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Effect of Phytohormones and Group Selective Reagents on Acid Phosphatase from *Cladosporium cladosporioides*

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Abstract: Acid phosphatase [EC 3.1.3.2] was isolated and characterized from *Cldosporium cladosporioides*. The activity was determined by using p-nitrophenyl phosphate (PNPP) as substrate. Gibberellic acid (GA_3), 6-benzylaminopurine (BAP), kinetin and 2,4-dichlorophenoxyacetic acid (2,4 D) induced the enzyme activity when included in the growth medium. GA_3 and BAP were the strongest inducers. However, indole acetic acid (IAA) did not show any effect on the enzyme activity. The effect of calmodulin antagonists on GA_3- BAP-induced acid phosphatase synthesis was also investigated. The calmodulin antagonists chlorpromazine, haloperidol, trifluoroperazine and quinacrine inhibited both GA_3- and BAP-induced synthesis of acid phosphatase. This leads to the suggestion that some calmodulin-controlled mechanism is involved in GA_3- and BAP-induced acid phosphatase synthesis. The enzyme was purified to homogeneity on the basis of polyacrylamide gel electrophoresis using ammonium sulfate (35-80 %), Sepharcryl S-200HR and Phenyl-Sepharose HP. The final specific activity was 203.8 U mg^{-1} with purification fold of 328.6. The divalent cations Ba^{2+}, Ca^{2+} and Sr^{2+} and Co^{2+} were strong activators whereas Zn^{+2} was a strong inhibitor. Ca^{2+} is required for activity and thermal stability of acid phosphatase. Citrate, borate and carbonate enhanced acid phosphatase. Bromide, arsenate, phosphate, sulfite, sulfate, fluoride, EDTA and EGTA inhibited the enzyme activity. N-bromosuccinimide (NBS), tetranitromethane (TNM), N-ethylmaleimide (NEM) and diethylpyrocarbonate (DEPC) inhibited acid phosphatase activity suggesting that tryptophenyl, cysteinyl and tyrosyl and histidyl residues taking part in the catalytic activity of acid phosphatase. Dithiothreitol (DTT), reduced glutathione (GHS), L-ascorbic acid and cysteine at 5 mM enhanced the enzyme activity. Triton X-100, Nonidet F40, Brij-35 and sodium oleate enhanced the acid phosphatase activity whereas sodium lauryl sulphate was inhibitor.

Key words: Fungi, acid phospatase, phytohormones, purification

INTRODUCTION

Acid phosphatase (EC 3.1.3.2) catalyzes the nonspecific hydrolysis of phosphate monoesters under acidic conditions (Roland *et al*., 1997). This enzyme is widely distributed in mammalian serum (Partanen, 2001), plants (Olczak *et al*., 1997; Ehsanpour and Amini, 2003; Prazeres *et al*., 2003) and in bacteria (Palacios *et al*., 2005).

Acid phosphatase has been detected in fungi, such as, *Aspergillus* (Han and Gallagher, 1987; Bernard *et al*., 2002), *Neurospora* (Han and Rossi, 1996), *Humicola lutea* (Aleksieva and Micheva-Viteva, 2000), *Penicillium* (Haas *et al*., 1991) and *Botrytis cinerea* (Roland *et al*., 1997). In fungi, acquisition of nutrients from the environment involves the secretion of an array of hydrolytic enzymes

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acting especially on different resources. The phosphatases, a generic designation for non-specific phosphoesterases, belong to a family of enzymes responsible for supplying inorganic phosphate (Pi) to the cell.

The behavior of acid phosphatase in the culture liquid (extracellular enzymes) and mycelial extract has been investigated in seven fungi grown as stationary cultures in a mineral medium (Reyes *et al.*, 1990).

Most enzymes posses one or more amino acids in their active sites involved in catalytic activity. Generally the existence of tryptophan, cysteine, histidine and arginine has been reported at or near the active site of enzymes (Roig and Kennedy, 1992). Several methods have been described in the literature for the identification of the catalytically essential amino acid residues of enzymes; determination of amino acids involved in catalysis by measuring the kinetic parameters of enzymes at different pH values; x-ray structural analysis and substrate specificity studies are some examples of these methods. In the cases where the enzyme is available in limited amounts; the chemical modification of the enzyme molecule by amino acid specific reagents seems to be one of the most convenient approaches for identification of amino acids at or near the catalytic center (Roig and Kennedy, 1992).

