedric and Smith (1968). Standard molecular weight markers used were α-lactalbumin (14,200), carbonic anhydrase (29,000), chicken egg albumin (45,000), bovine serum albumin (monomer, 66,000; dimer, 132,000) and urease (dimer, 240,000; tetramer, 480,000).

Isoelectric Focusing

Isoelectric focusing was conducted on 6% polyacrylamide gel with 1% ampholine according to the method of Wrigley (1968). Buffer used was 10 mM H_3PO_4 in upper chamber and 20 mM NaOH in lower chamber and two gels were used for each sample. After electrophoresis, gel without sample was sliced in 5 mm thickness and each slice was incubated in 2 ml of triple-distilled water for 24 hrs and then the pH was measured. The remaining gel was sliced, incubated as above and assayed for esterase activity.

Preparation of Antiserum

Purified esterase 6 (0.5 ml) and Freund's complete adjuvant (0.5 ml) were mixed and injected subcutaneously into a rabbit. Injections were made every other day for the 1st week, and the 4th injection was carried out 1 week later and a mixture of purified esterase 6 (0.5 ml) and Freund's incomplete adjuvant (0.5 ml) was injected 2 weeks after the 4th injection. Blood was collected 2 weeks after the 5th injection and centrifuged at 8,000 g for 10 min. The supernatant was used as antiserum against esterase 6 in the subsequent experiments.

Immunological Methods

Immunodiffusion was conducted by the method of Ochterlony (1949) with some modifications. After the interaction of antigen and antibody was carried out on 1% agarose gel buffered with 0.1 M barbital-lactate, the plate was stained with Amido black 10B and destained with 2% acetic acid.

Immunoelectrophoresis was carried out in 0.1 M barbital-lactate buffer (pH 8.6) at the voltage of 10 V/cm. Staining and destaining were performed in the same way as described above.

Rocket immunoelectrophoresis was performed according to the method of Laurell (1966). Antibody was added to a concentration of 3% in the same gel as described for immunodiffusion. Electrophoresis was carried out in 0.1 M barbital-lactate buffer (pH 8.6) at the voltage of 5 V/cm for 15 hrs. Staining and destaining were performed in the same way as described above.

Results

Biochemical Properties of Esterase

Various properties of the purified esterases (E2, E6 and E11) which were obtained from previous experiments were revealed as below.

Optimal Incubation Conditions: As shown in Figs. 1 and 2, E2, E6 and E11 showed their maximal activity at pH 6.0, pH 8.5 and pH 6.5 and at 50°C, 37°C and 40°C, respectively. A catalytic period of up to 4 hrs was found to be linear for E6 and E11, and that of up to 2 hrs was found to be linear for E2 (Fig. 3).

Heat Stability: Enzyme solution was preincubated at the various temperatures for 10 min and heat stability for esterase activity was measured. E2 maintained its normal activity until 50°C but E6 and E11 represented 70% and 45%, respectively. Thereafter, as the temperature goes higher, their activity decreased and

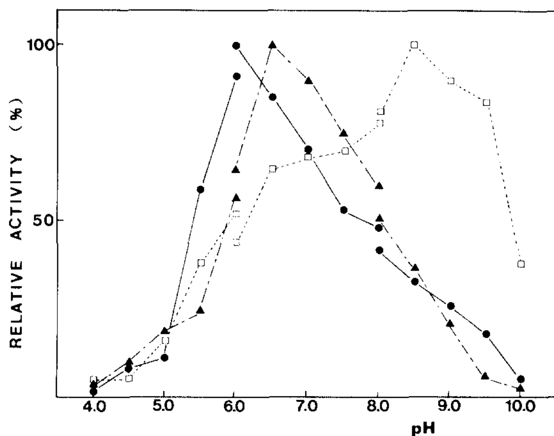

Fig. 1. The effect of pH on esterase activity of *P. rapae*. Enzyme activity in the pH 4.0 to 6.0 range carried out in 0.1 M acetate buffer; in the pH 6.0 to 8.0 range was carried out in phocphate buffer; in the pH 8.0 to 10.0 range carried out in glycine-NaOH buffer. ● —— ●, esterase 2; □ ······ □, esterase 6; ▲ ······ ▲, esterase 11.

