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Partial Characterization of Acid Phosphatase from Ginger (Zingiber Officinale) Rhizomes

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ABSTRACT

Acid phosphatase has been isolated and purified from ginger rhizome following conventional methods of protein purification with a molecular weight of 46kDa as judged by SDS-PAGE. The enzyme was found to be more stable around pH 5-9 and it was less stable at acidic pH (3-4) and the alkaline pH (10-11). The enzyme was completely inactive when exposed to 900C for 30min. The Km value of acid phosphatase was found to be 0.083M calculated from Line weaver- Burk plot and showed broad substrate specificity. Of the metals tested, only Zn2+ at 10mM concentration inhibited the enzyme to an extent of 60% and the rest of the metals tested were ineffective either in activating or inhibiting the enzyme. The activity of the enzyme was unaffected when it was exposed to 0.1% urea and guanidine hydrochloride. It was, however, inactivated to an extent of 50% when exposed to SDS (0.1%).

KEYWORDS : Acid phosphatase, rhizome, Line weaver- Burk plot, specificity and denaturants

INTRODUCTION

Acid phosphatases (Orthophosporic-monoester phosphohydrolase, EC 3.1.3.2) are ubiquitous in a broad variety of animals, plants and microorganisms. In plants, they are abundant in storage organs such as seeds and tubers and distributed in different parts of the plants- bulbs, roots, stems, leaves, fruits aleurone layer coleoptiles and pollen (Biswas *et al*., 1991; Ferreira *et al*., 1998; Zhang *et al*., 2000; Shinano *et al*., 2001; Cirkovic, 2002.).

Acid phosphatases are involved in the metabolic processes of germination and maturation of plants and are constitutively expressed in seeds during germination, and their activities increase with germination to release the reserve materials for the growing embryo (Thomas, 1993). Some acid phosphatases could be involved in protein dephosphorylations and therefore in signalling pathways (Duff *et al.* 1994).

Ginger, a herb, belongs to the family- *Zingiberaceae* is native to Asia, is a common cooking spice and can be found in a variety of foods and drinks. It is also used as a medicine in Asian, Indian, and Arabic herbal traditions since ancient times and has been used to help treat arthritis, colic, diarrhoea, common cold, flu-like symptoms, headaches, painful menstrual periods and heart conditions.

Although the functional significance of phosphatases is well known in prokaryotes and animals, it may as well be applicable for plants. Extensive studies have been carried out on this enzyme from plant storage organs such as seeds and tubers, cotyledons, roots, leaves, fruits. However, not much work has been carried out on phosphatases of rhizomes for understanding their role. The present study, therefore, focuses on the partial characterization of acid phosphatase of ginger rhizome.

MATERIALS AND METHODS

Fresh ginger root (*Zingiber officinale*) was procured from local market, washed with distilled water several times and then stored in an air tight container placed in the refrigerator at 5^{0}C.

Preparation of ginger extract

Ginger extract was prepared by grinding small pieces of 10g of ginger with acid washed sand in a mortar and pestle with ice cold 100ml of 0.1M citrate buffer, pH 5.6 at 5^{0}C using tissue to buffer ratio of 1:10. The extract was then centrifuged at 12,000×g for 15 min at 4^{0}C.

Assay of Acid phosphatase (EC 3.1.3.2)

Acid phosphatase activity was determined according to the method of Naganna *et al.,* (1955) using β-glycero phosphate as the substrate.

To 5ml of buffered substrate (0.1M sodium *β-glycerophosphate in 0.1M sodium citrate buffer, pH 5.6) preincubated for 5 min at 37*^{0}C, 1ml of enzyme extract was added and the reaction was continued for 30min and then terminated by adding 2.5 ml of 10%TCA. The contents were filtered in cold condition and the amount of inorganic phosphate liberated was estimated by the modified method of Fiske and Subbarow (1925). In the controls, TCA was added prior to the addition of the extract. One unit of acid phosphatase activity was expressed as µmoles of phosphate liberated for 30 min under the experimental conditions.

Purification of Acid phosphatase

Acid phosphatase from the ginger rhizomes has been isolated and purified following the procedure described in our previous paper (Amrutha *et al*., 2015).

Protein estimation

Protein was estimated by the method of Lowry *et al*., (1951) using bovine serum albumin as the standard.

Molecular Weight by SDS-PAGE

SDS-PAGE was carried out by the method of Laemmli (1970) in slab gels. After electrophoretic run, proteins were fixed in glacial acetic acid 10% (v/v), methanol 30% (v/v) and were visualized using coomassie brilliant blue.

pH and Temperature stability of acid phosphatase

To determine the pH stability of acid phosphatase, enzyme (2ml in 0.1M buffer) was kept at 5^{0}C for 12h, aliquots were then taken and assayed for enzyme activity. Buffers used were sodium acetate, pH (3-4), sodium citrate pH (5-6) and Tris- HCl (pH 7-9).