This study describes the purification and characterization of acid phosphatase from *Cladosporium*. Also, chemical modification by NBS, NEM, TNM and DEPC has been carried out in order to obtain information regarding functional amino residues at the active site of the enzyme.

MATERIALS AND METHODS

Growth of the Fungus

Cladosporium cladosporioides was grown according to El-Shora and Salwa (2002) on a liquid medium containing the following components: corn steep liquor (CSL) 2%, $NH_4H_2PO_4$ 1.2%, KCl 0.07%, $MgSO_4 \cdot 7H_2O$ 0.05%, $FeSO_4 \cdot 7H_2O$ 0.001% and pH 4.5. The liquid cultures were usually grown for 3 days at 27°C in 100 mL medium in 250 Erlenmeyer flasks in an orbital incubator. Cultures were inoculated from stocks kept on malt extract agar plates.

Extraction of the Enzyme

Twenty grams of freeze-dried mycelium collected from 400 mL of fungal culture were pulverized with an electric mixer in an extraction buffer (100 mM Na acetate buffer, pH 7.0, 5 mM DTT). Extracts were filtered through gauze and clarified by centrifugation at 5000 rpm for 20 min at 4°C. The resulting supernatant was called the crude extract.

Purification of the Enzyme

The supernatant was adjusted to 35% saturation with solid ammonium sulfate. The precipitate formed by standing overnight at 4°C was removed by centrifugation. The supernatant was adjusted to 80% saturation with solid ammonium sulfate and allowed to stand overnight. The precipitate was collected by centrifugation, dissolved in a small volume of sodium phosphate buffer (pH 5.0) and dialyzed. The dialyzed enzyme solution was applied to Sephacryl S-200HR column (2×25 cm). The active fraction was pooled and applied to a column of Phenyl-Sepharose HP. After being washed with 5 bed volumes of the buffer, the column was eluted with a continuous linear gradient formed of the buffer and 1.5 M NaCl. The active fraction was pooled for determination of some properties of purified acid phosphatase.

SDS-Polyacrelamide Gel Electrophoresis

The purity of acid phosphatase from *Cldosporium cladosporioides* was analyzed by 3-10% discontinuous SDS-polyacrylamide gel electrophoresis (Laemmli, 1970).

Determination of Acid Phosphatase Activity

Acid phosphatase was assayed according to the method of Granjeiro *et al*. (2003). The reaction mixture in a final volume of 1 mL, contained 100 mM sodium acetate buffer (pH 5.0), 5 mM p-nitrophenylphosphate (PNPP) and enzyme. After 10 min of incubation at 37°C, the reaction was stopped by the addition of 1 mL of 1 M NaOH. Acid phosphatase activity was measured as the release of PNPP monitored at 405 nm, using a molar extinction coefficient of 18,000 M^{-1} cm^{-1}. In the protection studies, enzyme activity was assayed by measuring the amount of phosphate released. For inorganic phosphate determinations, the assay conditions were the same as described for PNPP, except that reactions were terminated by adding 1 mL of 3% (w/v) ammonium molybdate (in 200 mM acetate buffer, pH 4.0) followed by 0.1 mL of 120 mM ascorbic acid (in 200 mM acetate buffer, pH 4.0). The absorbance of the resulting color was read at 700 nm, after 30 min at room temperature. The amount of inorganic phosphate released was calculated using a molar extinction coefficient of 4000 M^{-1} cm^{-1} (Ames, 1966). All the experiments were performed twice and conducted in triplicate with standard error.

Protein Determination

After scanning at 280 nm, the tubes with significant absorbance were pooled and a quantitative protein was determined by the Coomassie Blue G-250 method (Bradford, 1976).

