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Oocyte fertilization triggers acid phosphatase activity during *Rhodnius prolixus* embryogenesis

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Abstract

Acid phosphatase activity, previously identified in *Rhodnius prolixus* oocytes, was studied during egg development. Fertilized eggs exhibited a five fold increase of total acid phosphatase activity during the first days of development. In contrast non-fertilized oviposited eggs showed no activation of this enzyme. An optimum pH of 4.0 for pNPP hydrolysis in a saturable linear reaction and a strong inhibition by lysosomal acid phosphatase inhibitors such as NaF (10 mM) and Na^+/K^+ tartrate (0.5 mM) are the major biochemical properties of this enzyme. Fractionation of egg homogenates through gel filtration chromatography revealed a single peak of activity with a molecular mass of 94 kDa. The role of this enzyme in VT dephosphorylation was next evaluated. Western blots probed with anti-phosphoserine polyclonal antibody demonstrated that VT phosphoaminoacid content decreases during egg development. In vivo dephosphorylation during egg development was confirmed by following the removal of ^{32}P from ^{32}P-VT in metabolically labeled eggs. Vitellin was the only phosphorylated molecule able to inhibit pNPPase activity of partially purified acid phosphatase. These data indicate that acid phosphatase activation follows oocyte fertilization and this enzyme seems to be involved in VT dephosphorylation.

Keywords: Yolk degradation; *Rhodnius prolixus*; Acid phosphatase; Embryogenesis; Fertilization

1. Introduction

Vitellogenin (VG) is an hemolymphatic lipoglycophosphoprotein stored in growing oocytes during oogenesis. Once inside the oocytes it is conventionally named Vitellin (VT) and is the main component of yolk platelets (Sappington and Raikhel, 1998). After oocyte fertilization several different VT processing mechanisms will be triggered. VT will then be used as the building blocks for developing embryos. Several enzymes involved in this process have been characterized in the past few years (Fagotto, 1990; Yamamoto and Takahashi, 1993; Izumi et al., 1994; Liu et al., 1996; Cho et al., 1999). Although most of those studies have addressed the identification, purification and/or cloning and characterization of VT proteolytic systems in arthropod developing eggs.

The presence of covalently bound phosphate on VTs was soon recognized as a remarkable feature of those proteins (Allerton and Perlmann, 1965). Nowadays data concerning their physical-chemical properties, aminoacid sequencing and cDNA sequences are available. Thus it became clear that every VT molecule from any organism contains some degree of covalently bound phosphate in its structure (Byrne et al., 1989; Sappington and Rhaikhel, 1998). Dephosphorylation-coupled digestion of proteic substrates is a recurrent theme in cell biology. Acid phosphatases (EC 3.1.3.2) are widely distributed enzymes and occur as multiple forms and isozymes. These enzymes are able to catalyze the hydrolysis of orthophosphoric monoester in a wide range of

Abbreviations: SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate; VT, vitellin; VG, vitellogenin; pNPP, *para*-nitrophenyl phosphate; pNP, *para*-nitrophenol; pCMB, para-hydroxymercuribenzoic acid; Pi, potassium phosphate; NaF, sodium fluoride; NaCl, sodium chloride; EDTA, ethylenediaminetetraacetic acid; ECL, enhanced chemiluminescence.

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substrates. Their association with digestive process was early recognized since the first localization of their activities in hepatic lysosomes through electron microscopy techniques (Essner, 1972; Holt and Hicks, 1961; Daems, 1962). Following these early observations, acid phosphatase activity was demonstrated in various types of lysosomes (Novikoff, 1963). The role of acid phosphatase in processing of yolk during development was first recognized by Lemanski and Aldoroty (1974) working with amphibian embryos. In addition acidification of yolk platelets during *Xenopus* (Fagotto and Maxfield, 1994), sea urchin (Mallya et al., 1992), and tick development (Fagotto, 1991) was demonstrated to correlate with yolk utilization. The presence of an acid phosphatase activity during egg development might therefore be a general requirement for different VT processing systems. It is tempting to speculate that phosphatases should be involved in VT digestion later in egg development.

Yolk platelet-associated hydrolases such as cathepsin D and acid phosphatase have already been identified in the oocytes of *Rhodnius prolixus* (Nussenzveig et al., 1992). Furthermore, VT processing and degradation during embryogenesis has been demonstrated (Oliveira et al., 1989). *Rhodnius* VT contains up to 38% of its phosphate moiety covalently bound to the polypeptide backbone (Masuda and Oliveira, 1985). However, neither the fate of the acid phosphatase activities found in the oocytes nor their ability to recognize VT as a substrate have been studied. In the present study we followed the fate of an oocyte associated acid phosphatase activity in both fertilized and non-fertilized eggs of this insect.

2. Materials and methods

2.1. Chemicals

Bovine serum albumin, phosphoserine, mannose 6-phosphate, ATP, molecular weight standards, glycine, acrylamide, bis-acrylamide, TEMED, *p*-Nitrophenyl Phosphate, p-CMB, levamisole, tetramisole, sodium acetate, pepstatin A, SBTI, antipain, leupeptin and PMSF were purchased from Sigma Chemical Company (St. Louis, MO, USA). Phenylarsine oxide was purchased from Calbiochem–Novabiochem Corp. (La Jolla, CA, USA), and rabbit polyclonal anti-phosphoserine antibody was from Zymed Laboratories Inc. (San Francisco, CA, USA). All other chemicals were from analytical grade.