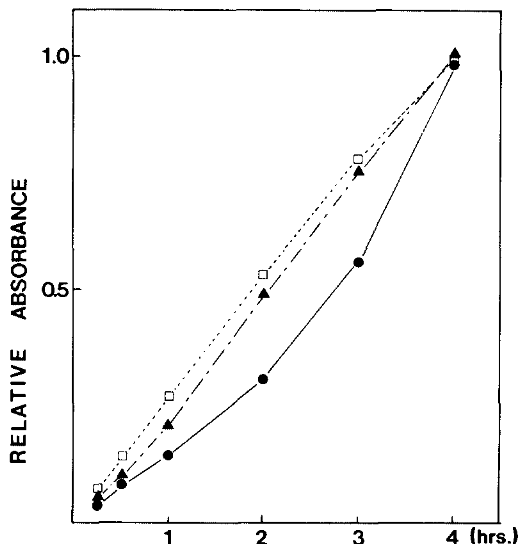

Fig. 3. The effect of time lapse on esterase activity of *P. rapae*. ● —— ●, esterase 2; □ ······ □, esterase 6; ▲ ······ ▲, esterase 11.

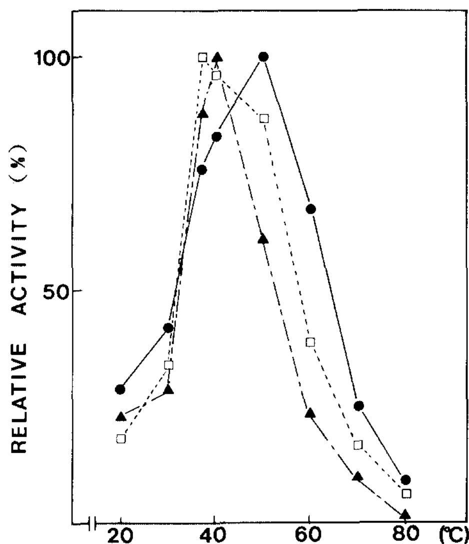

Fig. 2. The effect of temperature on esterase activity of *P. rapae*. ● —— ●, esterase 2; □ ······ □, esterase 6; ▲ ······ ▲, esterase 11.

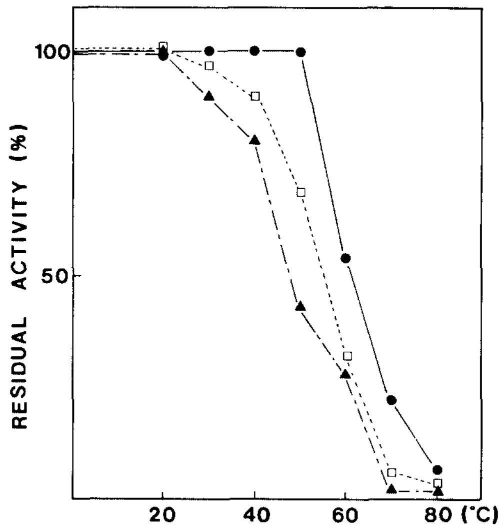

Fig. 4. Heat stability of esterase. The enzyme was incubated at the various temperatures for 10 min, immediately cooled and assayed about residual activity. ● —— ●, esterase 2; □ ······ □, esterase 6; ▲ ······ ▲, esterase 11.

finally showed no esterase activity above 80°C (Fig. 4).

Km Value: The substrate affinity of esterase was measured by altering the concentration of α-naphthylacetate. Fig. 5 depicts the results by the Lineweaver-Burk plot. Km values of E2, E6 and E11 were calculated as 6.89×10^{-4}M, 3.19×10^{-4}M, and 3.69×10^{-4}M, respectively.

Molecular Weight: The molecular weights of E2, E6 and E11 were estimated to be 42 KD,

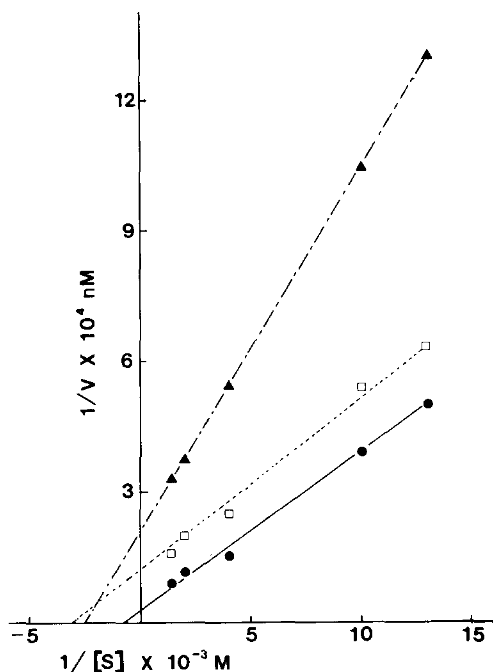

Fig. 5. Lineweaver-Burk plots of concentration of α-naphthylacetate against rate of hydrolysis by esterase of *P. rapae*. ● —— ●, esterase 2; □ ······ □, esterase 6; ▲ ······ ▲, esterase 11.

