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Purification and Characterization of Acid Phosphatase from the Egg of the Lady Beetle, *Harmonia axyridis* (Coccinellidae: Coleoptera)

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ABSTRACT Acid phosphatase (AP) in the egg of the lady beetle, *Harmonia axyridis*, was purified and characterized. Ammonium sulfate precipitation, CM column and isoelectrofocusing (IEF) were applied to purify an estimated molecular weight of 66 kDa AP. The purity was checked by SDS PAGE, native PAGE and Western blot. AP was detected in the hemolymph of the female and the egg, but not in the male on the blotting. Km of AP for a substrate, *p*-nitrophenyl phosphate (*p*-NPP), was 1.64×10^{-4} M. AP had the optimum enzymatic activity at pH 3.5. In inhibition tests performed with various chemicals, ammonium molybdate suppressed 99% of the enzyme activity of AP even at the concentration of 5×10^{-4} mM. AP was stable up to 50°C.

Key words : *Harmonia*, Acid phosphatase, Western blot, Optimum pH

Introduction

The insect egg had well controlled systems utilizing the yolk proteins, vitellins, for constructing new structures and organs of the developing embryo. It was known that the compactly packed vitellins were degraded in a similar manner of the protein digestion in the lysosome. The major yolk protein degrading proteinase of cathepsin, and acid phosphatase (AP), which were present in the lysosome of a cell also, were incorporated into the growing oocyte during oogenesis (Izumi et al., 1994; Ribolla et al., 2001).

AP was found to remove phosphate molecules from the vitellins and was supposed to do some roles on yolk protein digestion (Izumi et al., 1994; Fialho et al., 2002). AP was very active at strongly acidic conditions of pH 3-5. Though several insects were testified the presence of the enzyme in the egg, the precise roles of AP was not clearly proved yet.

In the dipteran system of *Musca domestica* (Ribolla et al., 2001), both cathepsin and AP were synthesized via different pathway from that of yolk proteins, which was synthesized mainly in the fat body (Kunkel and Nordin, 1985; Raikhel and Dhadialla, 1992). Previously, we found that yolk proteins of *H. axyridis* were present in the hemolymph of the female (Hong and Park, 1996a), suggesting a possibility that the proteins were synthesized in the fat body. In this paper, AP from the egg of *H. axyridis* was purified and characterized.

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Materials and Methods

Animals

The adult lady beetle, *H. axyridis*, was collected from the field and reared under a photoperiod of 16L : 8D at 25°C. The adult was provided with an artificial diet of fresh chicken's liver and sugar (80 : 20, v/v; Hong and Park, 1996a). Eggs were collected every day and stored at −20°C.

Spectrophotometric assay

AP activity was checked with a substrate of *p*-NPP in glycine-HCl buffer (pH 3.5). The reaction was kept for 3 min and stopped by adding 2 M NaOH. The enzyme activity was measured at 415 nm.

Electrophoresis

Native and SDS polyacrylamide gel electrophoresis (PAGE) was performed following the method of Laemmli (1970). Proteins were stained with Coomassie brilliant blue R−250. AP band on the gel was detected in 50 mM glycine−HCl (pH 3.5) buffer containing 5 mM *a*−NP and 5 mM Fast blue TR.

Purification of AP

1) Ammonium sulfate precipitation

The refrigerated eggs were mixed with 1.5 times of the volume of distilled water and homogenized in a 1.5 ml centrifuge tube. The pellet was removed after centrifugation at 12,000 rpm for 10 mins. The supernatant was mixed with acetic acid in a ratio of 6 µl of acetic acid per 1 ml of the sample. One ml of the mixture was incubated for 6 hours at 30˚C. At the acidic condition, a large part of the vitellins could be digested and removed.

The digested solution was precipitated by adding granular ammonium sulfate at the final concentration of 75%. The pellet containing most part of AP was dissolved in 1 ml of 0.3 M Tris−HCl buffer (pH 8.8). The residual yolk proteins was removed by centrifugation at the final concentration of 45% ammonium sulfate. AP was saved in the supernatant. Granular ammonium sulfate was added to the supernatant up to 75% and AP was precipitated as a pellet after centrifugation.