Modification of Acid Phosphatase

The enzyme was preincubated with amino acid modifying reagents, which included NEM, NBS and TNM, in 200 mL of 300 mM mannitol and 20 mM Hepes-Tris (pH 7.5) (buffer) for 30 min at 25°C. Incubation with DEPC was done in 200 mL of 300 mM mannitol and 20 mM MES-/NaOH (pH 6.0) (Boivin *et al*., 1997).

For DEPC the incubation medium contained ethanol at a final concentration of 1.5% (v/v). The pre-incubation reaction was stopped by diluting the mixture in buffer without substrate. The residual phosphatase activity was then quantified by adding PNPP.

Effect of Metals, Anions and Chelating Agents

Acid phosphatase was incubated with the anions (chloride salt), cations (sodium salts) or chelating agents at room temperature at the appointed concentrations for 30 min and the enzyme activities were determined.

Substrate Specificity

Substrate specificity was determined by using PNPP, ATP, ADP, AMP, glucose-1-phosphate (G-1-P), glucose-6-phosphate (G-6-P) and phenylphosphate (PP) at 5 mM.

Effect of Phytohormones

Hundred μM of GA_3, BAP, IAA, kinetin 2,4-D were added to culture medium for 72 h and then the enzyme activity was determined.

Statistical Analysis

All values are the mean of three measurements±SE.

RESULTS AND DISCUSSION

First of all we tried to test the possible stimulation of acid phosphatase synthesis by some phytohormones such as GA_3, BAP, kinetin, 2,4-D and IAA. It was found that the first four tested

Table 1: Effect of calmodulin antagonists on GA_3-induced acid phosphatase activity from *Cladosporium cladosporioides*

Additives	Relative activity (%)
Control	100±0.0
100 µmol GA_3	152±0.7
100 µmol BAP	126±0.9
100 µmol GA_3 + 0.5 mM Chlorpromazine	130±0.3
100 µmol GA_3 + 0.5 mM Haloperidol	116±0.5
100 µmol GA_3 + 0.5 mM Quinacrine	116±0.5
100 µmol BAP + 0.5 mM Chlorpromazine	120±0.4
100 µmol BAP + 0.5 mM Haloperidol	114±0.2
100 µmol BAP + 0.5 mM Quinacrine	114±0.2

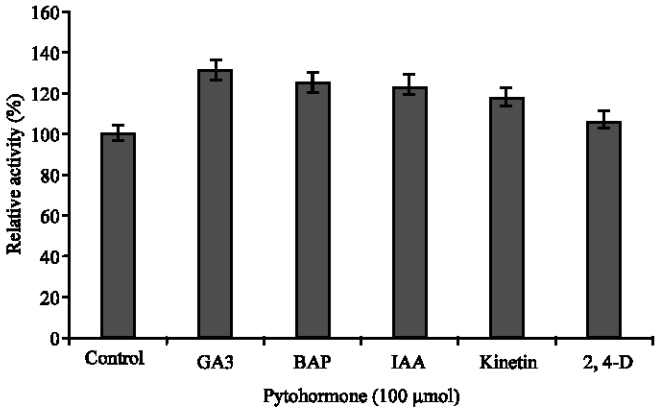

Fig. 1: Effect of plant growth regulators on phosphatase activity

phytohormones induced acid phosphatase activity with different rates when each individual compound was included in the growth medium of the fungus (Fig. 1). GA_3 and BAP were the most stimulators of acid phosphatase synthesis; therefore they were used in the next experiment. However, IAA did not show any effect on the enzyme activity. The stimulation of GA_3 is in agreement with the results obtained for other enzymes such as phosphoenolpyruvate carboxylase (El-Shora, 1993; Bihzad and El-Shora, 1996) and NADG-glutamate synthase (El-Shora, 2001) and phenylalanine ammonia-lyase (El-Shora, 2002). In support, 2,4-D expressed marked increase in synthesis of other enzymes like soluble RNA polymerase and chromatin-bound RNA polymerase (Guifoyla *et al*., 1975) NAD-oxidase (Brightman *et al*., 1988) and peroxidase (Chen and Poltanick, 1991). 2,4-D showed a very pronounced stimulation of RNA synthesis and resulted in an increase in translatable mRNA (Zurfluh and Guilfoyle, 1982). Thus, it seems likely that 2,4-D is controlling synthesis or translation of mRNA required for synthesis of the enzyme protein. However, additional work will be needed to establish these points beyond question.