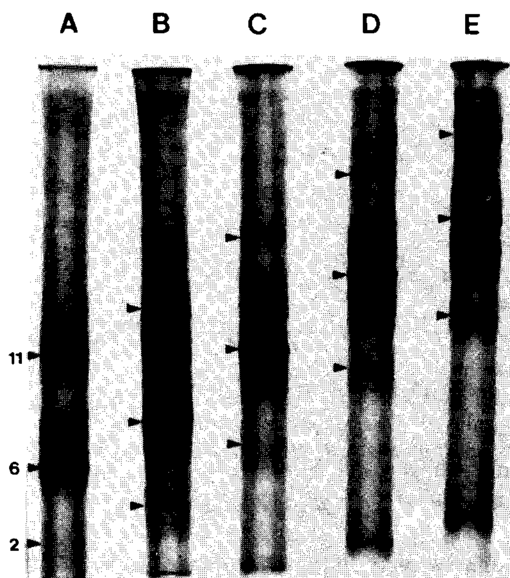

Fig. 6. The effect of different polyacrylamide concentrations on the electrophoretical migration of whole body esterases. A, B, C, D, and E; 5, 6, 7, 8, and 9% gel, respectively.

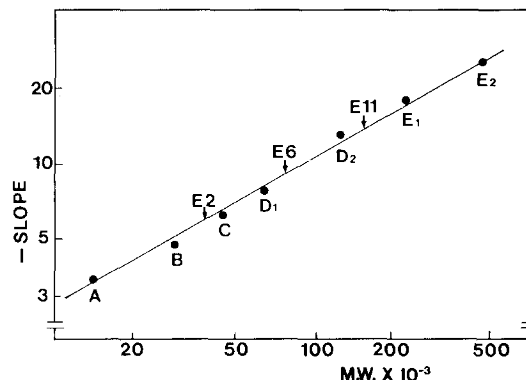

Fig. 7. Determination of M. W. for esterase isozymes under native conditions according to the method of Hedrick and Smith (1968). Marker proteins used were A, α-lactalbumin (14,200); B, carbonic anhydrase (29,000); C, chicken egg albumin (45,000); D_1 and D_2, bovine serum albumin, monomer and dimer (66,000 and 132,000); E_1 and E_2, urease, dimer and tetramer (240,000 and 480,000). E2, esterase 2; E6, esterase 6; E11, esterase 11.

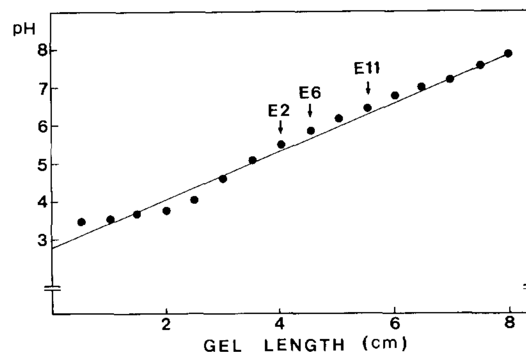

Fig. 8. Determination of isoelectric point of the purified esterases by the method of Wrigley (1968). E2, esterase 2; E6, esterase 6; E11, esterase 11.

81 KD and 174 KD, respectively (Figs. 6 and 7).

Isoelectric Point: The isoelectric points of E2, E6 and E11 were estimated to be pH 5.54, pH 5.89 and pH 6.50, respectively (Fig. 8).

Characterization of the Purified Esterases

None of the purified esterases tested were inhibited by eserine, and all of them were inhibited by only less than 10% by *p*-hydroxymecuribenzoic acid (PHMB) but inhibited about 90% by paraoxon, indicating that all of them were carboxylesterases (Table 1).

