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SOME PHYTOCHEMICAL AND ENZYMATIC STUDIES ON SEEDS OF WILD OATS AND COMMON LAMBSQUARTERS ASSOCIATED WITH WHEAT CROP

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ABSTRACT

Acid phosphatases (EC 3.1.3.2) catalyze the hydrolysis of various phosphomonoesters at pH below 6.0. These enzymes from two sources, Avena fatua (wild oats) and Chnopodium album (common lambsquarters) seeds were partially purified by chromatography on DEAE-Cellulose and Sephadex G-100 columns to make an appropriate comparison of their properties and characteristics. Both enzymes had obtained specific activity in range of 460-1000 units/mg of protein with approximately 20-40 fold purification and had overall recovery of about 60 %. Both enzymes were found fully stable at pH 5-6 and 20-37^{o} C. Both enzymes showed pH optima of 5.6, but the temperature optima of 55^{o} C for C. album seeds and 60^{o} C for A. factua seeds were obtained. The K_m of enzymes against substrate, p.nitrophenyl phosphate was 0.3mM and 0.23mM, respectively. Substrate specificity indicated that p.nitrophenyl phosphate, phenyl phosphate, ATP and sodium pyrophosphate were found good substrates. Kinetic parameters suggested that the enzyme from C. album was more active than that of A. fatua. Phytochemical analysis of weed seed extracts was carried out. Alkaloids, saponins, glycosides, terpenoids, steroids, flavonoids and tannins were detected. Tannins and flavinoids were in high concentration while alkaloids and saponins were present in low concentrations. Flavonoids are water soluble polyphenolic molecules thought to provide antioxidant effects and these plants may be used to protect oxidative damage of biomolecules in human body.

Key words: Acid phosphatases, alkaloids and tennins*, Avena fatua,* characterization*, Chenopodium album*, Purification.

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seeds of wild oats and common lambsquarters associated with wheat crop. Pak. J. Weed Sci. Res. 22(2): 211-226.

INTRODUCTION

There are more than 30 weed species in the wheat fields of Pakistan (Qureshi and Bhatti, 2001; Siddiqui and Bajwa, 2001). Among these, *Avena fatua* and *Chenopodium album* are found to be the most frequently occurring weeds. They reduce the wheat grain yield by 30-60% in the fields. There is a dire need of herbicides to control the weeds of wheat (Bibi *et al*., 2008; Cheema *et al*., 2006; Usman *et al*., 2010). Other species like *Phalaris minor, Vicia sativa, Galium aparine, Rumex dentatus, Lathyrus aphaca, Carthamus oxycantha, Convolvulus arvensis* and *Euphorbia sp.* in association with wheat crop plant have also ethno-botanical importance. These weeds have been extensively used in herbal medicine and also offering feeding, sheltering, refuge and breeding niches to beneficial arthropods. Their seeds dormancy and irregular germination throughout the growing season are the most important features contributing to the persistence of weeds.

Avena fatua is very dangerous weed grown in wheat, oat and barley crops which lowers the quality of these crops through competing for their resources. Various diseases and pests such as fruit fly and smut are associated with this self growing weed. *A. fatua* seeds are nerve tonic, stimulant and laxative (Hussain *et al.,* 2006).

Chenopodium album grows very fast in wheat fields. It is commonly used for food and medicinal values (Yadav *et al.,* 2007). Its leaves are cooked like spinach. Its black seeds are rich in proteins, alkali and alkaline earth metal ions together with some vitamins. It is used for cough and abdominal pain. *Chenopodium album* has antipruritic and antinociceptic activities (Yadav *et al.,* 2007). The literature survey (Abbas *et al.,* 2012) revealed that varieties of natural products are present in seeds of two plants namely *C. album* (dicot) and *A. fatua* (monocot). These secondary metabolites act as natural antioxidants which prevent free radical oxidation of macromolecules in cells and have special pharmacological activities (Walton and Brown, 1998; Quiroga *et al.,* 2001).

