

ACID PHOSPHATASE ACTIVITY IN UNGERMINATED CONIDIA FROM *Colletotrichum graminicola* AS DETERMINED BY SPECTROPHOTOMETRIC AND CYTOCHEMICAL METHODS

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ABSTRACT

The acid phosphatase in ungerminated conidia from *Colletotrichum graminicola*, a corn pathogen, was investigated using spectrophotometric and cytochemical methods. Acid phosphatase activity was studied in a homogenate obtained by fragmentation of ungerminated conidia. With *p*-nitrophenylphosphate as substrate, the apparent V_{max} and K_M were 1,000 nmol *p*-nitrophenol/mg of protein/min and 0.631 mM, respectively. The pH and temperature optima were 5.5 and 60°C, respectively. A cytochemical ultrastructural assay showed deposition of the reaction product inside vacuoles but not extracellularly on the cell surface. The permeabilization of conidia with Triton X-100 increased acid phosphatase activity eight fold. Compared to other procedures, our method was fast, easy to perform and gave consistent results.

Key words: Acid phosphatase, permeabilization, vacuoles, conidia

INTRODUCTION

Acid phosphatase is a widely distributed enzyme [1, 21] that has been studied in several fungi [5,10-13,21]. This enzyme releases inorganic phosphate, a key metabolite for cellular development [1], from phosphate esters [12, 22,23]. The optimum pH of the enzyme is acidic [8,12,22]. Acid phosphatase has been detected in vacuoles, vesicles [2,17,18] and other intracellular [10,16] and extracellular [17] structures of fungi and yeasts. In *Colletotrichum graminicola*, the enzyme has been detected in the vacuolar system of ungerminated conidia and during germination [19,20]. In this case, the reaction product was deposited on the vacuolar membrane where it served as a marker for the internalization of lipid bodies in a microautophagic process [20]. Advances in our knowledge about the physiological role of acid phosphatase in *C. graminicola* are dependent on new strategies of investigation. In this work, we compared cytochemical and spectrophotometric methods for the quantification of acid phosphatase activity in homogenates of conidia and in whole conidia which were or were not permeabilized with Triton X-100.

MATERIAL AND METHODS

Growth of C. graminicola

C. graminicola (Ces.) Wils., isolate CgM2 from corn, was provided by Dr. R. L. Nicholson (Host-Parasite Interaction Laboratory, Purdue University, West Lafayette, IN, USA). The fungus was grown on oatmeal agar at 22°C under continuous fluorescent light to induce sporulation.

Preparation of homogenate

Conidia were collected from the cultures and immediately resuspended in water. This suspension was centrifuged (1000 x g, 5 min) at room temperature and the pellet, was washed twice in water and once in 50 mM sodium acetate buffer, pH 5.5, before being resuspended in the same buffer. The preparation was then mixed with 500-1000 µm glass beads in an ice bath and fragmented in a vortex mixer at maximum speed for 1 min, followed by immediate cooling in an ice bath for 1 min (this procedure was repeated eight times). The glass beads were removed by sedimentation and the supernatant was centrifuged (1000 x g, 5 min, 0-4°C) to remove unbroken cells and cell debris. The supernatant, referred to hereafter as the homogenate, was collected for enzyme analysis and protein determination.

Acid phosphatase activity

Acid phosphatase activity was assayed by measuring *p*-nitrophenylphosphate (pNPP) hydrolysis [6]. The assay mixture contained sodium acetate buffer (50 mM, pH 5.5), 3 mM pNPP and 5-10 µg of protein from the homogenate, in a final volume of 325 µl. After 10 min at 37°C, the reaction was stopped by adding 1 ml of 0.2 M NaOH. The activity was expressed in nanomoles of *p*-nitrophenol (pNP) formed/mg of protein/min, based on an extinction coefficient of 18,400 M⁻¹ cm⁻¹ at 410 nm. The pH for optimum activity was

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determined using 50 mM sodium acetate buffers of different pH. The effect of temperature on enzyme activity was determined in the range between 20°C and 80°C. Kinetic parameters (V_{max} and K_M) were calculated from a Lineweaver-Burk plot of the reaction velocities obtained with different concentrations of substrate. Protein concentrations were determined according to Bradford [3].

