Isolation, Purification and Characterization of Acid Phosphatase from Germinating *Vigna radiata* Seeds.

[1]SADIA NADIR, [2]ASMA SAEED, [3]RUBINA NAZ, [3]AISHA SIDDIQUA, [3]MEHRIN SHERAZI, [4]SULTAN MEHMOOD WAZIR AND [1]AHMAD SAEED*
[1]*Department of Chemistry, University of Science and Technology, Bannu 28100, Pakistan.*
[2]*Department of Biological Sciences, Gomal University, Dera Ismail Khan 29050, Pakistan.*
[3]*Department of Chemistry, Gomal University, Dera Ismail Khan 29050, Pakistan.*
[4]*Department of Botany, University of Science and Technology, Bannu 28100, Pakistan.*
asaeedti@hotmail.com*

(Received on 25th October 2011, accepted in revised form 9th M arch 2012)

Summary: The acid phosphatase (EC 3.1.3.2) has been purified from germinating seeds of *vigna radiata* through ammonium sulphate precipitation, DEAE-Cellulose chromatography and concanavalin A-Sepharose 4B chromatography. The specific activity of 1291 nkat.mg^{-1}of protein was obtained with recovery of nearly 1%. About 222 times purification was achieved. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) resolved two bands of acid phosphatase corresponding to molecular weight of 29 kilo Dalton (kDa) and 18 kDa. The molecular weights of native enzyme determined by gel filtration on calibrated Sephadex G-100 column were found to be 29 kDa and 18 kDa The apparent K_m value of 29 kDa isoenzyme with p-nitrophenyl phosphate (pNPP) as substrate was 0.3 mM and V_{max} was 1336 nmol.sec^{-1}.mg^{-1} of protein. The optimal pH for this enzyme was 5.5 and pH stability was 4-7. It had optimum temperature of 50^{o}C and temperature stability was 0- 50^{o}C. The enzyme hydrolysed various phosphorylated compounds non-specifically. It was competitively inhibited by phosphate, vanadate while fluoride showed non-competitive inhibition and molybdate exhibited an inhibition of mixed type. It was found insensitive to tartrate and concluded that this enzyme was recognized as tartrate resistant acid phosphatase.

Keywords: Acid phosphatase; *Vigna radiata*; purification; characterization; seedlings; isoenzyme;

Introduction

Rainfed area crops including maize, millet and sorghum are the important cereal crops in Pakistan. These are cultivated in Khyber Pakhtunkhwa province of Pakistan where these form an important part of the diet of the rural population. In a study on rainfed area crop seeds for the determining the suitability of its diet, a number of seeds was found to contain different types of acid phosphatase activity [1,2]. The function of phosphatases is not clearly established but it is possibily involved in the mobilization of phosphate reserves of the seeds. Most of the phosphate in a seed exists as inositol hexaphosphate (phytic acid) and the concentration of free phosphate is very low to limit metabolic processes, the enzyme must be present to catalyse the hydrolysis of reserved phosphate organic compounds. Acid phosphatases are supposed to be involved in the hydrolysis of these compounds to release inorganic phosphate and appear to function in response to phosphate deficiency [3]. Acid phosphatases also appear to function in response to salt stress [4] and water deficit [5]. Moreover, changes in specific isozymes of phosphatase under phosphate deficiency are also involved [6-8]. Some studies have demonstrated that in germinating seeds, phosphate deficiency can cause an increase in extacellular phosphatase activity either due to *de novo* synthesis of enzyme protein or activation of the enzyme by imbibition. This seems to be involved in the hydrolysis of macromolecular organic phosphates in soil to release soluble inorganic phosphates which are then absorbed by the plant through root [9].

Acid phosphatases (EC. 3.1.3.2) are group of enzymes that catalyse the hydrolysis of many phosphate monoesters and show pH optima on acid side pH 5-6 [10]. These enzymes are ubiquitous in nature and widely distributed in plants and animals. Plant acid phosphatases are present in various plant tissues and cell organellae. These often occur in multiple forms and can differ in molecular weight, substrate specificity, sensitivity to inhibitors and some kinetic properties [11]. Acid phosphatases have been examined in leaves [12], roots [9,11], seeds [1,13,14] and tubers 15,16].