Seeds are the source of several enzymes (Olczak *et al.,* 1997; Kaneko *et al.,* 1990). During the germination of seeds, mostly enzymes are activated and mobilize the store food reserves through their catalytic activities. The acid phosphatase is one of these enzymes which releases inorganic phosphate from reserved phosphate organic compounds in response to phosphate deficiency (Duff *et al.,* 1994), salt stress (Pan, 1987) and water deficit (Barrett-Lennard *et al.,* 1982). In seeds and seedlings, increased acid phosphatase activity

either due to protein synthesis or by imbibition, catalyzes the hydrolysis of many phosphate esters of sugars and phosphorylated compounds to release inorganic phosphate for growth and development (Akiyama and Suzuki, 1981; Hoehamer *et al.,* 2005) while in roots, increased secretion of extracellular acid phosphatases act on insoluble organic phosphate compounds in soils to convert into soluble inorganic phosphate to be taken up by plants (Panara *et al.,* 1990) in growing stage of seeds.

Acid phosphatases (EC 3.1.3.2) catalyzing the hydrolysis of various phosphomonoesters at pH below 6.0, have been extensively studied in leaves, roots and seeds (Tejera-Garcia *et al.,* 2004). Similarly, some studies on the purification and characterization of acid phosphatase enzymes have also been done on the plant seedlings (Gonnety *et al.,* 2004; Tabaldi *et al.,* 2008).

The present study deals with isolation of acid phosphatase enzyme from seeds of weeds viz. *A.fatua* (*wild oat),* and *Chenopodium album (*common lambsquarters) and its biochemical properties. In addition to this, phytochemical constituents present in these seeds are also investigated qualitatively and quantitatively.

MATERIALS AND METHODS

Seeds of wheat weeds *A. fatua* and *C.album* were obtained from Ayub Agriculture Research Institute, Faisalabad Pakistan.

Enzyme Assays

The acid phosphatase activity was estimated according to the method of Nadir *et al.* (2012). The reaction mixture consisted of 950µl of 4mM substrate in 0.1M acetate buffer pH 5.5 and 50µl of the enzyme. After incubation for 5 min at 37^{o} C, the reaction was stopped by the addition of 4 ml 0.1M KOH. The yellow color obtained, was measured at 405 nm. One unit of enzyme was defined as the amount of enzyme which catalyses the hydrolysis of 1 nano mole of substrate p.nitrophenyl phosphate under above assay conditions. The molar extinction coefficient of 17600 $M^{-1}cm^{-1}$ was used and specific activity as units per mg of protein.

To determine pH optima, acid phosphatase activities were assayed at various pH values from 4 to 6.2. The pH range was covered with citrate buffer. Similarly, the activities were determined at different temperatures ranging from 35 to 70^{o} C to study optimum temperature.

The pH stability of the enzyme was studied in 0.1M citrate buffer pH range 4 to 6.2. After 1h pre-incubation at 37^{o} C, aliquots were taken and residual enzyme activities were assayed as described above.

The kinetic constants such as Michaelis-Menten constant (K_m), maximal velocity constant (V_{max}) and specificity constant (V_{max}/ K_m) were determined at pH 5.6 at 37° C. The rate of hydrolysis of p.nitrophenyl phosphate was measured under standard assay procedure. These kinetic constants were estimated from Lineweaver-Burk plot using substrate concentrations ranging from 0.05- 4 mM p.nitrophenyl phosphate. In substrate specificity study, the activities against other phosphorylated compounds were estimated by determination of released inorganic phosphate according to Black and Jones method (1983).

Thermal inactivation of each enzyme was determined at 37° C and at its optimal temperature (55°C for *C. album* and 60°C for *A. factual*). The separate enzyme solutions at pH 5.6 were pre-incubated at each temperature for upto100 min. The small portions were taken out at interval of 10 min and the remaining activity in each case was assayed as usual. Thermal denaturation study was carried out by pre-incubating each enzyme at temperatures from 35° C to 80° C. for about 10 min. The remaining enzyme activities were estimated as per routine procedure described above.

Protein determination

Method of Lowry *et al.* (1951) was used to estimate protein concentration. To 1ml of a protein solution, 0.5ml of 1N NaOH solution was added and mixture was shaken with vortex mixer. After 30 min, 5ml of a mixture containing 100 volumes of 2% Na_2CO_3, 1 volume of 2% sodium tartrate and 1 volume of 1% $CuSO_4$, was added. After 15 min 0.5 ml diluted Folin-Ciocalteu reagent (1/1, v: v) was added and the content was mixed vigorously. After 30 min the absorption was measured at 720 nm. The amount of protein was calculated from the standard curve using bovine serum albumin as standard protein, while protein concentrations of the column effluent fractions were estimated by measuring their absorption at 280 nm.