Table 1. The effect of inhibitors on the purified esterase activity

Enzyme	% inhibition of esterase activity		
	Eserine*	PHMB**	Paraoxon*
Esterase 2	0.0	8.4	80.8
Esterase 6	0.0	6.5	94.3
Esterase 11	0.0	7.1	87.5

*At 1×10^{-5}M concentration.
**At 1×10^{-4}M concentration.

Relationships among the Purified Esterases

As shown in Fig. 9, precipitin arc formed only in E6 but not in both of E2 and E11. This suggested that there are no immunological relationships at least between E6 and E2 or E6 and E11.

Quantitative Change of Esterase 6 during Development

A continuous precipitin line formed from early 5th instar larvae to newly ecdysed pupae (Fig. 10) and the concentration of E6 from both of

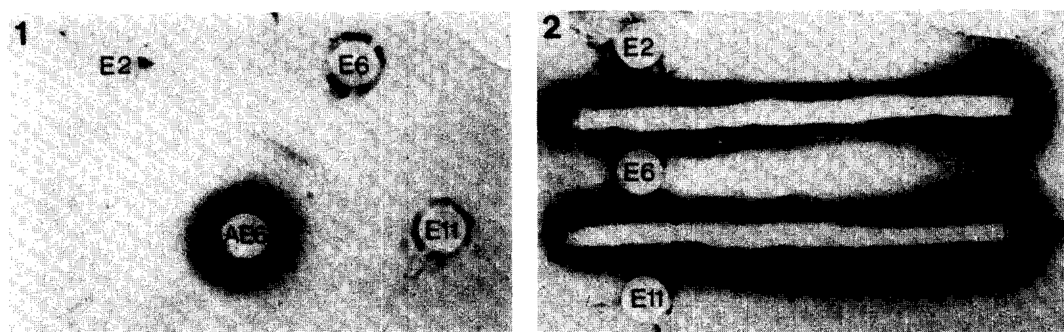

Fig. 9. Immunological relationship among the purified esterases. 1, double immunodiffusion; 2, immunoelectrophoresis. AE6, antiserum against purified esterase 6; E2, esterase 2; E6, esterase 6; E11, esterase 11.

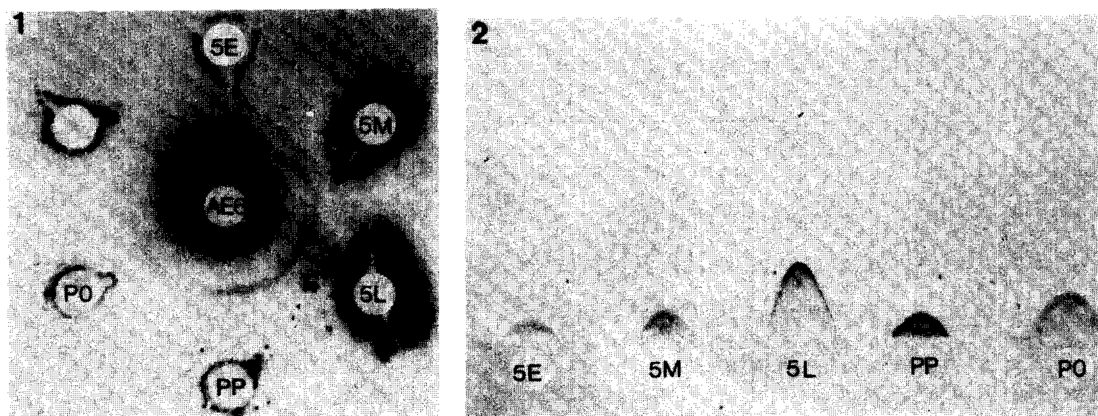

Fig. 10. Immunological assay of whole body during development reacted with anti-esterase 6. 1, double immunodiffusion; 2, rocket immunoelectrophoresis. AE6, antiserum against purified esterase 6; 5E, early 5th instar larvae; 5M, mid 5th instar larvae; 5L, late 5th instar larvae; PP, prepupae; P0, newly ecdysed pupae.

midgut and whole body during development was highest at the late 5th instar larval stage (Fig. 11).

Quantitative Difference and the Distribution of Esterase 6 in Each Organ

As shown in Fig. 12, precipitin line appeared in midgut, foregut and silk gland but not in haemolymph, cuticle, hindgut, malpighian tubule and fat body. The concentration of E6 at late 5th instar larval stage was highest in midgut followed by foregut and silk gland.

