

カイコ血液中の酸性フォスファターゼ・アイソザイムの新変異型

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A novel variant of acid phosphatase isozyme from hemolymph of the silkworm, *Bombyx mori*

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ABSTRACT

Four electrophoretic variants of acid phosphatase have been already reported in the silkworm hemolymph. We found an electrophoretic variant (type E) which had been designated O (null) type since the distinct band had not been detected. The band E was undiscernible when the substrate (α -naphthyl phosphate) and chromogen (Fast blue B) were poured simultaneously on the gel after electrophoresis according to the former procedure. However, if the gel was incubated with α -NP then Fast blue B was added, a distinct band E appeared. Quantitative analyses showed that Fast blue B inhibited the isozyme E competitively but other isozymes noncompetitively. Several lines of experimental evidence suggest the isozyme E is not only a charge isomer but also an isozyme showing different kinetic properties with other isozymes. By crossing experiments isozymes A-E were considered to be controlled by codominant alleles, Bph^A-Bph^E, respectively.

1. INTRODUCTION

Acid phosphatase polymorphism and genetic control have been reported in some insects. Ogita and Kasai (1965) studied the developmental change and genetic control of acid phosphatases in the house fly, *Musca domestica*. The gene-enzyme system of the major acid phosphatase, ACPH-1 in the fruitflies, *Drosophila melanogaster* and *D. simulans* was discovered and mapped by MacIntyre (1966). Genetic and biochemical studies were carried out by using naturally occurring quantitative variants of ACPH-1 (Johns and Postlethwait, 1985). Concerning *Drosophila virilis*, the properties of purified acid phosphatase allozymes were compared (Narise and Tominaga, 1987).

In the silkworm, *Bombyx mori*, Yoshitake and Akiyama (1964) found four electrophoretic variants, A, B, C, D and a null mutant, O of hemolymph acid phosphatase. They revealed that these isozymes are controlled by codominant alleles. Subsequently, linkage analysis located these alleles at 18.2 map unit on the chromosome 23 (Fujimori et al., 1984).

Recently, we found an electrophoretic variant, E from the silkworm

hemolymph. This might be identical with O (null) type by Yoshitake and Akiyama (1964).

The present paper describes conditions under which band E can be detected after electrophoresis together with the mode of inheritance and the specificity of this isozyme.

2. MATERIALS AND METHODS

Animal

Silkworms, *Bombyx mori* were reared on mulberry leaves at about 24°C. Five inbred strains, Daikojo (A), Tsunomata (B), Spain (B and C), C124 (D) and Daizo (E, formerly O) were used. Hemolymph was collected in a chilled tube by cutting the abdominal legs at the 4 or 5 days after the 4th ecdysis, stored at -40°C and thawed before use.

Chemicals

p-Nitrophenylphosphoric acid disodium salt, 1-naphthyl-phosphoric acid disodium salt, sodium dodecyl sulphate, agar powder, acrylamide, N, N'-methylenebisacrylamide and N, N, N', N'-tetramethyl ethylenediamine were purchased from Nakarai Chemicals, Japan. Fast blue B was obtained from Merck, FRG.

Electrophoresis

Agar and polyacrylamide gels were utilized for thin layer electrophoresis and essentially the same results were obtained by both methods.

Agar gel electrophoresis was performed according to Ogita (1964) and Eguchi (1968). A constant voltage of 13 V/cm was applied for 90-150 min at 4°C. After electrophoresis, a substrate solution, 20 mg disodium α -naphthyl phosphate (α -NP) in 20 ml of acetate buffer, pH 4.7 was poured on a gel plate, incubated for about 30 min at 30°C, then Fast blue B solution (20 mg/20 ml) was added.

In the case of polyacrylamide gel electrophoresis, 7% gel in Tris-HCl buffer, pH 8.8 was used. Electrophoresis was carried out at a constant voltage of 18 V/cm for 150 min at 4°C.