Preparation of extracts and partial purifications

Acid phosphatases were purified from two sources by a procedure that was slightly modified as described by Baig *et al*. (1986). The seeds were crushed in powder form and the crude enzyme was extracted by shaking the weed seed powder in 0.01 M Tris-HCl buffer pH 7.0 (1 g powder/10 ml buffer) for 1 h. It was then centrifuged at 4000-5000 rpm (Rotor JA-14) for 30 min. The clear supernatant, thus obtained was used for partial purification of the enzyme. The supernatant was dialysed against above buffer with several times replacement of new buffer until the ionic strength of dialysate was the same as that of buffer. The dialysate was centrifuged at same speed to remove precipitated protein. The 25 ml of supernatant sample was applied to DEAE-Cellulose column (2x20 cm).

The column was eluted buffer. Five ml/10 min each fraction was collected. When all unbound protein was washed out from the column, a linear sodium chloride salt gradient from 0 to 0.5 M (100 ml buffer +100 ml NaCl in buffer) was applied with same speed. The fractions representing the enzyme peak were pooled, concentrated to few ml by ultrafiltration and applied on to Sephadex G-100 column (1.8x 60 cm). The column was eluted with Tris buffer at flow rate 40 ml/h and 5 ml fractions were collected. The active fractions were combined and stored for further study.

Preparation of alcoholic extracts.

The seeds were ground to powder and suspended (20g) in 100 ml of 80 % methanol. It was left for one week to complete the extraction process. Every day the mixture was vigorously mixed and then it was filtered through Whatman filter paper No.1. The filtrate was concentrated under vacuum distillation until dry mass is obtained. To perform tests, 0.1 g dry mass was suspended in 10 ml of 10% dimethyl suloxide.

Phytochemical screening

Following chemical tests were carried out for qualitative determination of constituents on the methanolic extract using standard procedures.

Tannins

A volume of 0.5 ml of extract was mixed with water and heated. To it, few drops of 1% $FeCl_3$ were added. The appearance of a blue color indicated the tannins (Trease and Evans, 1989).

Saponins

Up to 0.5ml of extract was mixed with water and shaken vigorously. Formation of persistant froth indicated the saponins (Kumar *et al*., 2009).

Flavonoids

To 0.5ml of extracts, 1 ml of sodium hydroxide was added. Yellow color was formed which becomes colorless on the addition of dilute acid, indicated the presence of flavonoids. (Roopashree *et al.,* 2008).

Steriods

To 0.5ml of extract, few drops of H_2SO_4 were added followed by adding 1ml of acetic anhydride. The appearance of blue-green coloration indicated the presence of steroids (Kokate, 1994).

Terpenoids

To 0.5ml of extract, few drops of chloroform were added and then conc.H_2SO_4 was run down the sides of test tube slowly so that two layers are formed. The formation of a reddish brown ring at the junction of the two layers indicated the presence of terpenoids (Evans, 1997).

Glycosides

To 0.5ml of extract containing glacial acetic acid, few drops of $FeCl_3$ were added and then conc.H_2SO_4 was run down the sides of test tube slowly. The appearance of a purple ring at the junction of the two layers indicated the presence of glycosides (Sofowara, 1984).

Alkaloids

The extract was heated with 2% H_2SO_4. On cooling, Dragendroff's reagent was added orange red precipitation was observed, showing the presence of alkaloids (Sofowora, 1982).

Quantitative determination of the chemical constituents

Quantitative determination of alkaloids, tannins and saponins was done by using methods as described by Harborne (1973), Van-Burden and Robinson (1981), Obadoni and Ochuko (2002), respectively. For these tests, fat free seed powder was prepared. Two g of seed powder were defatted with 100 ml of diethyl ether using a soxhlet apparatus for 2 h. For flavonoids determination, a method of Bohm and Kocipai-Abyazan (1994) was employed. Ten grams of the seed sample was extracted with 100 ml of 80 % aqueous methanol and solution was filtered through Whatman filter paper No 42. The filtrate was evaporated to dryness and weighed to a constant weight.