2.2. Insects and eggs

Insects were taken from a colony of *Rhodnius prolixus* maintained at 28 °C and 70% relative humidity. Animals were fed on rabbit blood at 3-weeks intervals. Fertilized and non-fertilized mated females fed on rabbit blood six days beforehand were allowed to lay eggs for a period of 6 h. These eggs were grouped and kept for the desired length of time under the same conditions used for rearing the colony. To obtain samples along the days of embryogenesis and days after oviposition, a group of fertilized and non-fertilized eggs was collected everyday at the same time, stored at −18 °C, and up to the day of hatching, for further analysis. Eggs were homogenized with a plastic pestle in 20 mM acetate buffer pH 4.0, 10 mM DTT, 10 mM EDTA. The homogenates were frozen, thawed and used for kinetic analysis. This preparation is referred to as egg homogenate. Protein content of egg homogenate was determined by the method of Lowry (1951) using bovine serum albumin as standard.

2.3. p- Nitrophenylphosphate hydrolysis

Unless otherwise stated, pNPP hydrolysis was measured at 37 °C as follows: the reaction medium contained 5 mM pNPP, 20 mM sodium acetate pH 4.0, 10 mM DTT, 10 mM EDTA and egg homogenate with a final protein concentration between 0.04–0.3 mg. All the reactions were done using microplates, initiated by the addition of pNPP and stopped at different times with 10% of total volume with 1 N NaOH. The amount of p-nitrophenol (pNP) formed was read at 405 nm in a ELISA reader (Thermomax microplate reader, Molecular Devices). Specific activity is expressed as nmol of (pNP) released $mg^{-1}\times min^{-1}$. In the gel filtration experiments the results are expressed as pmol of (pNP) released. min^{-1}. Controls lacking homogenates were used as blanks and the extinction coefficient for p-nitrophenolate ion (1.75×10^4 M^{-1} cm^{-1}) was utilized for determination of enzyme specific activity (Sheng and Charbonneau, 1993). The optimum pH for pNPP hydrolysis was determined utilizing fertilized eggs from second and tenth days after oviposition. The reactions contained the following mixture of buffers: glycine, sodium acetate, sodium citrate, and Tris in 0.1 M final concentrations. The desired pH was adjusted in each medium from 3.0 to 9.0. In some experiments pNPP hydrolysis was measured in the presence or absence of different protein phosphatase modulators as described in Table 1.

2.4. Gel filtration chromatography on Superose 6HR (HPLC) and molecular mass determination of Rhodnius acid phosphatase

Eggs from second and tenth days of embryogenesis were obtained as described above. Fifty eggs were homogenized in 0.1 mM sodium acetate pH 5.0, 0.1 M NaCl. Gel filtration chromatography was then performed in a Superose 6HR column at room temperature with a flux of 0.5 ml/min. The column was previously equilib-

Table 1
Effect of phosphatase inhibitors on the acid phosphatase activity present in embryogenesis of *Rhodnius prolixus*

Effector	Second day	Tenth day
Control (without drug)	1.00±0.014	3.14±0.106
NaF 0.5mM	1.00±0.042	3.00±0.141
NaF 1mM	0.90±0.092	2.19±0.664
NaF 5mM	0.25±0.001	0.58±0.056
NaF 10mM	0.10±0.001	0.27±0.021
Zn^{2+} Sulphate 0.1 mM	1.02±0.001	2.83±0.001
Na^{+1}/K^{+1} Tartrate 0.5 mM	0.10±0.008	0.54±0.024
Na^{+1}/K^{+1} Tartrate 1 mM	0.09±0.033	0.45±0.035
Na^{+1}/K^{+1} Tartrate 5 mM	0.11±0.026	0.30±0.042
Na^{+1}/K^{+1} Tartrate 10 mM	0.10±0.001	0.26±0.001
pCMB 0.1 mM	0.95±0.106	2.61±0.021
pCMB 1 mM	0.95±0.005	2.54±0.374
Levamisole 1 mM	1.07±0.035	3.08±0.049
Levamisole 10 mM	1.39±0.141	3.48±0.134
Tetramisole 1 mM	1.25±0.056	2.86±0.282
Tetramisole 10 mM	2.18±0.473	3.87±0.007
Caffeine 0.5 mM	1.02±0.049	2.88±0.233
Caffeine 5 mM	1.03±0.077	2.89±0.367
Pi 1 mM	0.79±0.063	2.62±0.169
Pi 5 mM	0.53±0.070	1.91±0.021
Pi 10 mM	0.47±0.021	1.51±0.098
Pi 30 mM	0.25±0.001	0.93±0.113
Vanadate 0.5 mM	1.08±0.176	2.95±0.480
Vanadate 1 mM	0.96±0.014	2.63±0.208
Vanadate 2 mM	0.69±0.007	2.22±0.049
Phenylarsine oxide 0.1 mM	0.96±0.021	2.95±0.289

Eggs from the second and tenth days old eggs were both assayed against pNPP in the presence of different phosphatase inhibitors. The values obtained with in controls lacking inhibitors were considered 1.00 as reference. Each point represents the average and standard deviations of two different experiments, performed in duplicate, with two different egg homogenates for each day.

rated in the same buffer used for homogenization. 0.05 ml aliquots from each fraction were assayed for acid phosphatase activity using the reaction media described above in the presence or absence of 10 mM sodium fluoride or 10 mM Na^+/K^+ tartrate. After 1 h reactions were stopped and the microplate was read at 405 nm. The apparent molecular mass of acid phosphatase from eggs on different days was determined using the following proteins as standards: thyroglobulin (669 kDa), ferritin (442 kDa), catalase (232 kDa), aldolase (158 kDa), and ovalbumin (47 kDa). Whenever indicated pNPPase activity of partially purified acid phosphatase was also assayed in the presence of different phosphorylated molecules such as: phosphoserine, mannose 6-phosphate, ATP and VT purified from *R. prolixus* oocytes (Silva-Neto et al., 1996).