To determine the temperature stability of acid phosphatase, enzyme (2ml in 0.1M buffer) was incubated for 30min at different temperatures (20-100^{0}C). After cooling for 2h, enzyme activities were determined from the aliquots.

Effect of substrate on acid phosphatase activity

Acid phosphatase activity was determined at different concentrations of sodium β-glycerophosphate (0-02-.0.14 M). Km value was calculated using Lineweaver- Burk plot.

Substrate specificity

Substrate specificity of acid phosphatase was determined by incubating enzyme with various phosphorylated substrates-tetrasodium pyrophosphate, disodium phenyl phosphate, ATP, NADP, Glucose 1-phosphate, glucose 6-phosphate, Fructose-6-phosphate, fructose1,6 bisphosphate, phoshoenol pyruate (10mM) at 37 °C in 0.1M citrate buffer, pH 5.6 for 30 min. After incubation, TCA (10%) was added and inorganic phosphate in the filtrate was estimated quickly and at low temperatures.

Effect of denaturants on acid phosphatase activity

In order to determine the effect denaturants on acid phosphatase activity, the enzyme solutions were preincubated for 6h with the denaturants (urea, guanidine hydrochloride and SDS, each 0.1% at 5°C and then assayed for enzyme activity.

Effect of metals on acid phosphatase activity

To determine the effect of metal ions on acid phosphatase activity, enzyme solution was preincubated for 30 min with metal chlorides (final concentration 0.01M) at 5°C and assayed for acid phosphatase activity.

RESULTS AND DISCUSSION

Acid phosphatase from the ginger was purified following conventional methods of protein purification. Recoveries and relative purification at each step for a typical purification from 20 g of ginger is shown in Table-1. By this procedure, about 25 mg of the enzyme was obtained and the final yield of enzyme was about 27%.

When subjected to SDS-PAGE, acid phosphatase showed single band on 10% slab gels. From the plot of distance migrated in cm versus log molecular weight for standard proteins (Fig-1), the molecular weight of acid phosphatase was determined to be 46kDa.

pH and temperature stability

The stability of the purified acid phosphatase was tested at different pH and temperatures and the results obtained are shown in Fig. 2 and 3. The enzyme was found to be more stable around pH 5-9 and it was less stable at acidic pH (3-4) and the alkaline pH (10-11).

The acid phosphatase was stable up to 50°C and at 60°C there was a loss of about 33% of its activity. The enzyme was completely inactive when exposed to 90°C for 30min.

Table -1: Summary of purification of ginger acid phosphatase (20 g of ginger)

Preparation	Volume ml	Total protein mg	Total activity units	Specific activity units/mg protein	Yield%	Fold purification
Crude extract	200	530	112.9	0.21	100	1
60%Ammonium Sulphate	50	150	82	0.54	72.63	2.57
DEAE-Cellulose	20	45	50	1.11	44.28	5.28
Sephadex G-100	15	25	30.5	1.22	27.01	5.80

*Yield and fold purification were calculated on the basis of total activity units and specific activity units respectively.

Figure- 1: Molecular weight determination of ginger acid phosphatase by SDS-PAGE

Plot of distance migrated against log molecular weight of standard proteins (♦) and ginger acid phosphatase(■).

Standard proteins: Phosphorylase b , 97 kDa, Bovine serum albumin, 67 kDa, Ovalbumin, 44kDa, Chymotrypsinogen A, 25 kDa, Soyabean trypsin inhibitor, 20.1 kDa, Lysozyme,14kDa

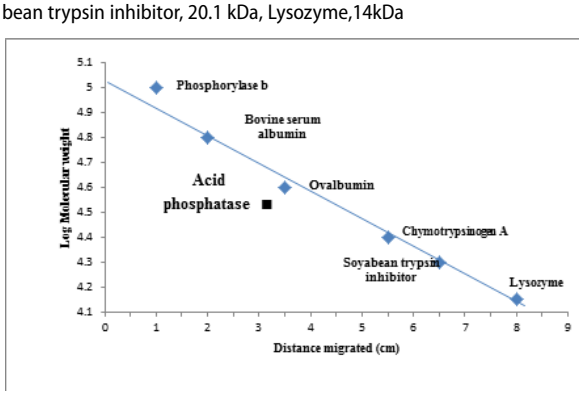


Figure-2: Stability of the purified acid phosphatase at different pH

Purified acid phosphatase (2 ml) was incubated for 12 h at 4°C in an appropriate buffer (10mM). Aliquots of the enzyme were diluted with sodium citrate buffer,0.1M, pH 5.6 and assayed for acid phosphatase activity using sodium β-glycerophosphate as the substrate. Inorganic phosphate values are expressed as µmoles of phosphate liberated per mg protein.