RESULTS AND DISCUSSION

Acid phosphatases from two sources viz. *A.fatua and C. album* seeds were partially purified by chromatography on DEAE-Cellulose and Sephadex G-100 columns to make an appropriate comparison of their properties and characteristics.

Purification was obtained from DEAE-Cellulose column. The enzyme was completely bound to the top of the column while unwanted proteins were washed out with equilibration buffer. The acid phosphatase activity was eluted with linear gradient between 0.05M-0.25 M NaCl in the same buffer. Two to three times purification of the enzyme was achieved with 75-82 % yield with respect to starting material loaded the column in all two cases. The active fractions of anion exchange chromatography were subjected to Sephadex G-100 column chromatography. The acid phosphatase enzyme came out as one peak. Ten to thirteen times more purification was achieved from Sephadex G-100 chromatography. The specific activity was increased from 46 to 460 U/mg in case of *A.fatua* while specific activity was increased from 78.3 to 999 U/mg in case of *C.album* (Table-1). The typical patterns of chromatography are shown in Fig. 1. The final preparations had specific activity of 460-999 U/mg of protein with approximately 23-37 fold purification (S.A of the final step/ S.A of the initial step during purification) and had overall yields about 60 % (with respect to starting materials).

Catalytic properties of the enzyme

At 37°C, acid phosphatases from both sources presented stability over pH from 5 to 6 conserving at least more than 90 % of total activities and seemed to be stable at 4°. No activity loss could be detected even after incubation at 20° C for 24h. Both enzymes showed pH optima of 5.6 (Fig. 2). The temperature optima of 55° C for *Chenopodium album* seeds and 60° C for *A.fatua* seeds were obtained (Fig. 3).The bell shaped curves were seen in all cases. The thermal inactivation experiments indicated that at 37° C, the enzymes from both sources were found stable for 100 min (Fig. 4) but at their optimal temperatures, enzymes from *C. album* and *A.fatua* seeds were less stable with half lives of 46 and 63 min, respectively. The thermal denaturation showed that enzymes retained full activities at their optimal temperatures. Above these optima, their enzymatic activities decreased quickly as the temperatures increased (Fig.5). The enzymes appeared to be completely denatured at 80° C. The kinetic parameters of the two enzymes were studied using p.nitrophenyl phosphate (Table-2). The K_m values were found in range of 0.2-0.3mM. The activity of acid phosphatases toward various substrates is shown in Table-3.The p.nitrophenyl phosphate, phenyl phosphate, ATP and sodium pyrophosphate were found good substrates. Among natural substrates, ATP had high specificity. In general, these enzymes hydrolyse large number of substrates and thus have broad specificity for substrates. Kinetic parameters suggested that the enzyme from *C. album* was more active than that of *A.fatua.*

The phytochemicals analyses of the plant seeds (Table-4) showed that the seeds contained flavinoids, saponins, tennins, steroids, glycosides, alkaloids and terpenoids. But quantitative determinations of these constituents (see in Materials and Methods) indicate that these were found rich in alkaloids, flavonoids and tannins (Table-5). Both seeds contained highest percentage of tannins (16-18%) followed by flavinoids (4-8%), alkaloids (2%) and saponins (less than 1%). The flavonoids as an important class secondary metabolites prevent free radical oxidation of molecules in the body (Cho *et al.,* 2004). So *A. fatua* and *C. album* may be used to prevent oxidative stress. Flavoanoids also possess many other activities such as anti-inflammatory and antiviral activities (Bbosa, 2010). Glycosides are reported to decrease the blood pressure (Nyarko and Addy, 1990). Phenolic compounds such as gallic, catechin have got much importance for their significance antioxidant properties, prevent oxidative damage and for the perfection of health toward various diseases (Li *et al*., 2013). Suchandra *et al.* (2007) also reported the phenolic compounds in *M. fragrans* which have great potential to scavenge DPPH free radicals and inhibit lipid peroxidation. Tannins are

polycyclic aromatic compounds and constitute active component of seeds cotyledon. These possess different anti-diarrheal activities (Enzo, 2007). Saponins act as anti-nutrient in seeds possess cholesterol lowering activity and deleterious properties (Akubugwo *et al*., 2007). Terpenoids have pharmaceutical applications such as artemisinin and taxol medicines are used for malaria (Goto *et al*., 2010). Steroidal compounds have great importance as sex hormones (Okwu, 2001). Similarly, alkaloids have got pharmacological importance as enzyme inhibitors and growth terminators (Nazrullacv *et al.,* 2001).