Enzyme Assay

Usually, phosphatase activity was measured by the rate of hydrolysis of p-nitrophenyl phosphate (p-NPP). The reaction mixture contained 0.5 ml of 10 mM p-NPP, 2 ml of 0.1 M acetate buffer, pH 4.7 and 0.5 ml of enzyme solution. After 30 min incubation at 30°C, the reaction was stopped by adding 2 ml 0.1 M NaOH. The color formed was measured at 400 nm.

When α -NP was employed as a substrate, the mixture of 0.2 ml enzyme, 0.2 ml of 10 mM α -NP and 0.8 ml of 0.1 M acetate buffer was incubated for 30 min at

30°C, then 0.8 ml of 4% sodium dodecyl sulphate (SDS) solution containing 0.2% Fast blue B was added and the amount of α -naphthol produced was determined by measuring absorption at 540 nm.

3. RESULTS

Electrophoresis of Hemolymph Acid Phosphatases

Zymograms of acid phosphatases on polyacrylamide gel are shown in Fig. 1; the electrophoretic mobility of hemolymph phosphatases showed variations among different silkworm strains. In a previous report (Yoshitake and Akiyama, 1964), phosphatase isozymes were designated A, B, C and D from the slowest to the fastest moving band toward anode, but we have reversed the naming according to the conventional nomenclature.

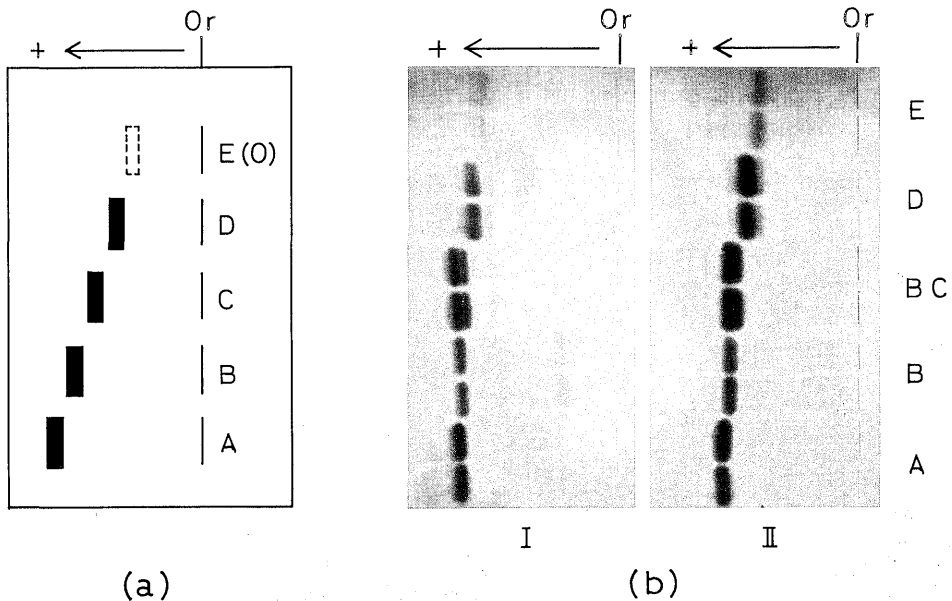


Fig. 1 (a) Diagram showing five acid phosphatase isozymes of the silkworm hemolymph by polyacrylamide gel electrophoresis. A symbol in parenthesis indicates the former name. (b) Photographs showing isozymes from distinct silkworm strains and comparison of the band E in different staining conditions. The 7% polyacrylamide gel, pH 8.8 on the glass plate (14×10 cm) was used. Electrophoresis was done at a constant voltage of 18 V/cm for 150 min at 4°C. After electrophoresis, the gel was incubated in the substrate solution, 50 mg disodium α -naphthylphosphate (α -NP) in 50 ml of acetate buffer, pH 4.7 for 15 min (b-I) or 30 min (b-II) at 37°C and then Fast blue B solution (100 mg/50 ml) was added.