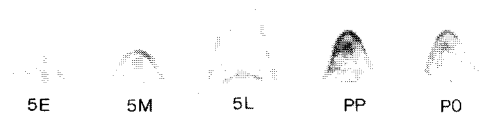

Fig. 11. Rocket immunoelectrophoresis of midgut during development reacted with anti-esterase 6. Stage as in Fig. 10.

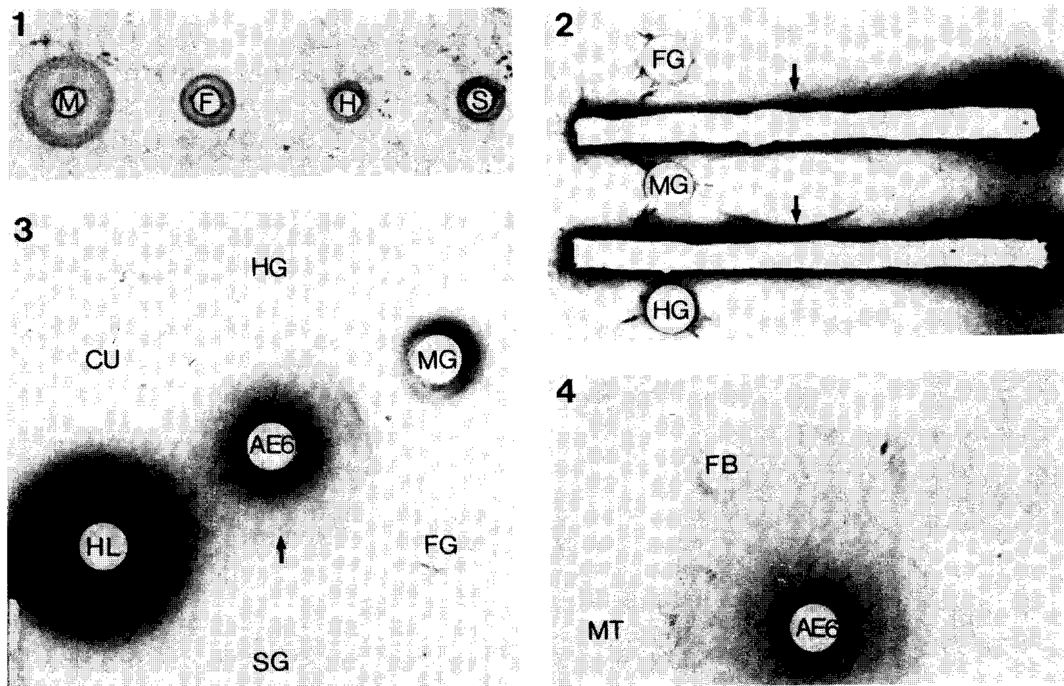

Fig. 12. Immunological assay of organs from late 5th instar larvae reacted with anti-esterase 6. 1, single radial immunodiffusion; 2, immunoelectrophoresis; 3 and 4, double immunodiffusion. AE6, antiserum against purified esterase 6; M and MG, midgut; F and FG, foregut; H and HG, hindgut; S and SG, silk gland; HL, haemolymph, CU, cuticle; MT, malpighian tubule; FB, fat body.

Discussion

It is generally accepted that lipases are involved in the utilization of triglycerides (TGL), the major yolk lipids, during insect embryogenesis (Salkeld, 1961; Guss and Krysan, 1972; Gadallah and Marei, 1973; Turunen, 1973; Geering and Oberline, 1975). After hatched from the egg, however, the larvae (particularly herbivores) become adapted to a different diet. Dietary leaf lipids may be very low, if at all, in TGL and high in other esters, suggesting that the insects need other hydrolases (Turunen,

1977). This substrate diversity probably accounts for the high degree of polymorphism and genetic variation found among insect esterases. In the previous study, three esterases from the late 5th instar larvae of *P. rapae* were purified and present experiments were performed to investigate the biochemical properties and physiological role of the enzymes.

Optimum pH of E6 was found to be 8.5 which is different from those of E2 and E11 being 6.0 and 6.5, respectively. Those of *L. dispar* (Kapin and Ahmad, 1980) and *P. americana* (Cook and Forgash, 1965) showed the optimum between 7.5-7.7.