2.5. SDS–polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gels (15×15×0.1 cm) were run in the presence of SDS (Laemmli, 1970) at a constant current of 20 mA. Gels were stained with Coomassie Brilliant Blue G, destained with a 7% acetic acid, 40% methanol solution, dried and exposed to X-ray film at −70 °C.

2.6. Metabolic labeling of eggs with ^{32}Pi

Carrier free ^{32}Pi was purchased from Comissão Nacional de Energia Nuclear (São Paulo, Brazil) and purified by ion-exchange chromatography on Dowex 1×-10 (de Meis and Masuda, 1974). Fertilized and non-fertilized adult females were fed with blood enriched with ^{32}Pi (1 μCi/female 0.2ml of blood) using a special feeder described by Garcia et al. (1975). Seven days later the eggs were collected and stored at −18 °C as described in Section 2.2.

2.7. Western blots

Total homogenates from 3 and 10-day old eggs were separated by SDS-PAGE (7.5%) and transferred at 180 mA for 90 min to a nitrocellulose membrane. The membrane was probed with a polyclonal antibody raised against phosphoserine and developed using the ECL system. The same membrane was stripped using 62.5 mM Tris–HCl pH 6.8, 2% SDS and 100 mM mercaptoethanol for 1 h at 56 °C, blocked overnight with TBS-Tween-Albumin, probed with polyclonal antibody raised against VT (Masuda and Oliveira, 1985) and developed using

ECL system. Others conditions as described in (Towbin et al., 1979). Densitometric analyses were performed using the QuantiScan (BioSoft) and Idrisi for Windows programs.

3. Results

3.1. pNPPase activity in fertilized and non-fertilized eggs of R. prolixus

When acid phosphatase was assayed in homogenates of fertilized eggs obtained from different days after oviposition, we noticed a five fold increase at 6 days after oocyte fertilization (Fig. 1, closed triangles). After day 6, pNPPase activity reaches a plateau and the enzyme is kept activated up to the end of embryogenesis. The majority of this activity is associated with embryo cells (data not shown). Non-mated females of *R. prolixus* produce and lay non-fertilized eggs (Davey, 1967). These eggs were also assayed for pNPPase activity during several days after oviposition, and we did not observe the same profile obtained with fertilized eggs. pNPPase activity remained unchanged during 14 days after oviposition (Fig. 1, open triangles). The above set of data indicates that activation of acid phosphatase is dependent on fertilization.

3.2. Characterization of an acid phosphatase activity in fertilized eggs of R. prolixus

The following experiments were performed with egg homogenates obtained from the second and tenth days after oviposition. The optimum pH for activity of *R. prolixus* acid phosphatase is 4.0 and it did not change during the days after oviposition (Fig. 2). Time and concentration dependence for pNPP hydrolysis are presented in Fig. 3. This reaction is saturable by pNPP as demonstrated on Fig. 3(A) and linear up to 60 min for the enzyme obtained from both days [Fig. 3(B)]. The effect of classical phosphatase inhibitors was evaluated in the next experiments (Table 1). Like other classical lysosomal phosphatases *Rhodnius* enzyme is inhibited by Na^+/K^+ tartrate and sodium fluoride, but not by p-chloromercuribenzoate (Araujo et al., 1976). These competitive inhibitors are described to block the activity of secreted enzymes (Mackenzie et al., 1980; Guranowski, 1990). Tyrosine phosphatase modulators such as zinc sulphate, caffeine, sodium orthovanadate and phenylarsine oxide did not significantly change enzyme activities (Table 1). Interestingly, levamisole and tetramisole activate this enzyme. This set of results indicates that the main acid phosphatase activity observed in fertilized eggs of *Rhodnius prolixus* is probably of lysosomal origin.

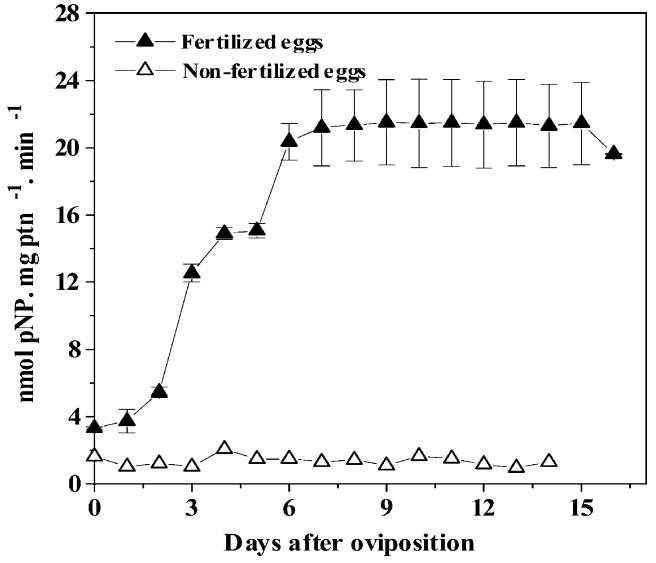

Fig. 1. Acid phosphatase activity in fertilized and non-fertilized eggs of *Rhodnius prolixus*. Three fertilized (—▲—) or non-fertilized (—△—) eggs per sample from day zero to day sixteen after oviposition were homogenized in 20 mM sodium acetate buffer pH 4.0, 10 mM DTT, 10 mM EDTA. Homogenates were immediately frozen. Samples were assayed for pNPP hydrolysis at 37 °C. After 60 min reactions were stopped with 1:10 vol of NaOH 1 N and pNP formed was read at 405 nm. The results are expressed in nmol pNP mg ptn^{-1} min^{-1}. Each point represent the mean±SE of three independent experiments.