Cytochemical assays

Acid phosphatase activity was detected using sodium β -glycerophosphate as substrate and cerium chloride as the acceptor [20]. The deposition of cerium phosphate (reaction product) was used to localize acid phosphatase activity. Control cells were incubated without substrate.

Permeabilization with Triton X-100

Conidia were collected from cultures and immediately resuspended in water. This suspension was washed by centrifugation (1000 x g, 5 min) once in water and three times in 50 mM sodium acetate, pH 5.5, followed by resuspension in the same buffer. The concentration of conidia was determined by turbidimetry at 570 nm with a standard spectrophotometer [6,11]. The measurements are proportional to the conidial concentration, and a reading of 0.7-0.8 corresponded to 1×10^6 - 1.5×10^6 conidia/mL counted in a Neubauer chamber. The optimal concentration of Triton X-100 was determined by incubating the conidia with different concentrations of detergent for 30 min at 37°C and then assaying the acid phosphatase activity. To determine the optimum temperature, conidial suspensions were incubated with 0.1% (v/v) Triton X-100 at different temperatures for 30 min before assaying acid phosphatase activity.

Acid phosphatase activity in permeabilized cells

Enzymatic activity was assayed using 300 μ l of permeabilized conidial suspension ($Abs_{570\text{ nm}} = 0.7-0.8$) and 3 mM pNPP. After 10 min at 37°C, the reaction was stopped by adding 1 ml of 0.2 M NaOH followed by centrifugation (1000 x g, 3 min). The activity was expressed as nmol pNP released per min for an absorbance of the conidial suspension of 0.7-0.8 at 570 nm [6]. The extracellular enzyme activity was determined in intact (non-permeabilized) conidia. All other conditions were identical to those used for permeabilized cells.

Statistical analysis

The results were expressed as the mean \pm SD of triplicates from two (n=6) or three (n=9) experiments. ANOVA and Student's *t* test were used to compare differences in the effect of Triton X-100 concentrations on conidial permeabilization, with the level of significance set at 5% ($P < 0.05$). Student's *t* test was used to compare differences in enzymatic activity between non-permeabilized and permeabilized conidia from three experiments done in triplicate. The level of significance was set at 1% ($P < 0.01$).

RESULTS

Acid phosphatase in cell homogenates

Cell fragmentation using glass beads was efficient but slow and laborious. Other methods for fragmenta-

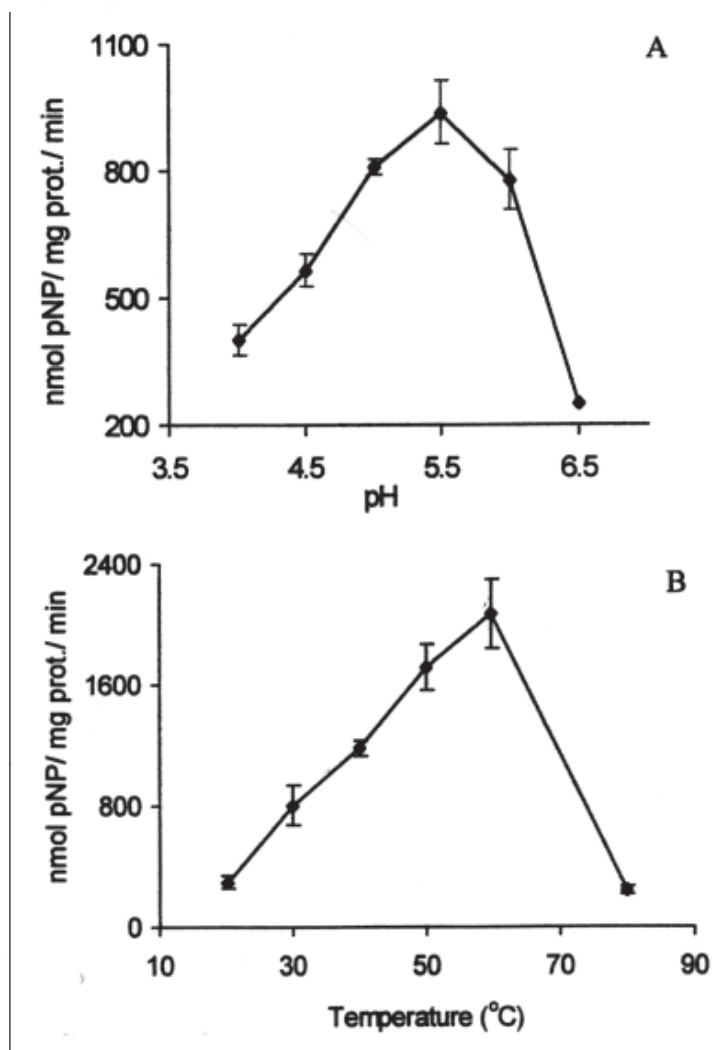


Figure 1. pH (A) and temperature (B) optima for acid phosphatase activity in homogenates of ungerminated conidia. Each point represents the mean \pm SD of two independent experiments done in triplicate.