The effect of calmodulin antagonists such as chlorpromazine, quinacrine and haloperidol at 0.5 mM on GA_3- BAP-induced acid phosphatase synthesis was investigated (Table 1). These calmodulin antagonists inhibited GA_3- and BAP-induced acid phosphatase synthesis. This leads us to suggest that some calmodulin-controlled mechanism is involved in GA_3- and BAP-induced acid phosphatase synthesis. Chlorpromazine and quinacrine inhibited formation of other fungal enzymes such as xylanase in *Trichoderma reesei* (Robert *et al*., 1998). It has been reported that calmodulin antagonists inhibited GA_3-enzyme secretion in barely aleurone layer (Obata *et al*., 1983). The phosphatase activity was assayed with PNPP as substrate. In the present work, the acid phosphatase was purified with ammonium sulfate precipitate at saturation 35-80%, Sepharose S-200HR and Phenyl Sepharose (Table 2). The specific activity was 203.8 U mg^{-1} with purification fold of 328.6. The

Table 2: Purification of acid phosphatase from *Cladosporium cladosporioides*

Steps	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1} protein)	Yield (%)	Purification fold
Crude extract	522.00	112.9	0.62	100.0	1.0
$(NH_4)_2SO_4$ (35-80%)	5.00	61.9	12.40	54.8	20.0
Sephacryl S-200HR	1.20	30.8	25.70	27.3	41.5
Phenyl-Sepharose HP	0.08	16.3	203.80	14.4	328.6

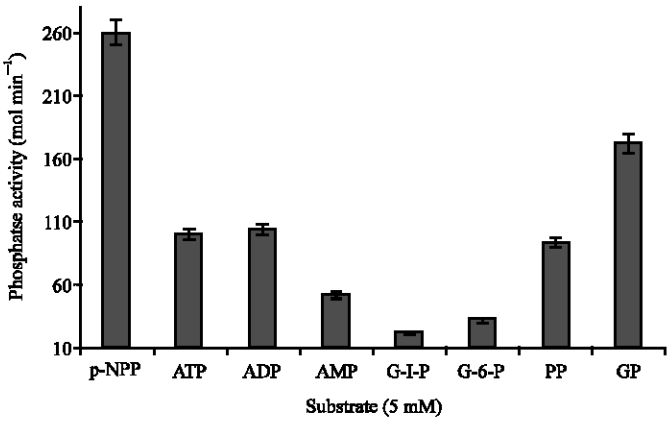

Fig. 2: Substrate specificity of acid phosphatase

specific activity obtained for the enzyme from *Cldosporium cladosporioides* in the present research is higher than 46.6 U mg^{-1} protein reported for the enzyme from kidney bean (Cashikar *et al*., 1997). Acid phosphatase of *Cladosporium* in the present investigation was purified to homogeneity (data not showed).

The purified acid phosphatase showed broad specificity, hydrolyzing a wide variety of substrates (Fig. 2). The substrates hydrolyzed at the highest rates were p-NPP and glycerophosphate followed by ATP and ADP. The enzyme showed less preference for other pyrophosphate, AMP, G-6-P and G-1-P. These results are in agreement with those of Wannet *et al*. (2000).

The effect of various cations on acid phosphatase activity was investigated at either 1 mM or 5 mM. The divalent cations Ca^{2+}, Ba^{2+}, Co^{2+} and Sr^{2+} were strong activators particularly at 5 mM (Fig. 3). These results are in consistent with those of Cashikar *et al*. (1997). Also, the enhancement of the activity of acid phosphatase by Co^{2+} is similar to the observation of Palacios *et al*. (2005). Only Zn^{+2} was a strong inhibitor and this support the results of other investigators (Abdallah *et al*. 1999; Han and Rossi, 1996). Monovalent cations seem to have no appreciable effect on the enzyme activity. In contrast, Na^{+}, Ca^{2+} and K^{+} were activators of acid phosphatase from other sources (Yenigün and Güvenilir, 2003). It seems likely that acid phosphatase from various sources responds differently to monovalent cations.