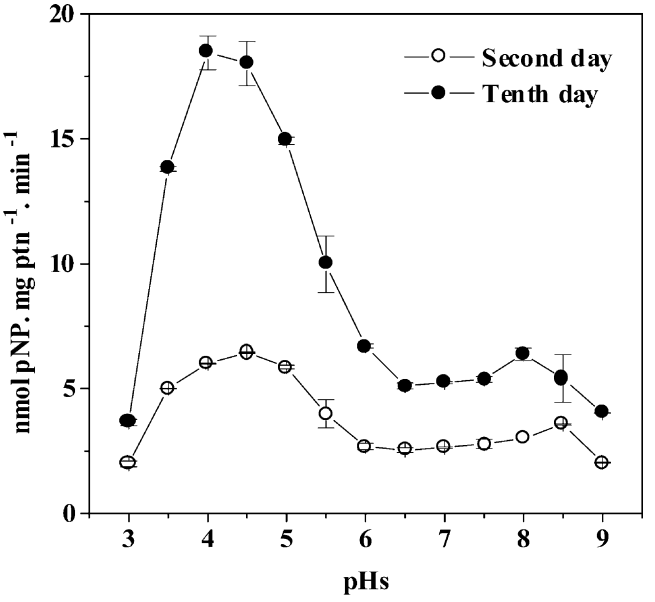

Fig. 2. pH dependence for the hydrolysis of p-NPP by acid phosphatase from *Rhodnius prolixus* during embryogenesis. p-nitrophenylphophatase activity was measured at the second (—○—) and tenth (—●—) days after oviposition. Enzyme solutions were prepared with buffers at a final concentration of 0.1 M. The incubation medium contained 60 μg of total protein and 5 mM pNPP, and reactions were terminated by addition of 1 N NaOH after 60 min at 37 °C. The results are expressed in nmol pNP mg ptn^{-1} min^{-1}. Each point represent the mean±SE of three independent experiments.

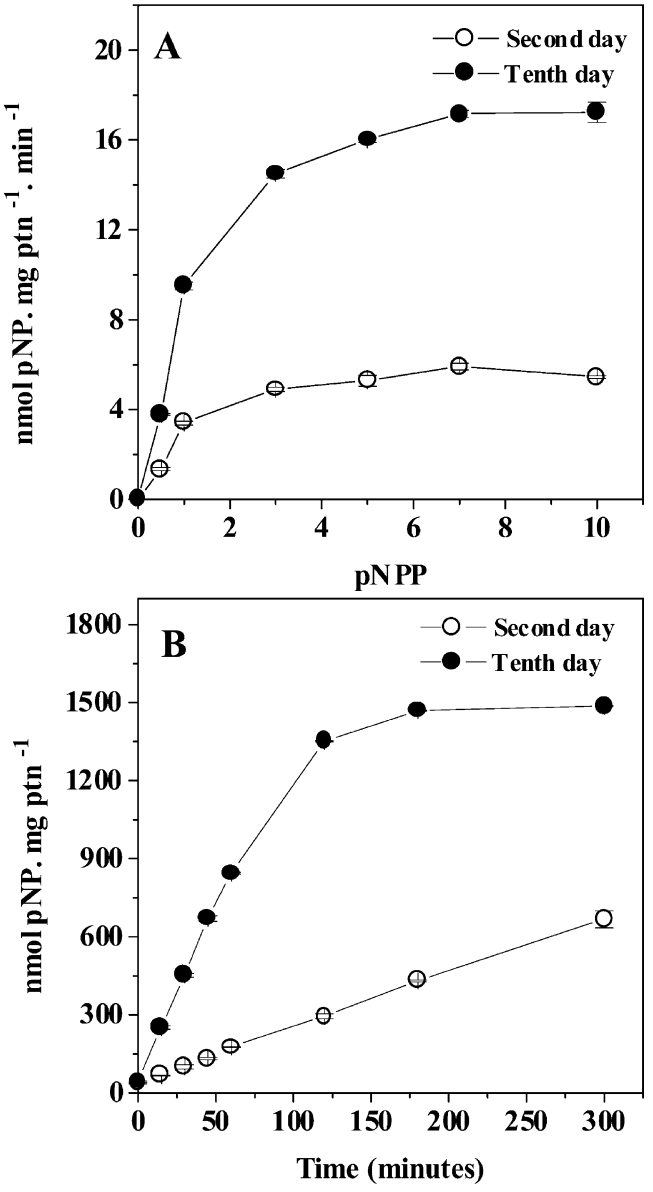

Fig. 3. Kinetic analysis of acid phosphatase activity in fertilized eggs of *Rhodnius prolixus*. Two (—○—) and ten (—●—) day old eggs were homogenized in 20 mM sodium acetate buffer pH 4.0 plus 10 mM DTT, 10 mM EDTA. To determine the concentration dependence (A) and the time course dependence (B) for pNPP hydrolysis, the reaction media contained 40 μg of total protein. Different concentrations of pNPP (0, 0.5, 1, 3, 5, 7 and 10 mM) were used and after 60 min the reactions were stopped with NaOH solution. The time course were determined at different times (0, 15, 30, 45, 60, 120, 180 and 300 minutes) and the reactions were stopped as described above. The results from each point represent the mean±SE of three independent experiments.