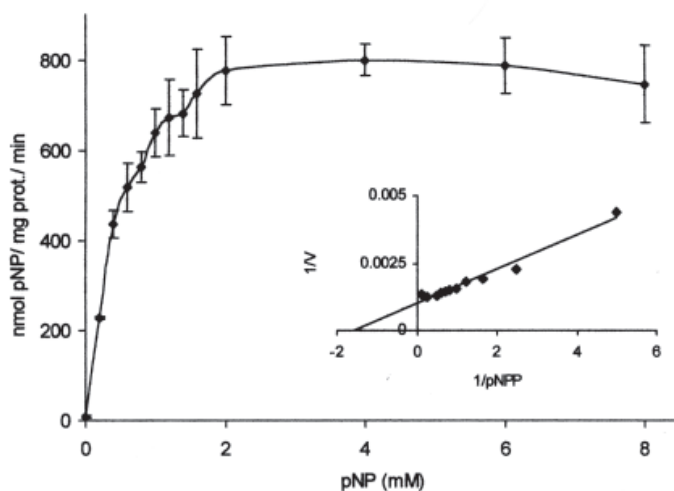


Figure 2. Kinetics of acid phosphatase activity in a homogenate of ungerminated conidia. Each point represents the mean \pm SD of two independent experiments done in triplicate. Inset - The V_{max} and K_M were 1000 nmol pNP / mg prot. / min and 0.631 mM pNPP, respectively.