2) CM column chromatography

Mini CM column (3 cm in length, 1.5 cm in width) was used to remove the degraded yolk proteins by adsorption. The column was equilibrated with 20 mM sodium monophosphate containing 0.2 M KCl and was loaded with the semi−purified ammonium sulfate precipitate, which was dissolved in the equilibrium buffer. The elute was collected and condensed by the saturated ammonium sulfate at 4˚C. Ammonium sulfate was removed by dialysis in 20 mM sodium phosphate buffer (pH 7.0) for 12 hr at 4˚C.

3) Isoelectrofocusing (IEF)

The CM sample was isoelectrofocused with the mixture of ampholytes of pH 3−10 and pH 5−8 (4 : 1, v/v) for 3 hr (Rotofor Prep IEF Cell, BIO−RAD, U.S.A.). Each about 0.5 ml of 20 fractions was collected simultaneously under vacuum. Fractions were tested for AP activity by PAGE and by a microplate reader (Automated Microplate Reader ELx808, BIO−TEK, U.S.A.).

4) Production of polyclonal antibodies and Western blot

The purified AP was injected to rabbits. Polyclonal antibodies made were used for detecting AP by Western blot. The procedures of the blotting (Towbin et al., 1979), was slightly modified (Park and Goodman, 1993). Briefly here, nitrocellulose paper (NCP, 0.2 µm, BIO−RAD) was used for the transfer of proteins after PAGE. Secondary antibodies were anti−rabbit mouse horse radish peroxidase conjugated antibody, Sigma U.S.A. The band was formed by 4−chloro−1−naphtol.

5) Characterization of AP

Michaelis constant (km) was measured by a double reciprocal plot. AP inhibitors tested were 0.08 mM and 0.04 mM potassium fluoride, 0.08 and 2 mM tartaric acid, 0.5 µm ammonium molybdate, 1.5 mM and 25 mM mercuric acid, and 3 mM and 50 mM oxalic acid in 50 mM glycine−HCl buffer (pH 3.5).

pH optimum of AP was measured by the spectrophotometer using *p*−NPP as the substrate. The buffers for measuring the pH optimum were 50 mM glycine−HCl (pH 2−3.5), 50 mM acetic acid−NaOH (pH 4−5.5), 50 mM Na_2HPO_4−NaH_2PO_4 (pH 6), 50 mM Tris−HCl (pH 7−8), and 50 mM glycine−NaOH (pH 9). The enzyme reaction was kept for 3 min at 30˚C and stopped by adding 2 M NaOH.

Heat stability was measured by incubating the enzyme for 30 min at 5, 25, 40, 50, 60 and 70˚C. Temperature optimum of AP was checked at 5, 15, 25, 35, 45, 55, 65, 75 and 85˚C.

Results and Discussion

Purification of AP

Vitellins were major egg proteins of *H. axyridis* (Hong and Park,1996a, b) and caused difficulty in purifying the minor quantity protein of AP. Before the final purification step of IEF, acid digestion, ammonium sulfate precipitation and CM adsorption (data were not given) were applied to remove the vitellins.

IEF spectrum showed one single sharp peak of AP activity. Most of the enzyme activity was in the eluted fractions of 5, 6 and 7 from the anode (Fig. 1). AP stained native gel was matched well with the above spectrophotometric result, showing a single band in fractions of 5, 6 and 7, respectively (Fig. 2). Coomassie blue stained native gel had one clear band in the

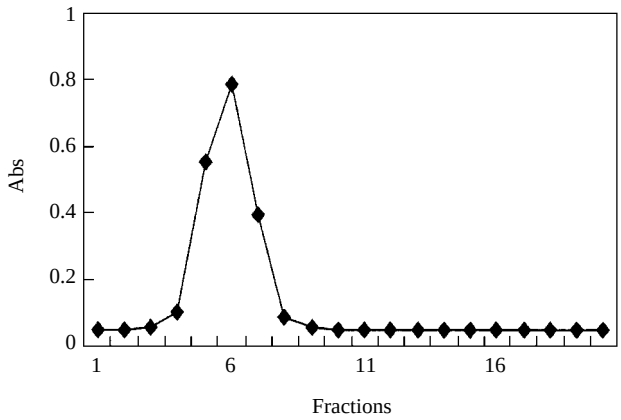

Fig. 1. Isoelectrofocusing profiles of AP of *H. axyridis* egg. The focusing was done at 12 watts for 3 hours. Absorbances was measured at 415 nm. The substrate was *p*−NPP.