CONCLUSION

For the first time, partial purification of acid phosphatases from two sources, *A. fatua and C. album* seeds was reported. The enzymes were purified to specific activity of 460-1000 U/mg of protein with 20-40 fold purification and had overall recovery of about 60 %. Both plants contained relatively high concentrations of tannins and flavinoids while alkaloids and saponins were present in low concentrations. The antioxidant activity may be expected due to the presence of higher phenolic contents. Thus these plants may be used to prevent oxidative stress and their bioactive phytochemical constituents may be considered on medicinal merit.

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Table-1. Purification steps of Avena fatua and Chenopodium album acid phosphatases.

Purification steps	*Avena factua*			*Chenopodium album*		
	S.A (U/mg)	P.F	Act.Rec. (%)	S.A (U/mg)	P.F	Act.Rec. (%)
Crude extract	20	1	100	27	1	100
DEAE-Cellulose chromatography	46	2.3	82	78.3	2.9	75
Sephadex G-100 chromatography	460	23	63	999	37	57

S.A. specific activity; P.F. purification factor; Act. Rec. recovery

Table-2. Kinetic parameters of enzymes from two sources towards p.nitrophenyl phosphate

	Avena fatua	*Chenopodium album*
K_m	0.23 mM	0.3 mM
V_{max}	510 nmol.sec^{-1}mg^{-1}	1023nmol.sec^{-1}mg^{-1}
V_{max}/ K_m	2217	3410

Table-3. Substrate specificity of acid phosphatases from *Avena fatua and Chenopodium album*

Substrates	(% Activity) *Avena fatua*	(% Activity) *Chenopodium album*
pNPP	100	100
Phenyl phosphate	92	97
Glucose-1-phsphate	2	10
Glucose-6-phsphate	12	14
Fructose-1-phsphate	4	8
Fructose-6-phsphate	15	18
Sodium pyrophosphate	67	75
Adeosine tri phosphate	112	128
Sodium phytate	22	31

The enzyme activity was expressed as a percent of that of the same enzyme towards p.nitrophenyl phosphate as 100

Table-4. Qualitative phytochemical constituents of weeds seeds

Weed plants	Flavinoids	Saponins	Tannins	Steroids	Glycosides	Alkaloids	Terpenoids
Avena fatua	+	+	+	-	+	+	+
Chenopodium album	+	+	+	-	+	+	+

Table-5. Quantitative (percent) phytochemical constituents of seeds of weed plants

Weed plants	Alkaloids	Saponins	Flavinoids	Tannins
Avena fatua	1.91	0.73	4.1	17.93
Chenopodium album	2.23	0.83	8.7	16.14