The effect of various anions on acid phosphatase was tested at 5 mM. Carbonate, borate and citrate enhanced acid phosphatase whereas bromide, arsenate, sulfate, fluoride, phosphate, sulfite inhibited the enzyme (Fig. 4). Nitrate showed no remarkable effect. These results are in agreement with those of other investigators (Straker and Mitchell, 1986; Colón *et al*., 1992; Cashikar *et al*., 1997).

The chelating agents EDTA and EGTA at different concentrations (0.2-1.0 mM) inhibited acid phosphatase from *Cldosporium cladosporioides* when they are included in the assay medium (Fig. 5). These compounds inhibited phosphatases from other microorganisms such as *Lactobacillus pentosus (*Palacios *et al*., 2005). However, the enzyme from *Agaricus bisporus* was unaffected by EDTA (Wannet *et al*., 2000). The inhibition of acid phosphatase activity by EDTA and EGTA could be due

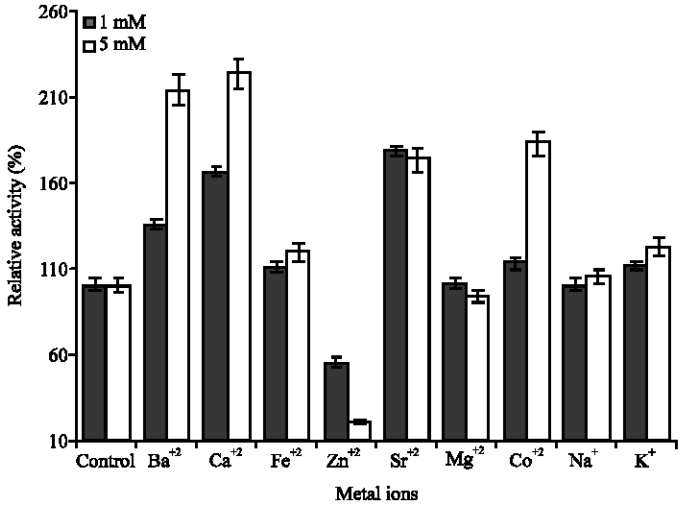

Fig. 3: Effect of various metal ions on acid phosphatase activity

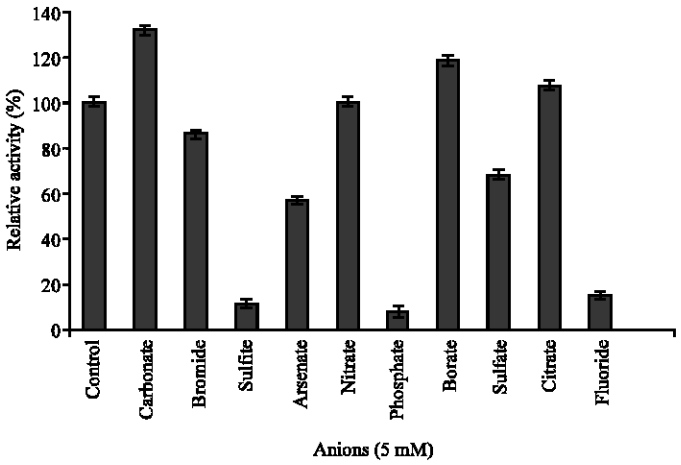

Fig. 4: Effect of anions on phosphatase activity

to their influence on the interfacial area between the substrate and the enzyme. The inhibition of acid phosphatase activity by the two chelating agent suggests that acid phosphatase is a metaloenzyme. Acid phosphatase was very heat labile in the absence of Ca^{2+} (Table 3). Pre-incubation at 70°C prior to the addition of PNPP effectively inactivated the enzyme. The presence of $CaCl_2$ during the incubation however, was sufficient to preserve 70% of the enzyme activity. Even preincubation at 30°C for 20 min without $CaCl_2$ significantly reduced the activity of the enzyme. Ca-chelator EGTA further reduced the enzyme activity to 15% of the control. The presence of excess $CaCl_2$ during the 30°C incubation partially protected the enzyme. Without a preincubation period (i.e., when substrate was added immediately after EGTA) the enzyme activity was reduced by only 20%.