Several attempts on the purification and characterization of acid phosphatase enzymes in seeds were made. Acid phosphatases from sunflower seeds [17], wheat germ [18, 19], soybean [20], caster bean seeds [14] and yellow lupin seeds [1] have been purified and characterized. Similarly, much studies have been done on the plant seedlings. Tso and Chen [21] reported for the existence of four groups of isoenzymes and for the isolation of group III isoenzyme from rice seedlings. Acid phosphatase

*To whom all correspondence should be addressed.

isoenzymes purified from cotton seedlings [22], garlic seedlings [23], common baen seedlings [24] and peanut seedlings [25] were also reported. Recently, the study on the presence of multiple acid phosphatase activity in seedlings of cucumber, radish and rocket salad [26] was undertaken.

The present work deals with the purification of acid phosphatase isoenzyme from germinating seeds of *vigna radiata* and its characterization with respect to kinetic parameters, pH dependence, optmum temperature, thermal stability, substrate specificity, molecular weight determination and the effect of metal ions and other substances on its activity

Results and Discussion

Acid Phosphatase Activity During Germination of Vigna radiata

Vagna radiata seeds were germinated in aired room at ambient temperature. Maximum specific acid phosphatase activity (6 nkat.mg^{-1} of protein) was obtained on the 4th day of germination (Table-1). Onward this day, the specific activity of acid phosphatase decreased gradually during the germination. The activity was about half of maximum specific activity (3.2 nkat.mg^{-1} of protein) on the 7th day of its germination. This result indicates that increase in activity of acid phosphatase is dependent on germination. Similar results have been obtained in peanut seedlings [25] and germinating maize seeds [27].

Table-1: Expression of acid phosphatase activities during vigna radiata seed germination.

Days	Activity (nkat / ml)	Protein concentration (mg / ml)	Specific activity (nkat.mg^{-1})
0	10.855	5.6	1.938
1	11.189	5.8	1.928

Enzyme Purification

Acid phosphatase was purified 222-fold from the seedlings of 3-4 days old *v. radiata*. The purification steps are presented in Table-2 and their elution profiles of chromatography are shown in Fig. 1. The enzyme was obtained with specific activity of 1291 nkat.mg^{-1} of protein and recovery of 0.7%. There was a great decrease in the total activity during ammonium sulphate precipitation at 70% saturation level and DEAE-Cellulose chromatography steps resulting in a very low recovery of enzyme. The enzyme might be glycoprotein as this was bound to Con-A column and was eluted with α-D-methyl mannoside.

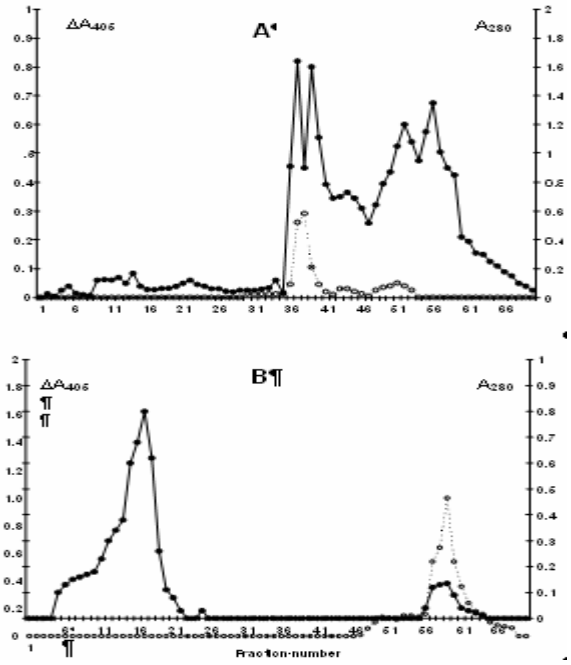


Fig. 1: Purification of acid phosphatase from germinating *v.radiata* seeds.
(A) Ion-exchange chromatography on DEAE Cellulose.
(B) Affinity chromatography on Con-A Sepharose 4B column. Ordinates: Protein at 280nm ((•—•); acid phosphatase activity, ΔA_{405}(o····o).

