カイコの胚発生におけるアルカリホスファターゼ活性の変動

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Changes in alkaline phosphatase activity during embryogenesis of *Bombyx mori*

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During embryogenesis of *Bombyx mori* the level of alkaline phosphatase activity increased biphasically, first until the middle stages then the final period shortly before hatching. The middle-stage enzyme resembled the late-stage one in some properties such as optimum pH (10.8) but differed in affinity toward the synthetic substrate p-nitrophenylphosphate and in substrate specificity. The properties of the latter-stage enzyme was similar to those of larval gut juice alkaline phosphatase.

In the diapausing eggs of *Bombyx mori* the rate of protein synthesis is suppressed to a low rate but increases during the first 20 to 24 hr of post-diapause development when is the stage preceding the onset of organogenesis (Saito *et al.*, 1982, 1983, 1984, 1985a, b). Also in the natural and artificial non-diapause eggs the rate of protein synthesis rises before the embryo begins organogenesis (Sonobe and Odake, 1986). The electrophoresis pattern of proteins synthesized *in vivo* (Sonobe and Odake, 1986; Saito *et al.*, 1986) or in a cell-free translation system of mRNAs (Saito *et al.*, 1985a) substantially remains unchanged during the augmentation of the protein synthesis rate, although a stage-specific (Saito *et al.*, 1986) and a mutant-specific product (Sonobe and Odake, 1986) can be detected.

In this connection, we are interested in the problem as to how the embryos show changes in the molecules that reflect the differentiation process during organogenesis. Alkaline phosphatases are among the most important enzymes serving as markers for such study because of the following criteria: 1. Their occurrence is often limited to the region of endoderm and thus to the stage of gastrulation in *Xenopus laevis* (Abe *et al.*, 1972), the sea urchin (Pfohl, 1975) and the ascidian *Ciona intestinalis* (Whittaker, 1977). 2. The activity change of the enzymes also correlates well with the mitotic cycles of embryos of the sea urchin (Karsenti *et al.*, 1987). 3. The enzymes

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are localized on the plasma membranes of cells lining the embryonic gut (Pfohl, 1975) and with the surface microvilli of the oocyte (Le Goascogne *et al.*, 1986), suggesting that they are involved in the membrane architecture. 4. Some of alkaline phosphatases can attack phosphorylated proteins and may have a role in the control of other enzymes and functional proteins through dephosphorylation (review by Ingebritsen and Cohen, 1983).

As to the silkworm eggs, histochemical study has shown that alkaline (as well as acid) phosphatase activity is expressed in the endoderm of the embryo at the stage of blastokinensis (Sugai, 1957). The fluctuation in activity of this enzyme during embryogenesis has been reported (Ito *et al.*, 1954; Chino, 1961). In the present study, we re-examine the assay conditions of this enzyme for the egg homogenate, and extend the study of its developmental changes in embryogenic as well as diapausing eggs. Also we compared some kinetic properties of this enzyme with those of larval enzymes (Eguchi *et al.*, 1972a, b; Eguchi and Suzuki, 1973a, b).

Materials and Methods

Eggs: Fertilized eggs of a bivoltine race, N124, deposited within 30 min were pooled and kept at 25°C to let them enter diapause. Some of the batches were transferred to 5°C at 42 hr after oviposition. After 40 days of chilling the eggs were treated with hot HCl (spec. grav. 1.10, 48°C, 6 min) and then incubated at 25°C to resume development (referred to as post-diapause eggs). The remnant batches were treated with hot HCl (spec. grav. 1.075, 46°C, 6 min) at 20–22 hr after oviposition and kept at 25°C to allow the continuous development (artificial non-diapause eggs). Eggs were staged according to Takami and Kitazawa (1960).

Crude enzyme: Eggs (0.6 g) were ground with a bray and pestle in 4 ml 50 mM sodium borate buffer, pH 10.8, containing 2 mM $MgCl_2$. The broken egg-shells were discarded and the egg contents were homogenized in a motor-driven, Teflon-glass homogenizer (about 10 strokes). The homogenate was centrifuged at 1,000×g for 10 min. The supernatant was taken and the precipitate was extracted with 2 ml of the extraction buffer; the combined supernatant was used as enzyme preparation. All the procedures were done at 0 to 4°C. Gut juice was obtained from larvae of N124 at the 5th instar with electric shock, diluted with the above buffer and used as enzyme solution.