3.3. Fractionation of acid phosphatase from R. prolixus

Two and ten days following oviposition, eggs from fertilized females were subjected to gel filtration chromatography as shown in Fig. 4. A similar amount of total protein was used as shown by absorbance at 280 nm (Fig. 4, panels A and C). When fractions from both chromatographies were assayed for acid phosphatase activity using pNPP as substrate a single peak of activity was detected. Activity from 10-day eggs was higher than that from day two eggs. NaF and Na^+/K^+ tartrate inhibited more than 95% of both activities (Fig. 4, panels B and D). A native molecular mass of 94 kDa was found for this enzyme activity based on the migration of molecular mass standards (data not shown). The enzyme preparation obtained using this procedure is free from detectable traces of VT (data not shown). This set of experiments thus demonstrates that during egg development there is an activation of a single type of acid phosphatase activity likely of lysosomal origin. The next sets of experiments were designed to determine whether VT is dephosphorylated during egg development.

3.4. Vitellin dephosphorylation during embryogenesis

Western blotting analysis with the aid of polyclonal phosphoserine antibody (Fig. 5, panel A) revealed a decrease in VT phosphorylation level during development. The similar amount of VT in both lanes was confirmed with the use of an antibody against this protein (Fig. 5, panel B). Nitrocellulose membranes from figures 5A and B were densitometrically scanned and the ratio between the areas of phosphoserine-reactive bands and VT-reactive bands is presented on Figure 5C. This ratio corresponds to the total level of phosphoserine associated with VT on each day of egg development and is presented as a percent of phosphorylation [Fig. 5(C)] considering day 3 to be 100%. This strategy indicated a 20% decrease in VT phosphoserine content [Fig. 5(C)]. It also identified a 64 kDa VT-derived polypeptide (Oliveira et al., 1989) that is devoid of any phosphoserine (Fig. 5, panel A, open arrow). This result thus suggested that VT is dephosphorylated during embryogenesis. VT did not show any reactivity to phosphotyrosine-raised antibody (data not shown), suggesting that this phosphoaminoacid is absent or in a very low content in VT structure (Masuda and Oliveira, 1985). We decided to verify if eggs from fertilized and non-fertilized females metabolically labeled with ^{32}Pi were also dephosphorylated in physiological conditions. Females were fed with ^{32}Pi and soluble ^{32}P-proteins from laid eggs were separated by SDS-PAGE (Fig. 6, panels A and C). The level of VT labeling on each day after egg oviposition was then determined by autoradiography for both fertilized and non-fertilized eggs (Fig. 6, panels B and D). Densitometric scanning of lanes both from Coomassie Blue stained gels and autoradiographies were then conducted. The total area of each autoradiography profile was then divided by total area of Coomassie blue stained lane from each day after oviposition. Day 0 after

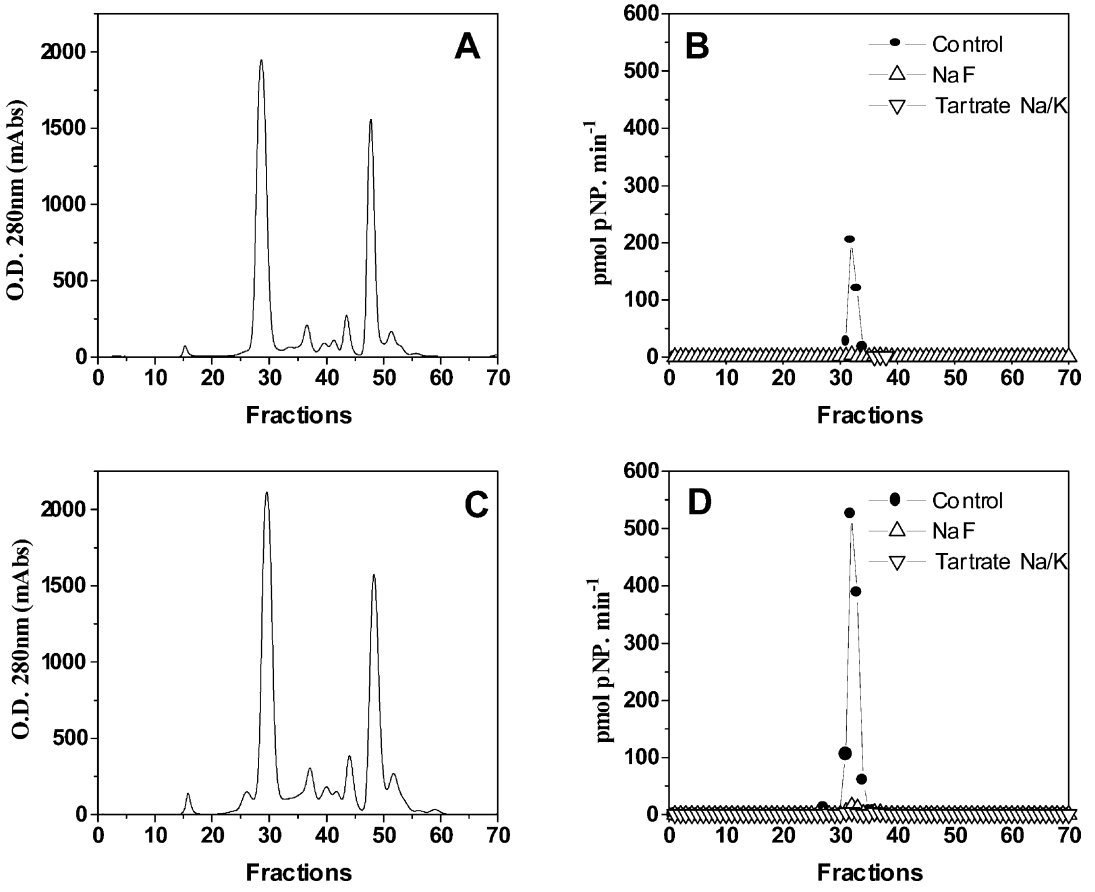

Fig. 4. Acid phosphatase activity after gel filtration chromatography of second and tenth day old eggs. Extracts of two (A) and ten day old eggs (C) were chromatographed in a Superose 6HR gel filtration column. (A) and (C) shows the elution profile from 2-day and 10-day old eggs respectively. Panels (B) and (D) show the pNPPase activity from eluted fractions in the presence or absence of 10 mM NaF or 10 mM Tartrate Na^+/K^+. Control (—●—), NaF (—△—) and Tartrate Na/K (—▽—). Other conditions as described in Section 2.