Enzyme activity in the presence of 2-10 M urea was studied and gradually decreased with increasing concentration of urea (Fig. 6). At higher concentrations, urea denatures the enzyme by causing a conformational change in the tertiary structure of the enzyme, which it was unable to bring about at a low concentration (Laidler and Bunting, 1973).

Table 3: Effect of Ca^{2+} and EGTA on thermal stability of acid phosphatase from *Cladosporium cladosporioides*

Treatments	Activity (Control %)
No preincubation, + Ca^{2+}	100
70°C + Ca^{2+}	70±0.4
70°C - Ca^{2+}	10±0.2
30°C + Ca^{2+}	95±0.3
30°C - Ca^{2+}	49±0.7
30°C + EGTA	15±0.8
30°C + EGTA + Ca^{2+}	55±0.9
No preincubation + EGTA	80±0.9

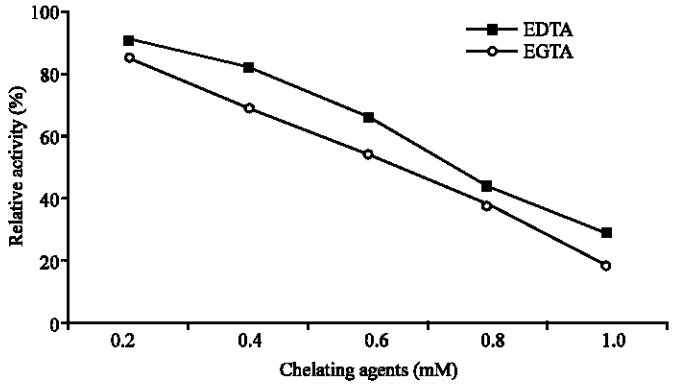

Fig. 5: Effect of chelating agents on phosphatase activity

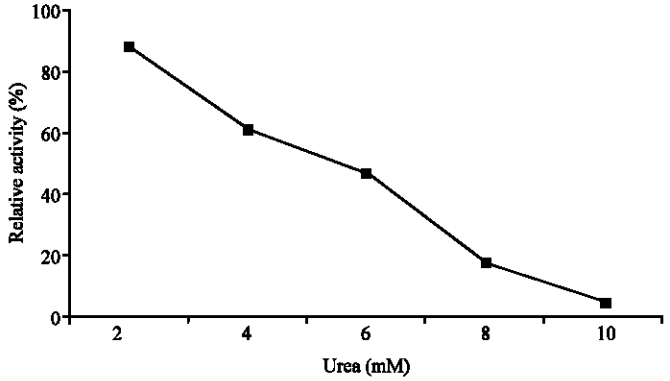

Fig. 6: Effect of urea on acid phosphatase activity

The identification of essential amino acid residues in the active site of an enzyme allows the evaluation of which roles of these amino acids play in the binding of substrates and in the catalytic mechanism. To determine which amino acid residues are involved in the catalytic mechanism of acid phosphatase, the enzyme was incubated at 25°C with different amino acid-modifying reagents namely NBS, TNM, NEM and DEPC at either 0.5 or 1 mM. Also, protection studies performed to determine the effect of substrate PNPP as substrate on induced inactivation of acid phosphatase by these reagents. The four tested reagents inactivated acid phosphatase activity. It is found that 0.5 and 1 mM of PNPP protected the enzyme with variable percentages against inactivation by the various reagents (Table 4). Thus, it seems likely that tryptophenyl, tyrosyl, cysteinyl and histidyl residues

Table 4: Protection of acid phosphatase from *Cladosporium cladosporioides* by PNPP against modification by NBS, TNM, DEPC and IAA

Incubation mixture		Relative activity (%)
Control		100±0.0
Enzyme (200 μg)	+ 1 mM NBS	20±0.3
	+ 1 mM TNM	10±0.6
	+ 1 mM DEPC	30±0.5
	+ 1 mM IAA	30±0.5
	+ 1 mM NBS + 0.5 mM PNPP	58±0.7
	+ 1 mM NBS + 1 mM PNPP	77±0.4
	+ 1 mM TNM + 0.5 mM PNPP	42±0.6
	+ 1 mM TNM + 1 mM PNPP	61±0.3
	+ 1 mM DEPC + 0.5 mM PNPP	49±0.8
	+ 1 mM DEPC + 0.1 mM PNPP	69±0.9
	+ 1 mM IAA + 0.5 mM PNPP	76±0.6
	+ 1 mM IAA + 1 mM PNPP	82±0.4