Buffers used were sodium acetate, 0.1M, pH (3-4), sodium citrate, 0.1M, pH (5-6) and Tris- HCl 0.1M, (pH 7-9).

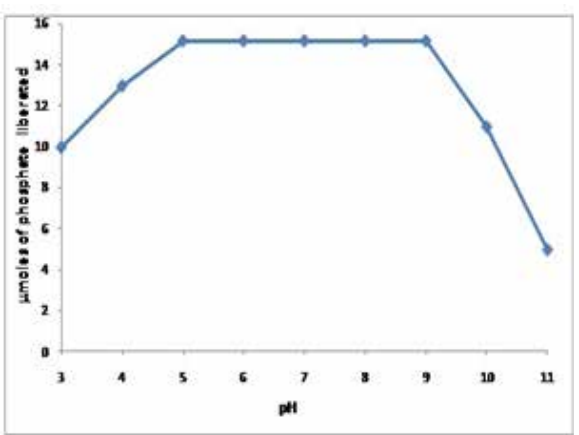
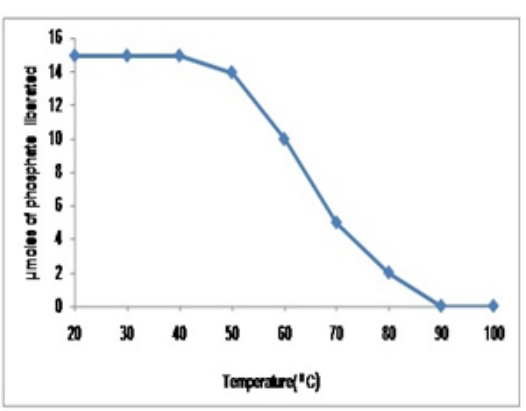


Figure-3: Stability of the purified acid phosphatase at different temperatures

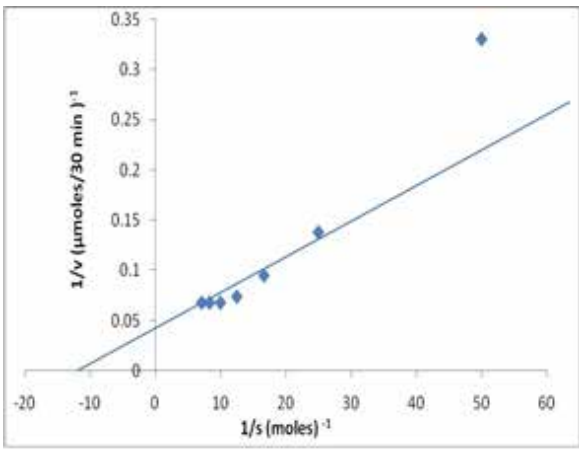


Effect of substrate on acid phosphatase activity

Acid phosphatase activity was determined varying its substrate concentration from 0.02 -0.16M. The results obtained are presented in the form of Line weaver- Burk plot (Fig-4). The Km value for acid phosphatase was determined to be 0.083M from the double reciprocal plot.

Figure-4: Effect of substrate concentration on acid phosphatase activity

0.1 ml of acid phosphatase was added to buffered substrate (5.9 ml) containing different concentrations of sodium β-glycerophosphate (0.02- 0.14 M) and incubated for 30 min at 37^{0}C and the reaction was terminated by the addition of 2.5 ml of 10% TCA. Inorganic phosphate was estimated in the filtrate by the colorimetric method.



Purified acid phosphatase (2 ml) in sodium citrate buffer, 0.1M, pH 5.6 was incubated for 30 min at different temperatures (20-100 ^{0}C), then kept at 4 ^{0}C for 2h, before enzyme activity was determined at 37 ^{0}C according to the method described.

A number of phosphorylated compounds were tested as substrates for acid phosphatase and the results are shown in Table-2. When compared to sodium β-glycerophosphate as the substrate, the enzyme caused hydrolysis of glucose-1-phosphate, glucose 6-phosphate, fructose 6- phosphate, fructose 1,6 -bisphosphate to an extent of 15%, 10%, 8% and 10% respectively. ATP, PEP, Tetrasodium pyrophosphate and NADP were hydrolysed to the extent of 260%, 180%, 75% and 65% respectively by the enzyme.

Table-2: Substrate specificity of purified ginger acid phosphatase

Substrate	Acid phosphatase activity(%)
Sodium β-glycerophosphate	100
Tetra sodium pyrophosphate	75.80
Glucose 1-phosphate	15.20
Glucose 6-phosphate	10.36
Fructose 6- phosphate	8.58
Fructose 1,6 bis phosphate	10.20
ATP	260.12
PEP	180.36
NADP	65.42

Acid phosphatase was incubated with various phosphorylated substrates (10mM) in sodium citrate buffer, 0.1M, pH 5.6 at 37 0C for 30 min and the released inorganic phosphate was determined.