oviposition corresponds to 100% of phosphorylation. Fig. 6, panel E, shows that the VT-bound phosphate is removed as eggs mature. Incubation of partially purified acid phosphatase with ^{32}P-VT led to a slightly dephosphorylation of the molecule (data not shown). ^{32}P-VT used in those experiments is metabolic labeled with low specific activity of ^{32}P otherwise egg development is abolished. Therefore in order to demonstrate the ability of the presently described acid phosphatase to recognize VT as a substrate in vitro the following experiment was conducted. pNPPase activity of partially purified acid phosphatase was assayed in the presence of different substrates containing covalently bound phosphate groups. Table 2 shows that despite the different phosphorylated molecules tested only VT was able to block pNPPase activity to a significant extent (70.9% of inhibition, 0.5 mM VT). This effect was not observed with the classically non-phosphorylated plasma protein, albumin. Therefore, this result indicates that phosphate groups linked to a proteic structure are the main structural feature recognized by *Rhodnius* acid phosphatase.

4. Discussion

Acid phosphatases (orthophosphoric-monoester phosphohydrolase (acid optimum) EC 3.1.3.2) are enzymes classically described by their ability to hydrolyze phosphate esters. They have been widely described in several mammalian tissues such as rat liver, human prostate, and human kidney (Himeno et al., 1989; Araujo et al., 1976; Vihko et al., 1993) as well as in isolated blood cells such as polymorphonuclear leukocytes (Smith and Peters, 1981). Their activity was also found to be associated with quite different unicellular organisms such as the parasitic protozoans *Trypanosoma brucei* and *Trypanosoma congolense* (Scheel et al., 1990; Tosomba et al., 1996), the fungi *Myxococcus xanthus* (Weinberg and Zusman, 1990), and during embryo development as in the case of amphibians (Lemanski and Aldoroty, 1974).

In invertebrates and especially in insects acid phosphatase has long been recognized as one of the main yolk-associated enzyme activities. Several reports are available in the literature ranging from the Dipteran

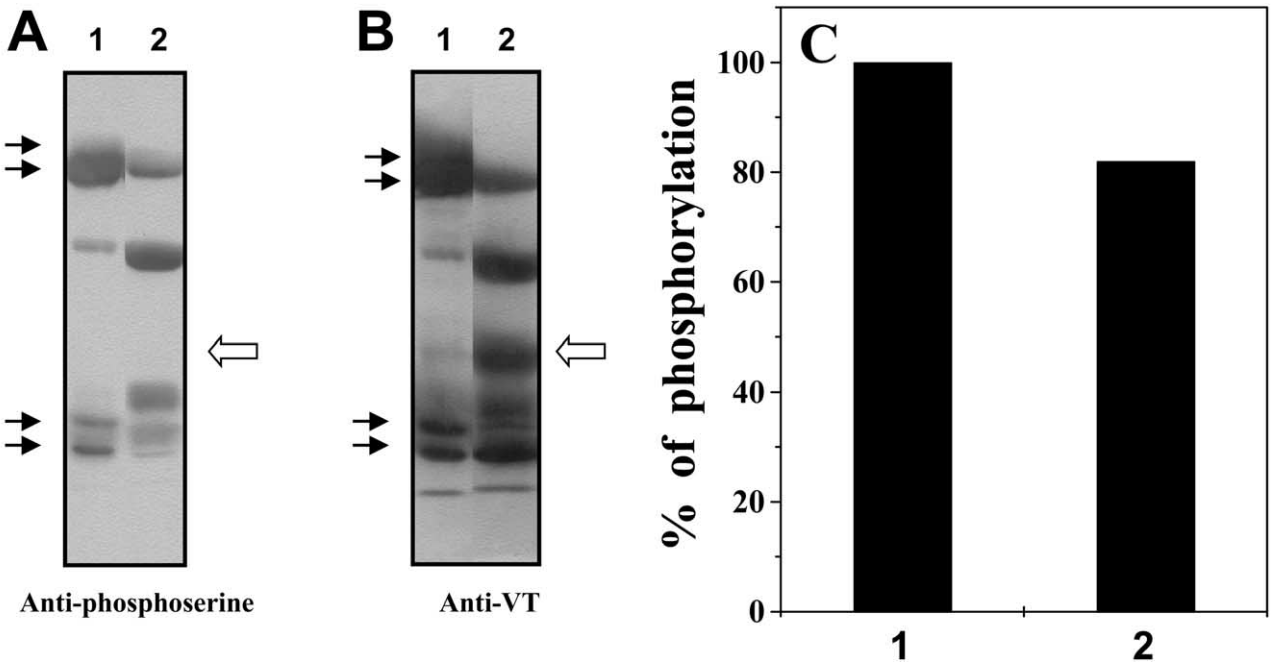

Fig. 5. Phosphoserine content of VT in different days after oviposition. Total homogenates (0.05 mg) from three (1) and ten (2) day old eggs were subjected to SDS-PAGE (7.5%), transferred to a nitrocellulose membrane and reacted with a polyclonal antibody against phosphoserine (panel A), the membrane was discharged as described in Material and methods and reacted with a polyclonal antibody against VT (panel B). In panel (C), densitometric analysis was conducted in both autoradiographies and the ratio between phosphoserine content and VT content in each lane was determined and compared. In this experiment the total level of phosphoserine associated to VT obtained from the third day of egg development was considered to be 100% for comparison purposes. Closed arrows indicate VT subunits. Open arrow indicate a 64 kDa VT fragment generated by proteolysis during egg development.