We found a new band E in the present study although this isozyme had been named the null variant, O in the above paper (refer to Fig. 1-a), since they failed to detect the activity on a gel in this variant. The band E was not discernible

when the substrate (α -NP) and chromogen (Fast blue B) were poured simultaneously on the gel plate after electrophoresis by the method of Yoshitake and Akiyama (1964).

However, if the gel plate was incubated with α -NP at 37°C then Fast blue B solution was poured on the plate, the distinct band E appeared. More than 30 min incubation was necessary for the detection of the clear E band (Fig. 1-b). Other isozymes were detectable as intense bands even if α -NP and Fast blue B were applied simultaneously.

Mode of Inheritance

Crossing experiments were carried out to determine the mode of inheritance of acid phosphatase isozymes, especially to clarify the relationship between genes controlling the novel isozyme and others. Comparison of zymograms of F₁ hybrids and parents showed that the offspring from single pair mating between different types produced phosphatases with both parental bands (data not shown).

In F₂ and backcross, segregation of each band was observed in individuals from a single moth; some examples are shown in Fig. 2. The segregation data of phosphatase isozymes are summarized in Table 1. The result reveals that the expressions of bands E, A and B are controlled by allelic genes Bph^E (blood phosphatase E), Bph^A and Bph^B, respectively. The χ^2 tests indicate good

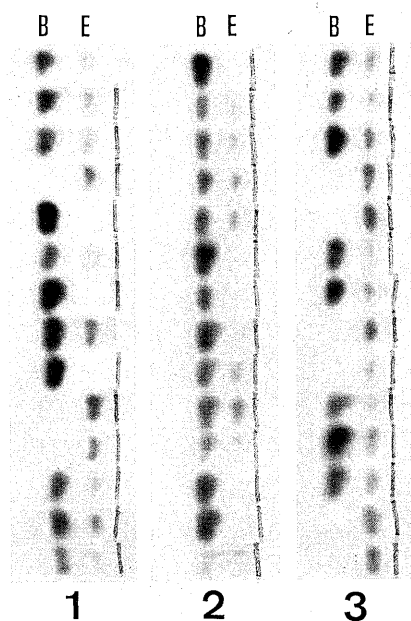


Fig. 2 Segregation of acid phosphatases E, B and EB in the F₂ generation on agar gels. Hemolymphs from individual larvae were electrophoresed at 13 V/cm for 120 min. The cathode is at the right side and the anode at the left. 1. (E×B) F₂, 2. (E×B)×B, 3. (E×B)×E.

Table 1. Crosses made to determine the mode of inheritance of hemolymph acid phosphatases A, B and E

Cross	Offspring					Total	χ^2	P
	E	EA	A	EB	B			
(E×A)F ₂	48	88	64	—	—	200	5.44	0.07
(E×A)×A	—	47	52	—	—	99	0.25	0.62
(E×A)×E	60	40	—	—	—	100	4.00	0.05
(E×B)F ₂	68	—	—	156	84	308	1.71	0.45
(E×B)×B	—	—	—	78	76	154	0.03	0.95
(E×B)×E	73	—	—	81	—	154	0.42	0.52

correspondences of the experimental results and expected ratios. Considering the result by Yoshitake and Akiyama (1964), acid phosphatase isozymes A–E are conceived to be controlled by codominant alleles Bph^A–Bph^E.

Analysis of Specificity of the Isozyme E

In order to investigate more quantitatively the specific property of the isozyme E, the following experiments were carried out. Instead of conventional method of phosphatase assay in which the reaction product, phosphoric acid or p-nitrophenol is determined, we attempted to measure the coloration of complex, α -naphthol and Fast blue B. Namely, we intended to analyze the enzyme reaction in a similar condition to that in the gel electrophoresis.