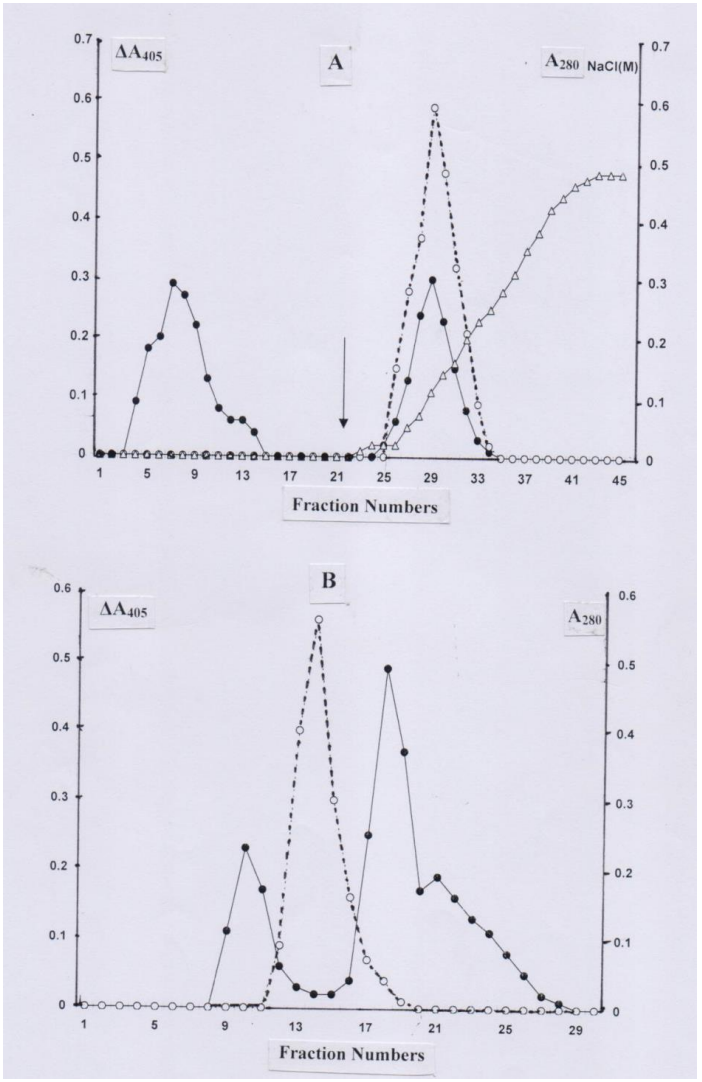

Figure 1 (A). DEAE-Cellulose chromatography

25 ml sample was applied and fractions of 5 ml were collected with flow rate of 30 ml/h. The arrow indicates the start of linear gradient. **(B).** Sephadex G-100 chromatography.
The column was eluted with buffer at flow rate of 10 ml/15 min and 5 ml fractions were collected. Ordinates: protein at 280 nm (•—•); acid phosphatase activity, ΔA_{405} (o···o) and NaCl linear gradient (Δ—Δ)

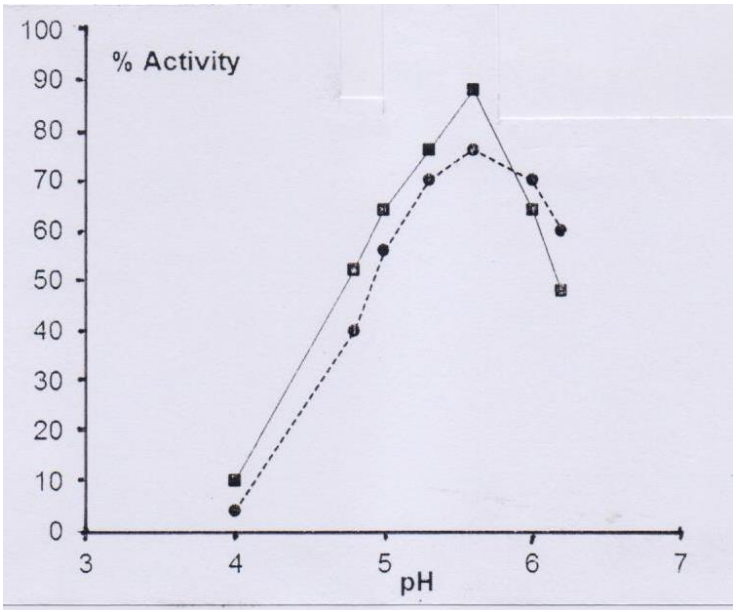

Figure 2 pH optima of acid phosphatases from *A. factua* and *C. album, A. factua* (**...●...**); *C. album* (□■□).