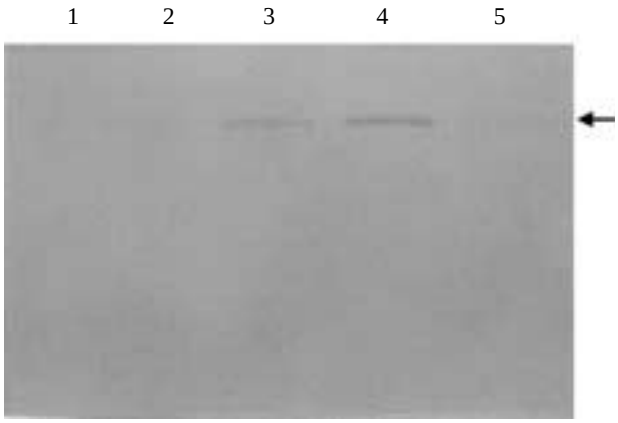

Fig. 2. Electrophoretic pattern of AP of the isoelectrofocused fractions. Samples were subjected under 9% native−PAGE at 100 volts for 2 hours. The band was stained with 5 mM *p*−NP and 5 mM Fast blue TR for 20 min. The arrow indicated AP band. Lane 1, 3rd fraction; Lane 2, 4th fraction; Lane 3, 5th fraction; Lane 4, 6th fraction; Lane 5, 7th fraction.

above 3 fractions (Fig. 3).

AP had an estimated molecular weight of 66 kDa (datum was not shown). Other insects studied had fairly high molecular weight AP also (Narise, 1984; Ribolla et al., 2001; Fialho et al., 2002). In *Drosophila*, AP was proposed to be present as a dimer (Narise, 1984).

Western blot

In various insects, yolk proteins and enzymes in the egg were synthesized and transported mainly from the fat body or the ovary, and or both, depending on the

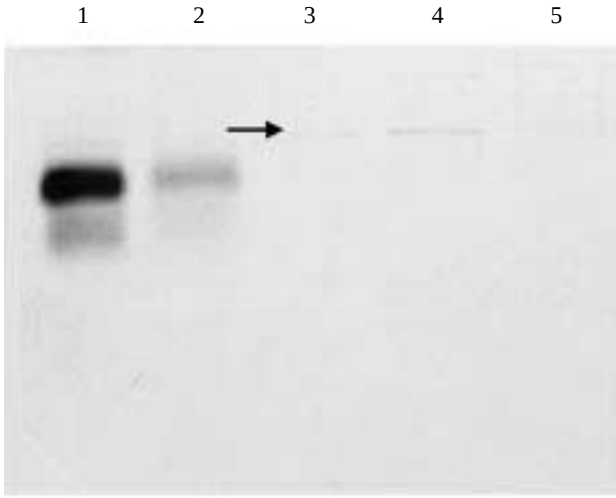

Fig. 3. Native PAGE analysis of the fractions of the isoelectrofocusing. Samples were subjected under 9% native−PAGE at 100 volts for 3 hours. Protein bands were stained with Coomassie brilliant blue R 250. The arrow indicated AP band. Lane 1, 3rd fraction; Lane 2, 4th fraction; Lane 3, 5th fraction; Lane 4, 6th fraction; Lane 5, 7th fraction.

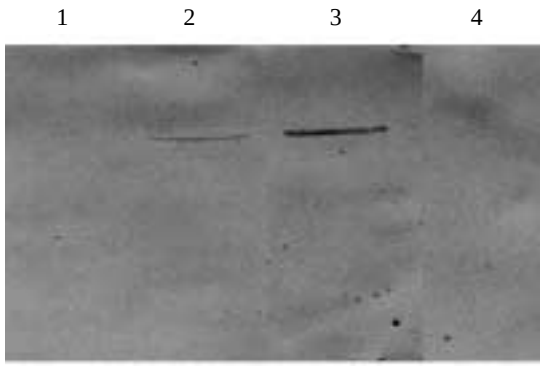

Fig. 4. Western blot of AP of *H. axyridis* egg. Primary antibodies were made against acid phophatase from a rabbit. Secondary antibodies were anti−rabbit goat antibodies conjugated with horse radish peroxidase (HRP). Lane 1, the fat body of the female adult; Lane 2, the hemolymph of the female adult; Lane 3, the egg; Lane 4, the hemolymph of the male adult.

ovariole type. In cell biology, knowing the organ of the synthesis of a protein or an enzyme could provide many preliminary informations on the traffics of the transportation and the localization of the transport in the egg.