species *Drosophila melanogaster* and *Drosophila virilis* (Giorgi and Jacob, 1977; Sawicki and MacIntyre, 1978; Narise, 1984), *Aedes aegyptii* (Raikhel, 1984; Raikhel and Lea, 1986), and *Musca domestica* (Ribolla et al., 1993) passing through Orthopteran species such as *Locusta migratoria*, *Blatella germanica*, *Ornithodoro moubata* (Petavy, 1986; Purcell et al., 1988, Fagotto, 1990); the coleopterans *Bombyx mori* and *Corcyra cephalonica* (Chino, 1961; Chaubey and Bhatt, 1988) and finally the hemipteran *Rhodnius prolixus* (Nussenzveig et al., 1992).

In the present study we report the dephosphorylation of the main yolk protein in the developing eggs of *Rhodnius prolixus*. We showed that on the first 6 days after oocyte fertilization there is an increase in the total acid phosphatase activity. This enzyme remains ten fold more active when compared to non-fertilized eggs until eclosion of the first instar larvae (Fig. 1). Oviposition of non-fertilized eggs did not result an increase in total acid phosphatase suggesting a link between fertilization and this enzyme activity (Fig. 1). This enzyme has an acid pH optimum in a pNPP saturable linear reaction (Figs. 2 and 3). Na^+/K^+ tartrate (5–10 mM), a classical inhibitor of secreted acid phosphatases (Vihko et al., 1993) was able to inhibit total enzyme activity from eggs from second and tenth days after oviposition (Table 1). In addition NaF is also a powerful inhibitor of *Rhodnius* eggs acid phosphatase. The activation of acid phosphatase activity in fertilized eggs is preserved even when eggs are fractionated through gel filtration column, which suggest that it does not occur due to the presence of low molecular weight effectors in eggs homogenates (Fig. 4). Na^+/K^+ tartrate and NaF also inhibited enzyme activity obtained from fractionated eggs. One question that should be raised here is whether the increase in total acid phosphatase activity is a consequence of the expression of regulatory molecules after fertilization or if the increase is due to the translation of maternally stored acid phosphatase mRNAs. This point should be clarified by future investigation.

The presence of acid phosphatase in this system led us to investigate if VT dephosphorylation occurs during egg development. VT content of phosphoserines was then evaluated with the aid of a polyclonal antibody. A decrease on total phosphoaminoacid content was also evident under these conditions (Fig. 5). This strategy pointed to a 64 kDa VT-derived polypeptide, which is virtually free of detectable phosphoaminoacids. The phosphorylation level of this stretch in intact VT should then be checked since it may likely contain the main sites, which are dephosphorylated as eggs mature. When developing eggs, obtained from ^{32}Pi fed females, were analyzed through SDS-PAGE followed by autoradiography, a significant decrease in ^{32}P content of VT was evi-