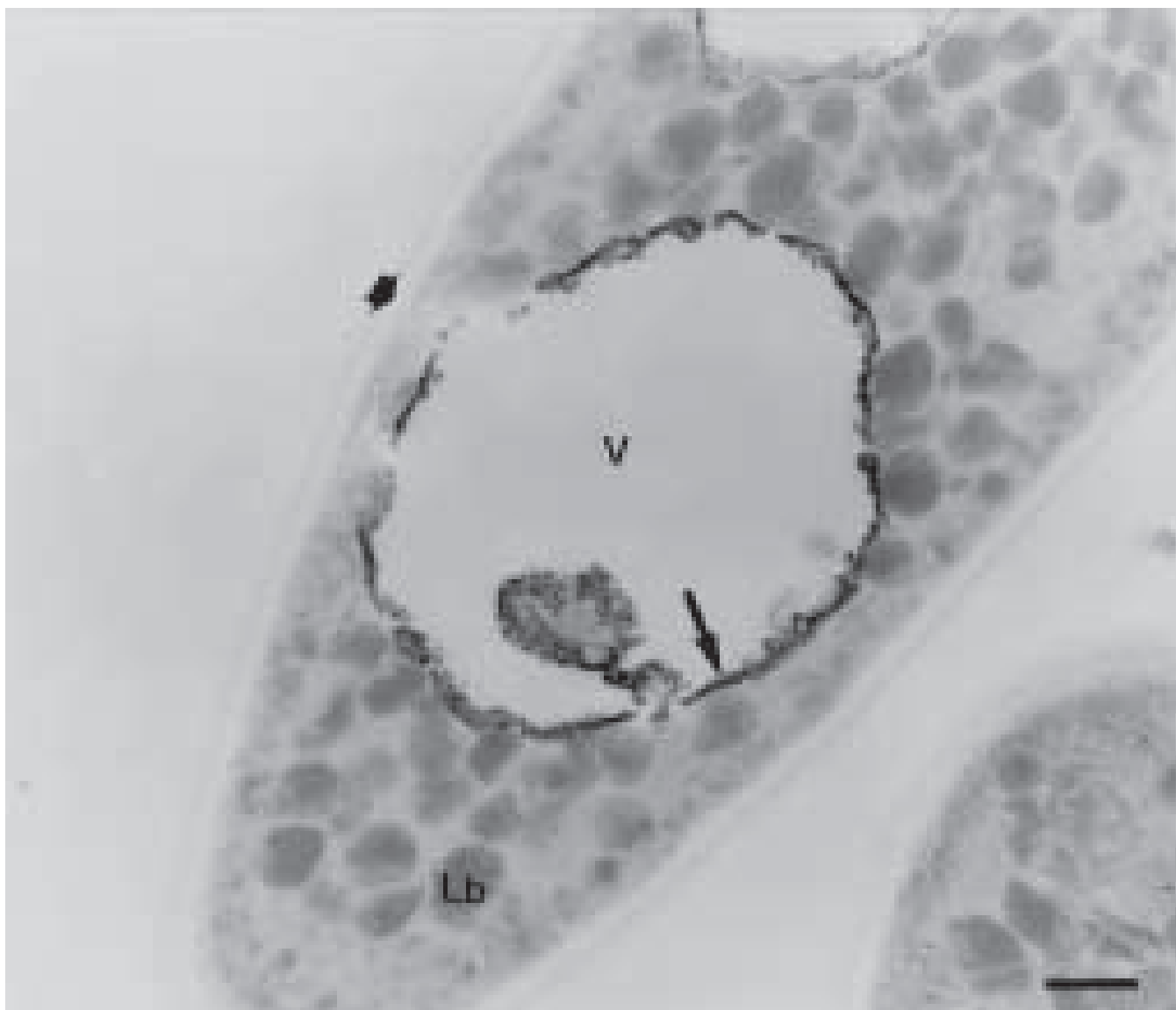


Figure 3. Section of an ungerminated conidium. The reaction product of acid phosphatase is shown (long arrow) inside a vacuole (V). Note the absence of reaction product in the cell wall (thick arrow). Lipid bodies (Lb) can be seen in the cytoplasm. Bar = 500 nm.

tions such as sonication were not effective. The extent of fragmentation was monitored by light microscopy.

The pH and temperature optima for acid phosphatase were 5.5 and 60°C (Fig. 1) respectively. A temperature of 37°C, which is more physiological and has been used for other fungi [12,23], was chosen for subsequent experiments. The effect of substrate concentration on enzyme activity is shown in Fig. 2. A Lineweaver-Burk plot gave apparent V_{\max} and K_M values of 1000 nmol pNP/mg protein/min and 0.631 mM, respectively (Fig. 2, inset).

Cytochemical localization of acid phosphatase

Reaction product was localized in the vacuoles and not on the cell surface (Fig. 3). This observation strongly suggests that the activity in the homogenate was released

predominantly from vacuoles. In control cells, no reaction product was observed on any cellular structure (data not shown).

Sodium β -glycerophosphate was used as substrate in the cytochemical assays (Fig. 3). pNPP was also tested but precipitated with cerium in the reaction medium. Nevertheless, reaction product was formed in the vacuoles in a manner similar to that with β -glycerophosphate (data not shown).

Intracellular and extracellular activities

Permeabilization allows substrates to pass into cells, and to become available to intracellular phosphatases [6,7,14]. Permeabilization with different concentrations (0.05-0.4%) of Triton X-100 was tested (Fig.4A) and no significant