AP was a maternally originated protein, but it was not clear whether AP was internalized into the egg in the form of mRNA or its product (protein), or both (Fialho et al., 2002). The egg and the female hemoly-

mph had one single band of AP with the same molecular weight, but the male hemolymph did not (Fig. 4), suggesting a possiblity that AP was female- and egg-specific protein.

Though the presence of AP in the female hemolymph indicated AP could be synthesized in the fat body and transported into the egg, we failed to testify the presence of AP in the fat body of the female (Fig. 4). The failure could be raised by the current technique limiting detection of small quantity of AP.

Biochemical characteristics of AP

The crystallized vitellin sphere was fused with a vesicle which was very similar functionally to a lysosome. The fusion of two differently originated vesicles could be thought as a final storage form of the vitellins (Ribolla et al., 2001), or as a start point of the vitellin digestion. Though the precise mechanism of the acidification of the vesicle in the egg was not known, the lysosome proteinase, cathepsin, was auto-activated at acidic condition and was proved to digest vitellin (Ribolla and de Bianchi, 1995; Ribolla et al., 2001).

Another enzyme in the egg, AP, was known to remove phosphate molecule from vitellin (Izumi et al., 1994; Fialho et al., 2002). AP of insect eggs showed very similar characteristics of lysosomal enzyme proved by inhibition tests, the optimum pH and the acidification of the yolk spear (Janssen et al., 1995; Ribolla et al., 2001; Fialho et al., 2002). However, the exact roles of AP on the process of yolk protein digestion was not known clearly.

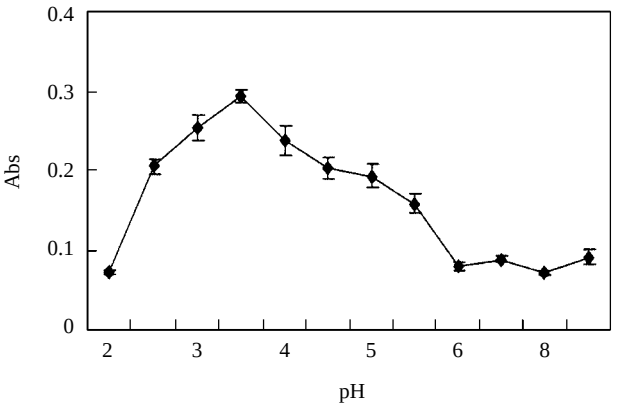

Fig. 5. Effect of pH on AP of *H. axyridis* egg. The buffers for measuring the pH optimum were 50 mM glycine-HCl (pH 2-3.5), 50 mM acetic acid-NaOH (pH 4-5.5), 50 mM Na_2HPO_4-NaH_2PO_4 (pH 6), 50 mM Tris-HCl (pH 7-8), and 50 mM glycine-NaOH (pH 9). The enzyme reaction was kept for 3 min at 30°C and stopped by adding 2 M NaOH. The substrate was *p*-NPP.

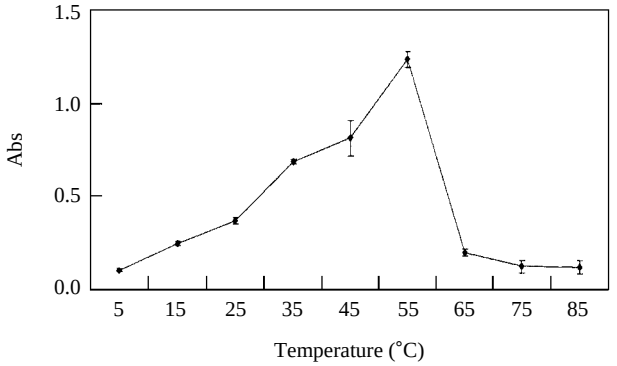

Fig. 6. Temperature optimum of AP of *H. axyridis* egg. The enzyme reaction was kept for 3 min at 30°C and stopped by adding 2 M NaOH. The substrate was *p*-NPP.