Optimum reaction temperatures for E2, E6 and E11 showed wide varieties (50°C for E2, 37°C for E6 and 40°C for E11, respectively). Optimum temperature of the esterase for *L. dispar* (Kapin and Ahmad, 1980), *C. tarsalis* (Houk and Hardy, 1981) and *M. persicae* (Sudderuddin and Tan, 1973) was also known to be 50°C, 30°C and 37°C, respectively.

E2 maintained its normal activity until 50°C, indicating that it is stable against heat but E6 and E11 reserved 70% and 45% of their activity at the same temperature, indicating that they are relatively unstable against heat.

Km values for the purified esterases were calculated to be 6.89×10^{-4}M (E2), 3.19×10^{-4}M (E6), and 3.69×10^{-4} M (E11), respectively. These values were different from those reported for *L. dispar* (Kapin and Ahmad, 1980; 4.25×10^{-5}M) and *M. persicae* (Devonshire, 1977; 1.31×10^{-4}M). Also, Hipps and Nelson (1974) reported that Km values for esterase of the midgut and gastric caecae of *P. americana* eluted from DEAE-cellulose column showed a wide range of the value from 10^{-4}M to 10^{-5}M.

The isoelectric points of E2, E6 and E11 were estimated to be pH 5.54, pH 5.89 and pH 6.50, respectively. These values are similar to those of major esterases from *C. tarsalis* (Houk and Hardy, 1981).

The molecular weights of E2, E6 and E11 were estimated to be 42 KD, 81 KD and 174 KD, respectively. Generally, the molecular weights of esterases have different values from the same species as well as other species. Danford and Beardmore (1979) reported that the molecular weight of esterase 6 in *D. melanogaster* was 90 KD composed of 4 subunits. According to Mane *et al.*, (1983), however, esterase 6 in the same species was shown to be glycoprotein and its molecular weight appeared to be 62 KD-65 KD.

None of the purified esterases were inhibited by eserine, an inhibitor of cholinesterases, which indicated that all of them were not cholinesterases. All of the purified esterases were slightly inhibited by only less than 10% by PHMB, an inhibitor of arylesterases and activator of acetylesterases, suggesting that none of them were arylasterases or acetylesterases. All of the purified esterases were greatly inhibited (about 90%) by paraoxon, an inhibitor of cholinesterases and carboxylesterases. Thus, E2, E6 and E11 could be classified as carboxylesterases (EC 3.1.1.1).

In the previous study, it was considered that the increasing rate of food consumption is correlated with the increase of esterase activity during larval development. Also, in zymogram pattern of esterase of the late 5th instar larval stage during which both of esterase activity and feeding activity is highest, E6 appeared as major band. Thus, antiserum against E6 was produced and immunological studies were carried out to elucidate the correlation between feeding activity and the concentration of E6 in whole body and midgut during development. The results showed that the concentration of E6 is highest at the late 5th instar larval stage during development particularly in midgut tissue. Therefore, it is considered that E6 is a hydrolase involved in the digestion of dietary lipids. These findings are in good accordance with those of study for *L. dispar* (Kapin and Ahmad, 1980). They reported that most of the esterases in midgut tissue are carboxylesterases and involved in the absorption and digestion of foods judging from the fact that esterase activity declines rapidly during apolysis and rises sharply immediately after ecdysis. Also, Tanada (1980) reported the localizations of high esterase activity associated with lipid droplets in the midgut epithelial cell of armyworm larva using a cytochemical technique and the electron microscopy.

Acknowledgements

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배추흰나비(*Pieris rapae* L.)의 esterase에 관한 연구
II. 생화학적 특성 및 면역학적 연구

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고려대학교 이과대학 생물학과*)

배추흰나비(*Pieris rapae* L.)로부터 정제된 3개의 esterase (E2, E6, E11)의 특성을 조사하였으며 면역학적 연구를 통해 E6의 생리적 역할을 추정하였다. 억제제의 연구결과, E2, E6, E11 모두 carboxylesterase (EC 3.1.1.1)임이 밝혀졌다. Km 값은 E2, E6 및 E11이 각각 6.89×10^{-4} M, 3.19×10^{-4} M, 3.69×10^{-4} M이었다. 분자량은 E2, E6 및 E11이 각각 42 KD, 81 KD, 174 KD이었고 등전점은 각각 pH 5.54, pH 5.89, pH 6.50이었다. 변태에 따른 E6의 농도는 5령 말기에서 가장 높았으며 특히 중장조직에서 높게 나타났다. 따라서 E6은 소화효소로 사료된다.