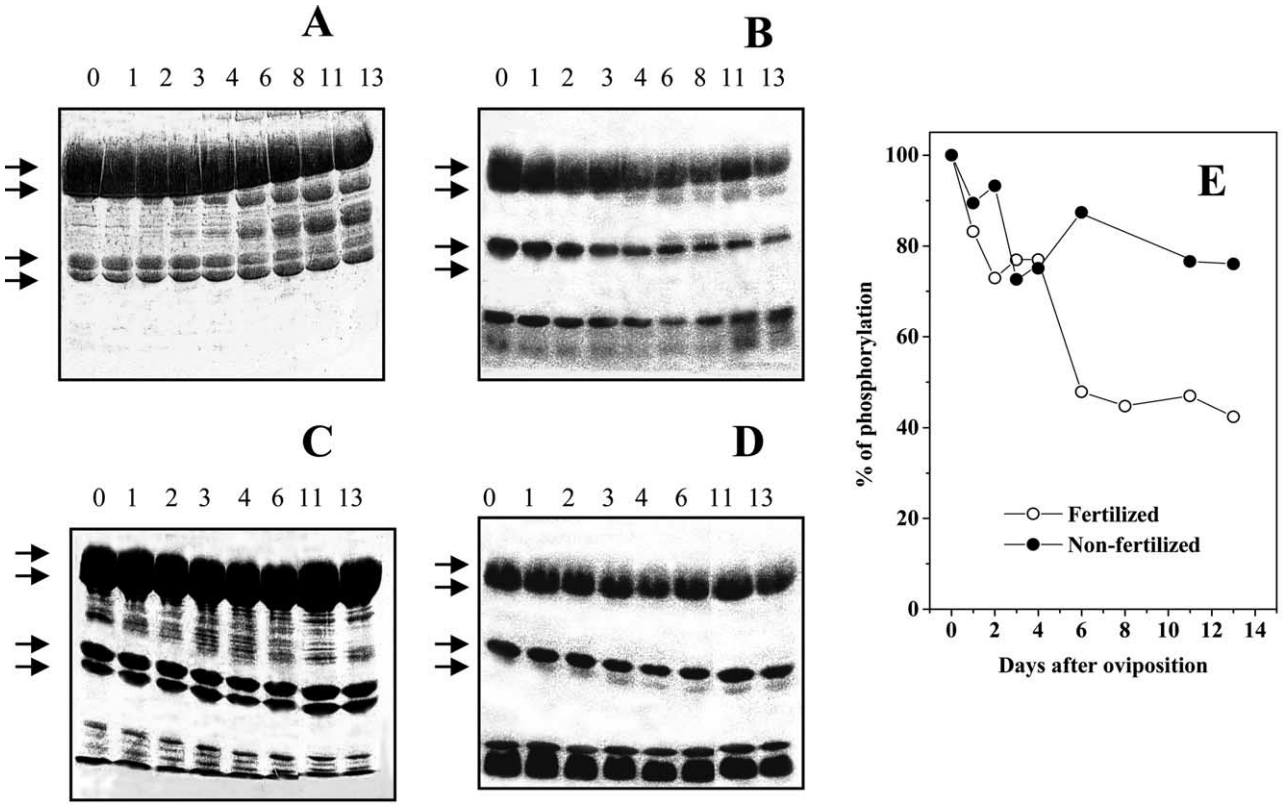

Fig. 6. VT dephosphorylation during egg development as determined by phosphate content of VT metabolically labeled with ^{32}P. Insects were fed with blood enriched with ^{32}Pi. Oviposited eggs were collected and homogenized on sucessive days after oviposition. Total homogenates from fertilized and non-fertilized eggs were then subjected to SDS-PAGE (7.5%). Panels (A) and (C) shows the gels stained with Coomassie Blue and panels (B) and (D) shows the ^{32}Pi labeled autoradiographies. Lanes 0–13: fertilized (A and B) and non-fertilized (C and D) eggs supernatants. The numbers represent the days after fertilization or oviposition. Both gels and autoradiographies were densitometrically scanned. The area of the profiles obtained for autoradiographies was then divided by that obtained for Coomassie Blue stained gels. The results are expressed using the value found for day zero as 100% of phosphorylation (panel E). The arrows indicate Vitellin subunits.

dent (Fig. 6). In fact, when we analyzed the capacity of partially purified acid phosphatase obtained as in Fig. 4 to dephosphorylate ^{32}P-VT the level of dephosphorylation was poor (data not shown). VT was the only phosphorylated molecule able to inhibit pNPP hydrolysis by acid phosphatase in vitro (Table 2). This result indicates that *Rhodnius* acid phosphatase specifically recognizes VT as a substrate.

Independent experimental approaches pointed to VT dephosphorylation during egg development. Immunological quantification of phosphoserines indicated a 20% dephosphorylation of VT (Fig. 5) from the third to the tenth day of development. Isotopic labeling of covalently bound phosphates suggested that a 50% overall decrease of phosphate content during the whole developmental period (Fig. 6). This discrepancy can be justified by the different techniques employed that can evaluate different moieties of phosphate on VT backbone (carbohydrates, phosphoaminoacids). In addition, the low level of covalent phosphate known to occur in invertebrate VTs diminishes the accuracy of the techniques employed in this study (Byrne et al., 1989; Silva-Neto et al., 1996). Nevertheless, both methods indicated that *Rhodnius* VT is dephosphorylated during embryogenesis. This result encourages the elaboration of an accurate map of VT phosphorylation sites during egg development.

In most non-mammalian oviparous organisms, one of the early events during embryo development is the initiation of proteolytic processing of VT. The regulation of this process is still unclear but a growing body of evidence (Liu and Nordin, 1998; Nordin et al., 1990; Fagotto, 1990; Mallya et al., 1992; Fagotto and Maxfield, 1994) indicates that pH may be one of the regulators of yolk degradation. Furthermore, granule restructuring is also linked with VT utilization. Although insect VTs contain covalently bound phosphate and carbohydrates, the roles of these substituents in VT's utilization have not been ascertained. In fact, several post-fertilization enzyme activities have been demonstrated during insect embryogenesis (Chaubey and Bhatt, 1988; Fagotto, 1991; Fialho et al., 1999). Some of them were shown to introduce major modifications in VT structure. *Rhodnius* acid phosphatase has previously been studied in the growing oocytes of this insect (Nussenzveig et al., 1992), and based on its association with yolk granules it was suggested that it participates in VT utilization.

Table 2
Effect of phosphorylated molecules and albumin on pNPPase activity of partially purified acid phosphatase from *Rhodnius prolixus* eggs

Conditions		Activity (nmol pNP min^{-1}±SD)
Control		0.361 (±0.004)
+Phosphoserine	0.1 mM	0.359 (±0.018)
	0.5 mM	0.354 (±0.001)
	1.0 mM	0.340 (±0.010)
+Mannose 6-phosphate	0.1 mM	0.364 (±0.012)
	0.5 mM	0.353 (±0.012)
	1.0 mM	0.346 (±0.009)
+ATP	0.1 mM	0.387 (±0.007)
	0.5 mM	0.365 (±0.015)
	1.0 mM	0.366 (±0.007)
+Albumin	0.1 mM	0.357 (±0.001)
	0.5 mM	0.361 (±0.006)
+Vitellin	0.05 mM	0.348 (±0.004)
	0.1 mM	0.301 (±0.015)
	0.5 mM	0.105 (±0.010)

Acid phosphatase activity was partially purified from developing eggs as described in Section 2 and assayed for pNPPase activity in the presence of either phosphoserine, mannose 6-phosphate, ATP, bovine albumin and Vitellin purified from oocytes. Controls assay were conducted using only 2 mM pNPP as substrate in the standard reaction media. Albumin was used as a negative control. The results are expressed in nmol pNP min^{-1} and represent the average of two experiments, performed in duplicate, with two different enzyme preparations.

Our results showed that the activation of VT directed acid phosphatase is a fertilization dependent event. This implies that phosphate removal is a part of a programmed pathway triggered by fertilization. To our knowledge this is the first study to establish a link between the major changes in the covalently bound VT phosphate moiety and an acid phosphatase activity in developing eggs of an insect. The localization of VT domains which are dephosphorylated after fertilization, together with the effects of this phosphate removal in its overall structure will thus increase our understanding of the role those reactions may play during insect embryo development.

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