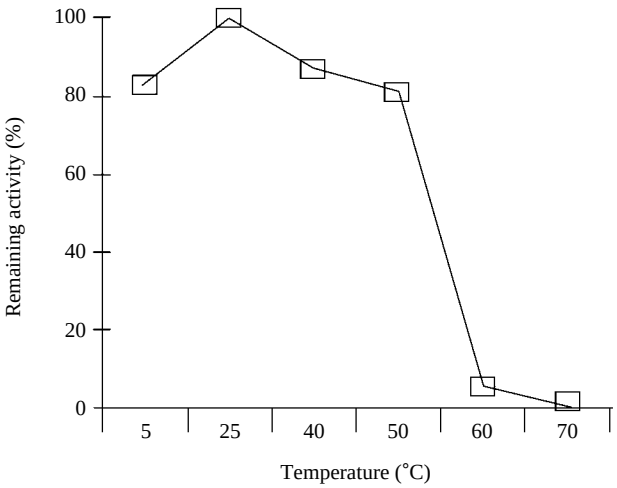

Fig. 7. Heat stability of AP of *H. axyridis* egg. The enzyme was pre-incubated for 30 min at the given temperatures. The enzyme reaction was kept for 3 min at 30°C and stopped by adding 2 M NaOH. The substrate was *p*-NPP.

The pH optimum of AP in several insects was in strongly acidic condition (Narise, 1984; Fialho et al., 2002). AP of the lady beetle kept fairly high activity at pH 2.5 through pH 5.5 and showed the maximum activity at pH 3.5 (Fig. 5), suggesting that AP could do something on the yolk protein digestion in the acidified condition.

The enzyme activity of AP increased gradually as temperature went on from 5°C to 55°C and had the plateau at 55°C. Above 55°C, the activity dropped sharply (Fig. 6). In 30 min incubation tested the heat stability of AP, the enzyme was stable at 5°C up to

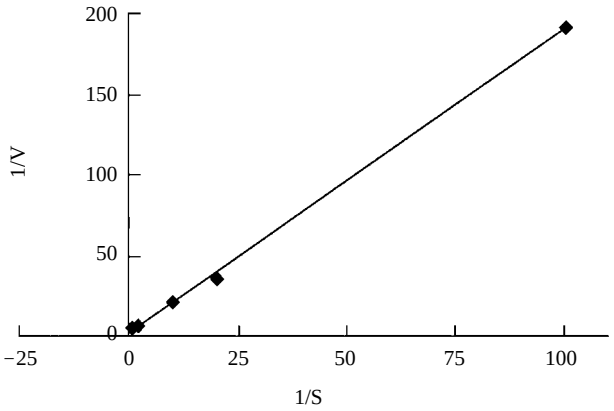

Fig. 8. Lineweaver−Burk plot of AP of *H. axyridis* egg.

Table 1. The effect of inhibitors on AP activity

Inhibitors	Concentration (mM)	Inhibition rate (%)
Oxalic acid	50	94
	3	39
Potassium fluoride	0.08	96
	0.04	43
Tartaric acid	2	84
	0.08	28
Mercuric chloride	25	80
	1.5	27
Ammonium molybdate	0.0005	99

50˚C and lost its activity suddenly at 60˚C. At 70˚C, the activity was almost gone (Fig. 7).

Michaelis constant (Km) of *Harmonia* AP was calculated as 1.64×10^{-4} M (Fig. 8). AP inhibitors, potassium fluoride, tartaric acid, mercuric chloride, oxalic acid and ammonium molybdate, showed various inhibition rate at the given concentrations. Ap activity was suppressed most strongly by ammonium molybdate (same result in *Drosophila* AP, Narise, 1984), showing 99% inhibition even at 5×10^{-4} mM (Table 1).

Phosphorylation and dephosphorylation of natural substrate molecules were well known cellular processes in controlling enzyme activity, protein assembly and dissembly, transport, sequestration, and degradation. Insect egg was a good model system understanding the biochemical modulation system in cell biology. Further characterization of *Harmonia* AP could expand our knowledge on the role of AP in the egg during oogenesis and embryogenesis.

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