Assay of alkaline phosphatase activity: A reaction mixture (total 2 ml) contained 25 mM p-nitrophenylphosphate (PNPP), 50 mM sodium borate buffer, pH 10.8, and enzyme (one tenth volume of the reaction mixture, about 2 mg crude protein). After 30 min of incubation at 37°C, the reaction was stopped by vigorous agitation with 2 ml of 1 N NaOH. The clear solution was directly used to measure the absorbance at 400 nm by using a Beckman UV-8 spectrophotometer. Appropriate blank values were subtracted. In some experiments the substrate PNPP was replaced by another substrate as specified. In this case the assay conditions were the same as above except that the reaction was stopped by adding trichloroacetic acid (final 5%). The reaction mixture was centrifuged at 1,000×g for 10 min, and an aliquot of supernatant was used for measurement of inorganic phosphate according to the method described previously (Eguchi *et al.*, 1972a). One unit of enzyme was defined as the amount which hydrolyzed 1 μmole of substrate per min. Protein was determined by the biuret method with bovine serum albumin as a standard (Ellman, 1962).

Results

Preliminary study from our laboratory has shown that developing eggs of *B. mori* exhibit marked activity of alkaline phosphatase both at the middle and late stages. Therefore, crude enzymes were prepared from day 5 and day 9 post-diapause eggs and assayed with PNPP as a substrate under the conditions as described in Materials and Methods, but pH of the reaction mixture was varied from 8.5 to 11.5. For both stages the highest activity was found at about 10.8 (data not shown). This value was similar to that for alkaline phosphatase in the larval gut juice either tested in the present study or reported in previous papers (Eguchi *et al.*, 1972b; Eguchi and Suzuki, 1873b). Both extracts showed a proportional increase in activity according to the increasing amounts of enzyme proteins (up to 4 mg crude protein) and in time course until 60 min. Thus, the assay was routinely done as specified in Materials and Methods throughout the present experiments. Results were expressed in terms of activity per mg protein.

Developmental changes in activity of alkaline phosphatase were investigated. As shown in Fig. 1, eggs shortly after oviposition contained a definite amount of activity. It then increased until 12 hr, showing a temporal minimum value around 21 hr and again a peak at 30 hr. The level gradually decreased as diapause deepened and finally became undetectable. When chilling was begun at 42 hr the activity decreased more slowly compared to the above eggs and again finally diminished.

Fig. 2 illustrates the enzyme activity in eggs treated with hot HCl at 21 hr (the artificial non-diapause state). The level increased soon after the acid treatment and showed a shoulder on days 4 to 5 (blastokinesis period, Stage 21). It further augmented rapidly after day 8.

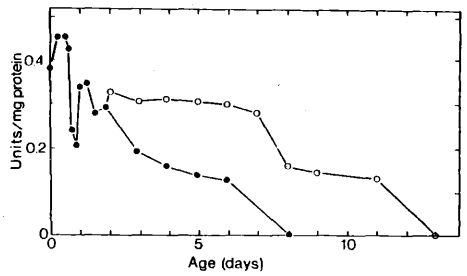

Fig. 1. Changes of alkaline phosphatase activity after oviposition. Eggs were allowed to enter diapause at 25°C (solid circles) or begun to be chilled at 5°C on day 2 (open circles). Ordinate, enzyme activity assayed with p-nitrophenylphosphate as a substrate. Abscissa, average age in days after oviposition (0 day). No activity was detected after day 8 or 13.

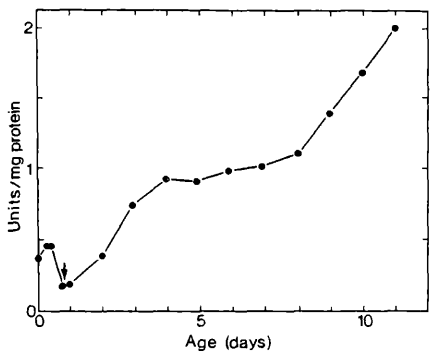

Fig. 2. Changes of alkaline phosphatase activity during embryogenesis of artificial non-diapause eggs. At 20–22 hr after oviposition eggs were treated with hot HCl and allowed to develop at 25°C. Majority hatched on day 11. For ordinate, see the legend to Fig. 1.

Post-diapause eggs after arousal by consecutive chilling and acid treatment were also tested. As shown in Fig. 3, enzyme activity was little detectable at the time of acid treatment but appeared as the development started.

There was again a shoulder on days 5 to 6 (blastokinesis period, Stage 21), and a rapid rise before hatching.

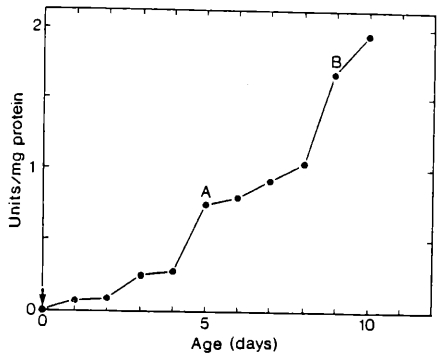

Fig. 3. Changes of alkaline phosphatase activity during embryogenesis of post-diapause eggs. Eggs were chilled at 5°C from day 2 after oviposition for 40 days and subjected to hot acid treatment (arrow). The day of the latter is set zero. For ordinate, see the legend to Fig. 1. See Table 1 for A and B.