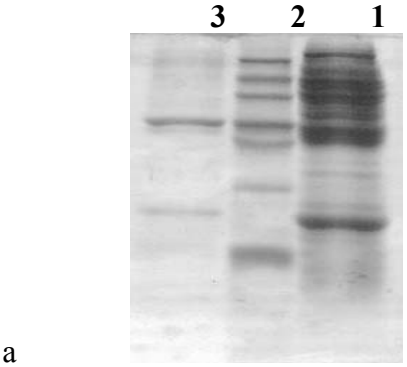
The homogeneity of the enzyme was checked on SDS- polyacrylamide gel eletrophoresis (12%). Two bands were detected and their molecular weight were estimated to be 29 kDa and 18 kDa (Fig. 2a, b).The gel filtration experiment of crude extract salted out from ammonium sulphate (30-60% saturation) showed one peak of acid phosphatase (V_e, 126 ml) but slight shoulder on the descending part of the peak (V_e, 135 ml) was observed. Their elution profile is shown in Fig. 3a and the apparent molecular weight of these two enzymes obtained on calibrated Sephadex G-100 column were estimated to be 29 kDa and 18 kDa (Fig. 3b) which supports that acid phosphatase from *v. radiata* has two isoenzymes with molecular weight of 29 and 18 kDa. Two isoenzymes were separated from each other by reverse-phase HPLC of purified enzyme preparation. Two peaks A and B in the ratio of 85 and 15% are shown in Fig. 4. When homogeneity of peak A was checked on SDS-PAGE, single band corresponding to 29 kDa was observed (Fig. 5a). The band of peak B corresponding to 18 kDa was also detected on SDS-PAGE (Fig. 5b). The most of the characterization was carried out with peak A.

Table-2: Various steps involved in purification of acid phosphatase from 130 g of seedlings of *v.radiata.*

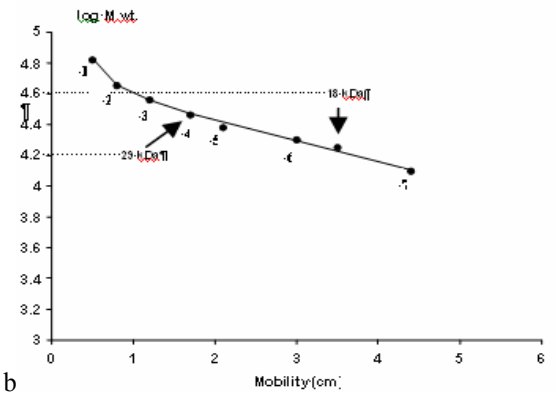
Steps	Vol. (ml)	T.Act. (nkat)	T.Prot[a]. (mg)	S.A (nkat.mg^{-1})	P.F.	Rec.%
Extract	**925**	**20700**	**3561**	**5.81**	**1**	**100**
Ammonium sulphate(30%saturation)	**935**	**19050**	**3500**	**5.44**	**0.94**	**92.0**
Ammonium sulphate(70%saturation)	**35**	**7310**	**306**	**23.89**	**4.11**	**35.3**
DEAE – Cellulose Chromatography (after pool, concentration & dialysis)	**12**	**473**	**6.5**	**72.77**	**12.52**	**2.3**
Con A- Sepharose 4B Chromatography	**4**	**155**	**0.12**	**1291.67**	**222.32**	**0.75**

Vol. volume; T.Act. total activity; Total prot. Total protein; S.A. specific activity; P.F. purification factor; Rec. recovery.

[a]Protein concentration was determined by Lowry method.



a



b

Fig. 2: Gel elecrophoresis and molecular weight estimation.

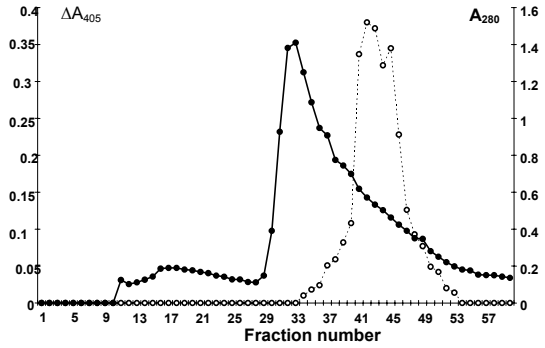
(a) SDS-polyacrylamide gel electrophoresis of acid phosphatases from germinating *v.radiata* seeds.

Lane 1 5µl non-bound protein peak sample from Con A column.

Lane 2 Protein markers used from top to bottom are (1) Albumin (66 kDa), (2)ovalbumin (45 kDa), (3)glycerolaldehyde-3-phosphate dehydrogenase (36 kDa), (4) carbonic anhydrase (29 kDa), (5) trypsinogen (24 kDa), (6) trypsin inhibitor (20.1 KDa) and (7)lactalbumin (14.2 kDa). Lane 3 5µl of acid phosphatase isoenzymes peak from mannoside elution.