Figure 3 shows the time course of α -NP hydrolysis by isozymes A and E in the

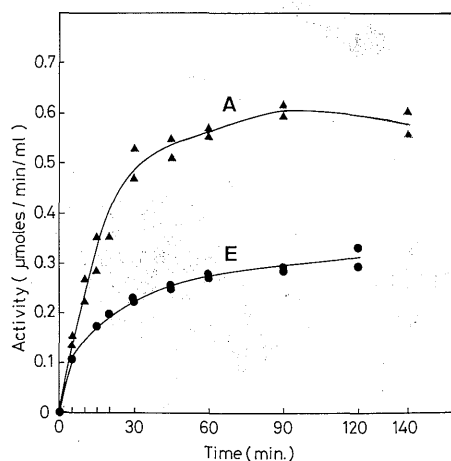


Fig. 3 Time course of α -NP hydrolysis by isozymes A and E in the presence of Fast blue B. The 0.1% Fast blue B was contained in the reaction mixture with α -NP and hemolymph. After incubation at 30°C for indicated times, the reaction was stopped by adding 4% SDS and the absorbance was measured at 540 nm.

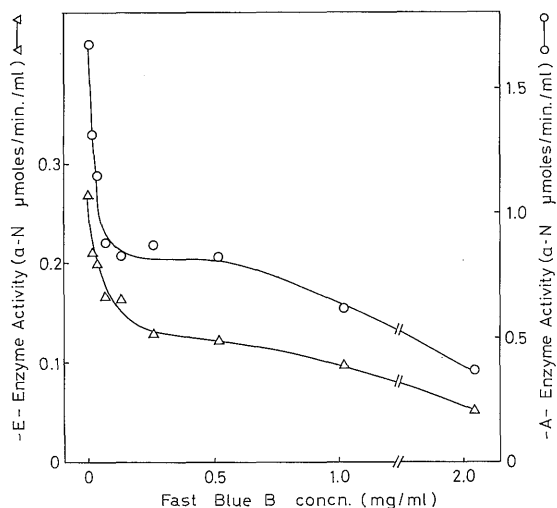


Fig. 4 Effect of Fast blue B on the phosphatase activity. Hemolymphs from the silkworm strains A and E were preincubated with various concentrations of Fast blue B for 15 min at 30°C, then 10 mM α -NP was added. After incubation for 30 min at 30°C, the reaction was terminated by adding 4% SDS containing 0.2% Fast blue B and measured at 540 nm. Enzyme activity was expressed as the rate of liberation of α -naphthol (α -N).

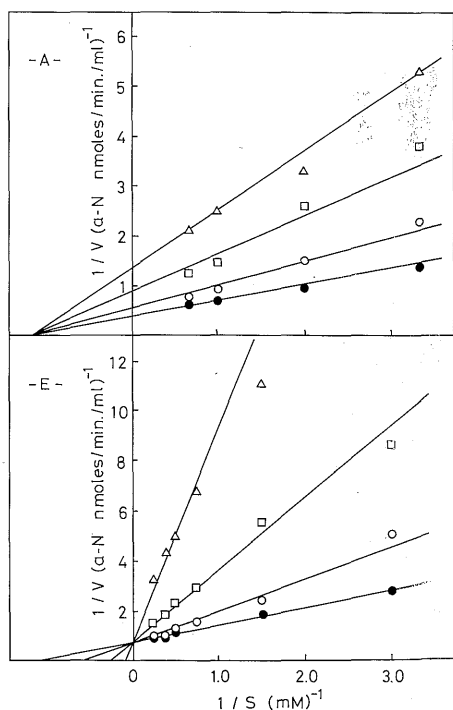


Fig. 5 Relationship between substrate (α -NP) concentration and phosphatase activity with or without inhibitor (Fast blue B). Lineweaver-Burk plots of the isozymes A and E are shown. Acid phosphatases, A and E were purified by ammonium sulphate fractionation, column chromatographies on DEAE-Sephacel and Sephacryl S-200. The enzyme activity was p-NP 0.918 μ mols/min/mg protein (A) and 5.04 μ mols/min/mg protein (E), respectively. ● without Fast blue B, □ 0.088 mM and Δ 0.438 mM Fast blue B. Isozyme E: ○ with 0.012 mM, □ 0.059 mM and Δ 0.292 mM Fast blue B.

reaction mixture containing Fast blue B. The reaction product increased almost linearly for 15 min in the isozyme A, whereas for the isozyme E, the decrease in reaction velocity occurred after about 5 min incubation. This result suggests the inhibitory effect of Fast blue B for acid phosphatases, since the reaction velocity increased linearly within 60 min in the reaction mixture without Fast blue B.