The biphasic increase of the alkaline phosphatase activity was confirmed in repeated experiments. This was also seen when the data were not normalized for protein content. These facts prompted us to compare the properties between the day 5 and the day 9 enzymes. The Michaelis-Menten kinetics showed that the affinity toward the substrate PNPP differed; the day 5 preparation showed a K_m value a little higher than that of the day 9 enzyme (Table 1). The enzymes at different stages also exhibited a slight deviation in relative activity against several substrates tested (Table 1). Both preparations had higher activity toward aromatic esters than naturally occurring esters of glycerol and glucose, and this was especially true for the day 9 enzyme. The latter was similar to larval gut jucie alkaline phosphatase in respect to K_m value and substrate specificity (see Table 1) in addition to the optimum pH.

Table 1. Comparison of some properties of alkaline phosphatses from day 5 and day 9 eggs

	Crude enzyme from eggs		Gut juice
	on day 5[a]	on day 9[a]	
K_m for p-nitrophenylphosphate (mM)[b]	6.81	3.72	3.20[c]
Relative activity vs. the substrate:[d]			
p-Nitrophenylphosphate	100	100	100[e]
α-Naphthylphosphate	125	101	106[e]
β-Glycerophosphate	99	55	42[e]
Glucose-1-phosphate	70	42	52[e]
Glucose-6-phosphate	64	50	45[e]

a) Post diapause eggs at days 5 and 9 (A and B in Fig. 3, respectively) were used.
b) Assayed under the standard conditions as in text except that substrate concentration was varied from 0 to 25 mM. The results were calculated for K_m as described by Hanes (1932).
c) Gut juice was obtained and the activity was assayed under the same conditions as those for eggs.
d) Activity was measured at a substrate concentration of 25 mM as in text.
e) Recalculated from published data (Eguchi *et al.*, 1972b; Eguchi and Suzuki, 1973b).

Discussion

In the developing silkworm eggs the alkaline phosphatase activity increased at two-step fashions, making a shoulder on days 4 to 6, and then more steeply toward hatching. A gradual increase of the activity during development of the silkworm eggs has previously suggested by Ito *et al.* (1954). The present assay under more optimum conditions showed that the apparent level at the shoulder was unexpectedly high being almost half the maximum It is yet uncertain whether or not the enzymes at different stages are of the same origin. As to the post-diapause eggs the first rise in activity was seen on day 2 after the acid treatment. This is Stage 17, the period when the endoderm begins to differentiate (Miya, 1976). Therefore, our present results are compatible with those by Sugai (1957) who found histologically that alkaline phosphatase appear specifically in the region of the endoderm. A two-step increase in activity of alkaline phosphatase has also been reported in the developing salamander ambryos (O'Day and Finnegan, 1970), although in these cases the initial rise was discussed based on the neural ectoderm differentiation.

The first augmentation of this enzyme activity was slower than that of overall protein synthesis rate in the developing silkworm eggs, since previous study has shown that the latter rate rises rapidly until the onset of organogenesis and levels off thereafter (see the citation in Introduction).

The high level of activity shortly before hatching of the eggs may be related to the digestive function of the larval alimentary canal preparatory to the trophic period as has been discussed previously (Chino, 1961). This inference is favored by our finding that the day 9 enzyme was similar to the larval gut juice counterpart in optimum pH, affinity to PNPP and substrate specificity (summarized in Table 1; see also Eguchi *et al.*, 1972b; Eguchi and Suzuki, 1973b). With optimum pH and substrate specificity, the enzyme of day 9 eggs resembled the midgut 'S' isozyme but not the midgut 'F' isozyme (Eguchi *et al.*, 1972a; Eguchi and Suzuki, 1973a).

In eggs just after oviposition was found to exist the alkaline phosphatase activity, which fell during the dormant periods (see Fig. 1). The functional significance of the up-and-down pattern in activity level seen until day 2 remains to be uncovered.

In summary, the present results established the complex developmental changes in activity of alkaline phosphatase, which are likely to mirror the endodermal differentiation, preparatory steps for the larval digestive system and so on. Therefore, this enzyme may serve as an intriguing marker for the processes underlying the development and diapause of *B. mori*.

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三原由美・斎藤　彰・古賀克己・坂口文吾：カイコの胚発生におけるアルカリホスファターゼ活性の変動

カイコ卵の胚発生において，アルカリホスファターゼ活性は中期に一旦上昇し，孵化直前にさらに著しく上昇するという二段階の発育変動パターンを示した。中期と後期の酵素の性質を比較したところ，これらは至適 pH などにおいて互いによく似ていたが，合成基質である p-ニトロフェニルリン酸への K_m 値や他の基質への相対活性において異なっていた。後期にみられる酵素は，幼虫消化液のものと類似していることが明らかとなった。