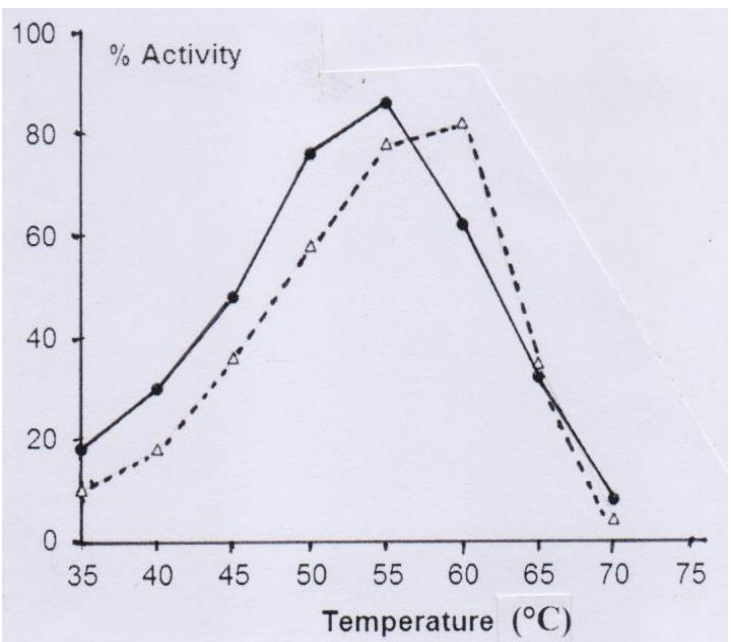

Figure 3. Temperature optima of acid phosphatases from *A. factua* and *C. album, A.* (**...Δ...**); *C. album* (□●□).

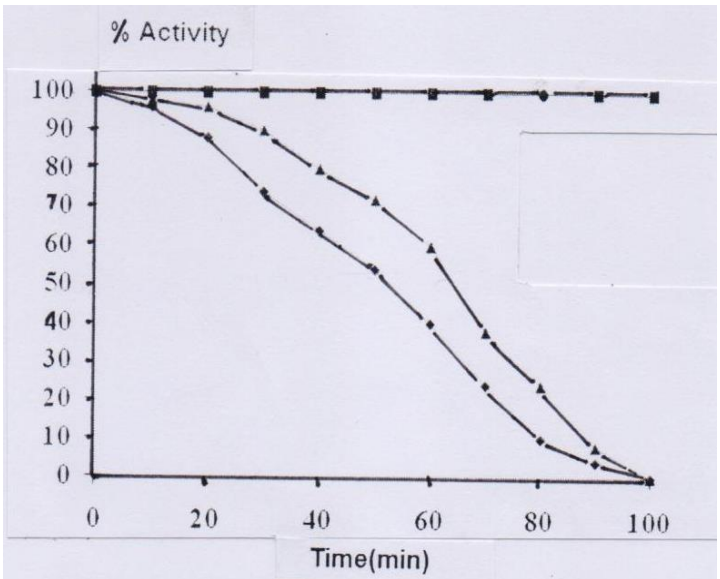

Figure 4. Thermal inactivation of acid phosphatases from *A. fatua* and *C. album. A. fatua* (□♦□) 37°C; *C. album* (□■□) 37°C. *A. fatua* (□▲ □) 60°C; *C. album* (□♦□) 55°C.

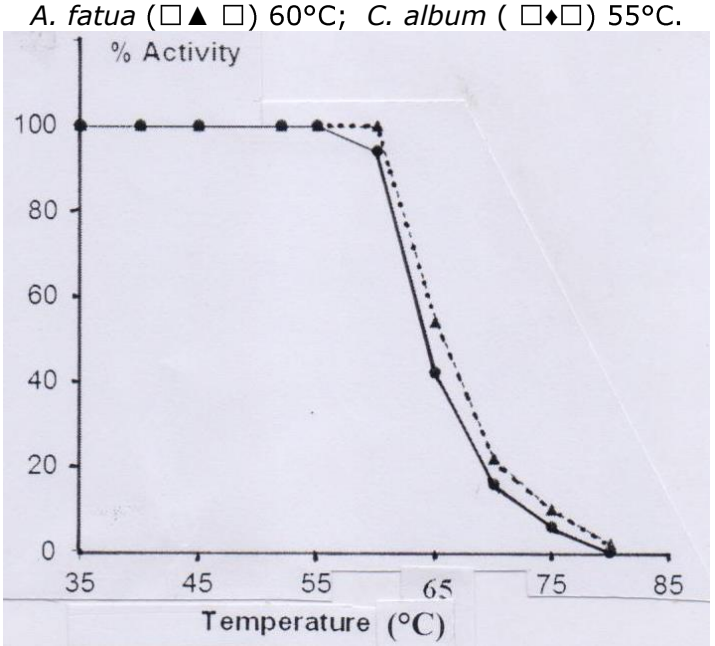

Figure 5. Thermal denaturation of acid phosphatases from *A. fatua* and *C. album. A. fatua* (**...▲...**); *C. album* (□●□)

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