Thus, the effect of Fast blue B on the phosphatase activity was examined. As shown in Fig. 4, the enzyme activities decreased with increasing concentration of Fast blue B; the effect was observed even in very low concentration of reagent.

Subsequently, the mode of inhibition of Fast blue B against different isozymes was compared. The relation between substrate concentration and enzyme activity was examined and the lineweaver-Burk plots are shown in Fig. 5. This figure indicates that Fast blue B inhibits the isozyme A noncompetitively, whereas the inhibition of the isozyme E seems to a competitive type. The inhibition pattern similar to A was seen in the isozyme B (data not shown).

4. DISCUSSION

In the present study, the specificity of the novel phosphatase isozyme E was examined and was compared with the properties of other isozymes. As mentioned above, this isozyme had been designated formerly the O type indicating the null mutant as well as electrophoretic variants of esterase in the silkworm (Eguchi et al., 1965; Eguchi and Yoshitake, 1966). On the other hand, null-activity mutants were induced for the acid phosphatase-1 system in *Drosophila melanogaster* using ethylmethanesulfonate as the mutagen (Bell et al., 1972) and partial characterization of these mutants was reported (Bell and MacIntyre, 1973).

The present study elucidated that the type O of hemolymph acid phosphatase of the silkworm is not a null variant but possesses an enzyme activity with noticeable characteristics. Using this larva, we found a distinct phosphatase band E by improving the enzyme detecting method. Moreover, we demonstrated that the chromogen, Fast blue B suppressed the phosphatase activity and the mode of inhibition of isozyme E was different from that of others. In addition to this characteristic, we found that the isozyme E is more labile in thermal and pH stability than other isozymes by using partially purified enzymes (unpublished data). To elucidate the structural and functional specificity of isozyme E in detail, the study on the purified isozymes seems to be necessary. We are performing the purification of E and A isozymes, but it appears to be difficult to purify the isozyme E owing to its instability.

Electrophoretic variants are widespread in the silkworm, *Bombyx mori* (Yoshitake and Akiyama, 1964; Eguchi et al., 1965, 1984; Eguchi and Yoshitake, 1967; Gamo, 1968, 1978, 1982) as well as other animals. It is noteworthy, however, that the phosphatase E is an isozyme showing different properties, that is, acid phosphatase isozymes would comprize heterogeneity not only in non-catalytic sites

but also in the catalytic site.

Acid phosphatases are widely distributed in various organisms but the physiological roles of this enzyme are not well understood. Biochemical study was performed using genetic variants of red cell acid phosphatase in man (Fisher and Harris, 1969). Yasbin et al. (1978) suggested the digestion of degenerating larval tissues of *Drosophila* during larval-pupal transition. Biochemical and genetic analysis in *Dictyostelium discoideum* showed the effectiveness of acid phosphatase isozymes as a marker for cell types (Loomis and Kuspa, 1984). The usefulness of serum acid phosphatase as a clinical tumor marker was investigated with advanced adenocarcinoma of the prostate (Maatman et al., 1984).

With regard to the silkworm, *Bombyx mori*, acid phosphatases were found to distribute in some tissues (Ishihara, 1957; Sridhara and Bhat, 1963; Kageyama et al., 1973; Seong et al., 1983) and marked changes in the gut (Eguchi and Iwamoto, 1975) and hemolymph (Eguchi, 1964) were observed during development.

At present, the function of acid phosphatases in the silkworm is not clear, but the comparison of structure and function between the isozyme E and others should afford valuable informations for the enzymatic studies of insects and other animals.

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