Stability of acid phosphatase towards denaturants

Acid phosphatase activity was unaffected when it was exposed to 0.1% urea and guanidine hydrochloride (Table-3). It was, however, inactivated to an extent of 50% when exposed to SDS (0.1%). The denaturants at 4M concentration completely inactivated the enzyme.

Effect of metals on acid phosphatase activity

The effect of various metal ions on acid phosphatase activity at pH 5.6 is shown in Table-4. Of various metal ions tested, Zn^{2+} inhibited the enzyme to an extent of 60% of its activity. The rest of the metals tested were ineffective either in activating or inhibiting the enzyme.

Table-3: Effect of denaturants on ginger acid phosphatase

Denaturant	Activity (Units / mg)
Control	14.62
Urea (0.1%)	14.04
Guanidine hydrochloride (0.1%)	13.70
SDS (0.1%)	7.25

Acid phosphatase was preincubated at 37 ^{0}C for 30 min with the denaturants (0.1%) and then the enzyme activity was assayed.

Table-4: Effect of metal ions on ginger acid phosphatase

Acid phosphatase was preincubated with various metal chlorides (each 10mM) at 37 ^{0}C for 30 min and then the enzyme activity was assayed.

Metals	µmoles of phosphate liberated
Control	14.74
Na^+	14.12
K^+	14.20
Mg^{2+}	13.96
Ca^{2+}	14.50
Ba^{2+}	14.34
Zn^{2+}	6.45
Fe^{3+}	14.24
Cu^{2+}	14.52
Mn^{2+}	14.25

Purified acid phosphatase was found to be stable from pH 5-9 and temperatures up to 50^{0}C. However, it lost its activity completely at 90^{0}C when incubated for a period of 30 min possibly due to thermal denaturation. Presence of sodium β- glycerophosphate as substrate under these conditions could not protect the enzyme from thermal denaturation. Acid phosphatases from peanut seedlings, soybean seeds, black gram seedlings, chick pea seeds showed maximal activity at temperatures 50-60^{0}C (Gonnety *et al.,* 2006, Ferreira *et al.,* 1998; Ullah & Gibson,1988; Kaur *et al.,* 2011).

The Km value of acid phosphatase as determined from Line weaver-Burk plot was found to be 0.083M for sodium β-glycerophosphate. Acid phosphatase from ginger is unique in that it preferentially used sodium β-glycerophosphate as substrate over p-nitrophenyl phosphate and disodium phenyl phosphate. This is unusual since majority of acid phosphatases prefer p-nitrophenyl phosphate as the substrate.

Acid phosphatase from ginger showed broad substrate specificity. It liberated inorganic phosphate from ATP, PEP, tetrasodium pyrophosphate and NADP to an extent of 260%, 180%, 75% and 65% when compared with sodium β-glycerophosphate as the substrate. It, however, caused less hydrolysis of other phosphorylated compounds tested

Of the metals tested, only Zn^{2+} at 10mM concentration inhibited the enzyme to an extent of 60%. It is well known that Zn^{2+} causes the inhibition of acid phosphatase from the seeds of *Vigna sinensis*, *Lagenaria siceraria* and chick pea (Biswas *et al.,* 1996; Koffi *et al.,* 2010; Kaur *et al.,* 2011).

The enzyme had lost 50% of its activity when exposed to 0.1% SDS for 30 min while urea and guanidine hydrochloride were without any effect on the enzyme activity. It has been reported that acid phos-

phatase activity is inhibited when exposed to SDS from pea nut seeds, *Lagenaria siceraria* seeds (Gonnety *et al.,* 2006; Koffi *et al.,* 2010). When the concentration of SDS was increased to higher level (4M), the enzyme activity was totally abolished.

Acid phosphatase of the ginger rhizome is a monomeric protein with a molecular weight 46kDa, active at pH 5.6, temperature 37-50^{0}C preferentially using sodium β-glycerophosphate as the substrate and significantly inhibited by SDS and Zn^{2+}.

With its broad substrate specificity, the enzyme is well suited to supply inorganic phosphate to cellular metabolism operating in the rhizome of ginger.

CONCLUSION

Acid phosphatase was found to be more stable around p^H 5-9 and stable up to 50^{0}C. The Km value of acid phosphatase was found to be 0.083M calculated from Line weaver- Burk plot and showed broad substrate specificity. Of the metals tested, only Zn^{2+} at 10mM concentration inhibited the enzyme to an extent of 60%. The activity of the enzyme was unaffected when it was exposed to 0.1% urea and guanidine hydrochloride.

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