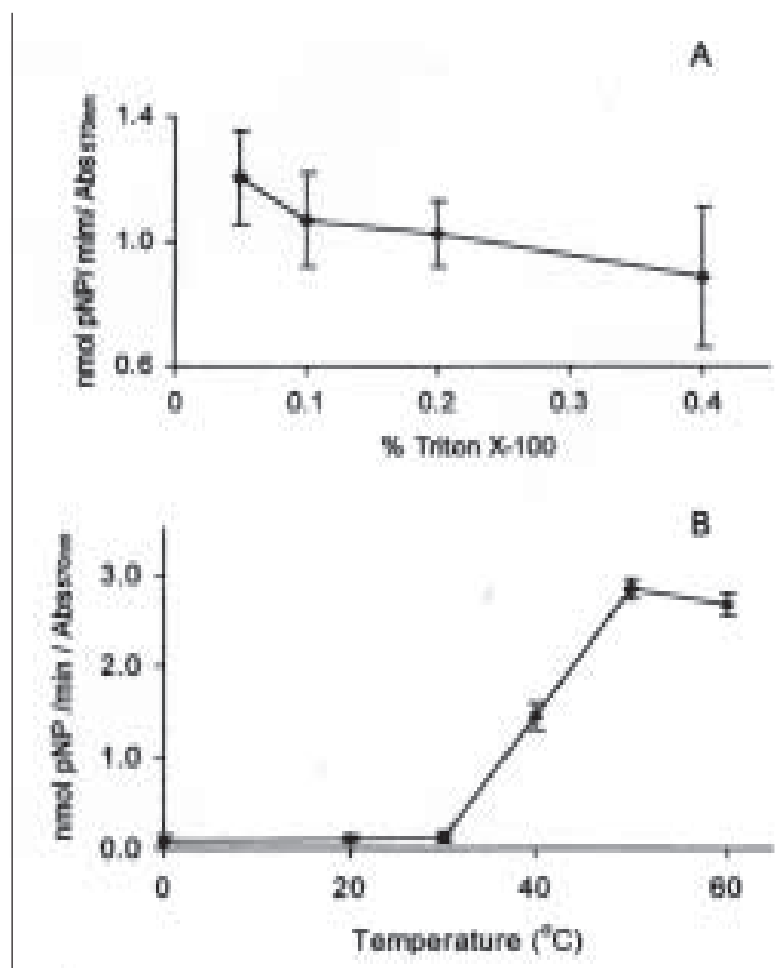


Figure 4. A. Effect of Triton X-100 concentration (%) on the permeability of conidia. B. Effect of temperature on the permeabilization of conidia by Triton X-100. Each point represents the mean \pm SD of two experiments, done in triplicate.

difference was observed by ANOVA ($P < 0.05$). Student's t test showed significant differences only between 0.05% and 0.4% Triton X-100 ($P < 0.05$). A concentration of 0.1% was used in most experiments, as recommended for *Candida lipolytica* [6] and *Yarrowia lipolytica* [7]. This concentration did not affect enzymatic activity in the homogenate (data not shown). The permeabilization was temperature dependent, with maximum activity at 50°C (Fig. 4B).

To compare the intra- and extracellular enzymatic activities, assays were done on permeabilized and intact (non-permeabilized) conidia. This approach has been used to localize intracellular acid phosphatase in *Neurospora crassa* [11]. Intact conidia showed low extracellular enzymatic activity (0.3 ± 0.13 nmol pNP/min/Abs_{570nm}) while eight-fold greater activity (2.5 ± 0.5 nmol pNP/min/Abs_{570nm}) was detected in permeabilized cells. These results are significantly different ($P < 0.001$, Student's t test). These findings and those shown in Figures 1-4 indicate that

permeabilization allowed the detection of intracellular acid phosphatase activity.

DISCUSSION

The presence of acid phosphatase activity detected here confirms a previous report of this enzyme in ungerminated conidia [20]. The acidic pH optimum of the enzyme was expected based on its vacuolar localization, as also shown for other fungi [8,12,21]. Fungi vacuoles are acidic organelles which are similar to lysosomes in animal cells [9]. The presence of acid phosphatase has been reported in the vacuoles of other fungi, including *Saccharomyces cerevisiae* [10,16], *Aspergillus flavus* [2], *Psilocybe cubensis* [17] and *Gigaspora margarita* [18]. The effect of temperature on activity was similar to acid phosphatases of other origins [4,5,21], and the elevated activity after incubation with Triton X-100 at high temperatures agreed with the thermostability observed in other fungi (8, 21-23).

Cell fragmentation is widely used to study intracellular acid phosphatase in fungi [6,8, 21,23]. However, cell disruption can be difficult, expensive and time consuming [6,15]. The mechanical disruption of *C. graminicola* conidia was difficult and laborious whereas permeabilization with Triton X-100 was simpler, easier to perform and faster. Cell permeabilization also avoids possible damage to cellular components during extraction and the enzyme can be studied closer to its natural state [14]. Considering the predominantly intravacuolar localization of this enzyme, cellular permeabilization is recommended for advanced studies of acid phosphatase in *C. graminicola* conidia.

ACKNOWLEDGMENTS

The authors thank Dr. Philip A. J. Gorin and Dr. Cloriz D. Faraco for their comments on this manuscript. This research was supported in part by grants from the Brazilian National Research Council (CNPq), PRONEX and FUNPAR.

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Received: November 19, 2000
Accepted: March 19, 2001