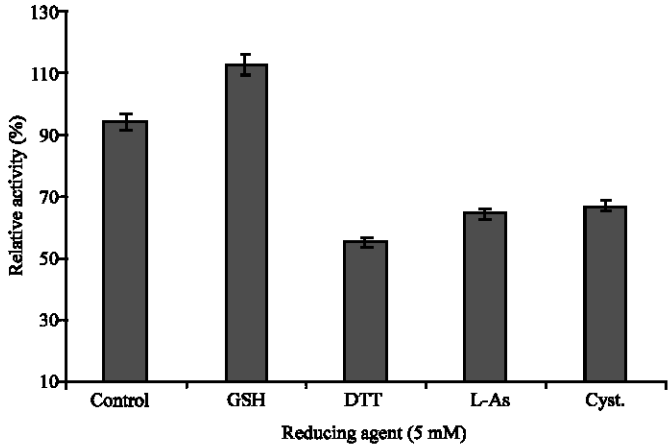

Fig. 7: Effect of reducing agents on phosphatase activity

respectively are essential for the catalytic activity of acid phosphatase from *Cldosporium cladosporioides*. These results are in harmony with those reported for acid phosphatase from *Ricinus communis* and *Bacillus stearothermophilus* (Granjeiro *et al*., 2003; Gote *et al*., 2006). Also, the cysteine residue is probably located in the active site since the protective compound PNPP resorted the enzyme activity. Lopez *et al*. (2000) working with different modifying sulfhydryl reagents, showed the presence of cysteine essential for a caterpillar venom activity on human factor V.

DTT, GSH, L-ascorbic acid and cysteine at 5 mM (Fig. 7), which may act as reducing agents, enhanced the enzyme activity. The enhancement of acid phosphatase by L-ascorbic acid is consistent with the results of Palacios *et al*. (2005) and Eunwha Son *et al*. (2007). These results support the suggestion that sulfhydryl groups could support the efficiency of enzyme catalysis.

The effects of chemical substances on the activity of an enzyme are often precise and specific. In the present study some surfactants were chosen for an evaluation of their effects on acid phosphatase activity (Table 5). The effects of 5% Triton X-100, Nonidet F40, Brij-35, sodium oleate and sodium lauryl sulphate were investigated. The first four surfactants caused remarkable increase in enzyme activity. The increase of acid phosphatase activity is caused by an improved accessibility for the substrate and the enhanced activity of the catalytic site of the enzyme due to its immobilization in the surfactant aggregates (Anikeeva and Egorov, 2000). These results are in agreement with the results reported for phosphatase from *Aspergillus ficuum* (Han and Gallagher, 1987; Youn *et al*., 1987).

Table 5: Effect of various detergents at 0.5% on acid phosphatase from *Cladosporium cladosporioides*

Detergent	Relative activity (Control %)
Control	100
Triton X-100	127.4±0.90
Brij-35	33.8±0.71
Nonidet F40	112.3±0.70
Sodium lauryl sulphate	76.0±0.40
Sodium oleate	119.0±0.40
Sodium dodecyl sulfate	21.6±0.30

However, the extent of stimulation by surfactants varies for the different enzymes (Kim *et al*., 1995). However, sodium lauryl sulfate inhibited the enzyme activity. The inhibition may be the result of a combined effect of factors such as the reduction in the hydrophobic interactions that play a crucial role in holding together the tertiary protein structure and a direct interaction with the protein molecule (Creighton, 1989; Kar *et al*., 2003).

On the basis of the above observations we can conclude, this work shows that production of acid phosphatase was induced by phytohormones GA_3, BAP, IAA and 2,4-D with various percentages. Also, tryptophenyl, cysteinyl, arginyl and histidyl residues are essential for the enzyme catalytic mechanism.

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