(b)Linear graph of log molecular weight versus mobility of stained bands of proteins markers 1 to 7 as mentioned in Fig.2a.

Arrows indicate the molecular weight of isoenzymes



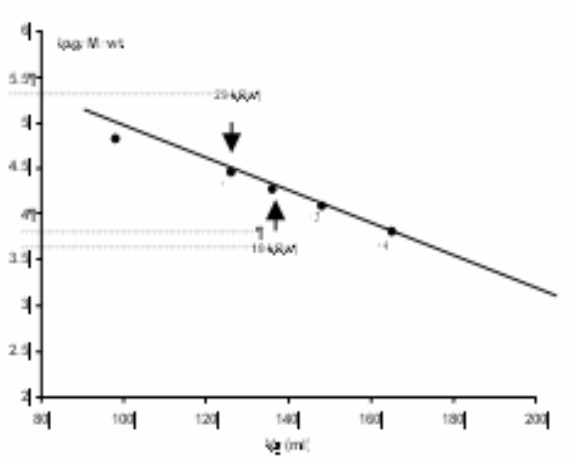


Fig. 3: Estimation of molecular weight of acid phosphatases on Sephadex **G-**100 column.

(a) Eluton profile from Sephadex G-100. Ordinates: Protein at 280nm ((•—•); acid phosphatase activity ΔA_{405}(o····o).

(b) Linear graph of log molecular weight versus eluton volumes of standard proteins.

The standard proteins are (1) Bovine serum albumin (M_r 66,000), V_e 98ml; (2) Carbonic anhydrase (M_r 29,000), V_e 126ml; (3) Cytochrome c (M_r 12,400), V_e 148ml; (4) Aprotinin (M_r 6,500). Arrows show the molecular weight of isoenzymes

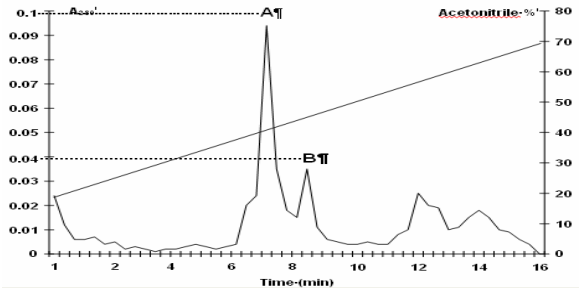


Fig. 4: Separation of acid phosphatase isoenzymes in reverse-phase chromatography**.**

Flow rate, 1ml/min; Back pressure, 420 psi; Absobance at 280 nm

(—); Elution gradient (—)· Peaks A and B are two isoenzymes.

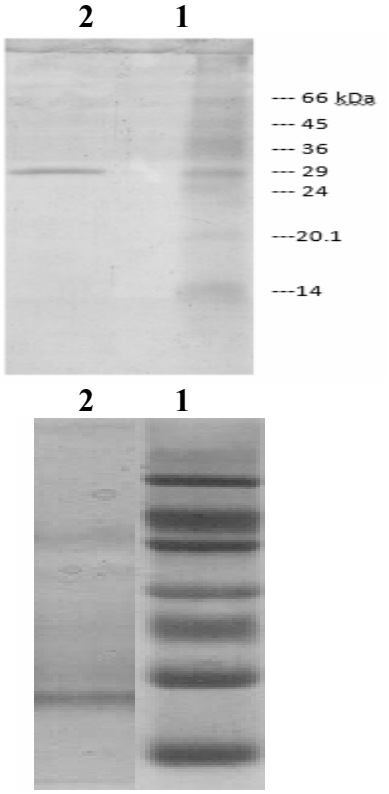


Fig. 5: Gel elecrophoresis of isoenzymes.

SDS-polyacrylamide gel electrophoresis of 29 kDa acid phosphatase

(Peak A) obtained from HPLC Lane 1 Protein markers as mentioned in Fig.2a.

Lane 2 10µl of isoenzyme (Peak A).

(a) SDS-polyacrylamide gel electrophoresis of 18 kDa acid phosphatase

(Peak B) obtained from HPLC.

Lane 1 Protein markers as mentioned in Fig.2a

Lane 2 10µl of isoenzyme (Peak B).

Kinetic Properties of an Acid Phosphatase from Vigna radiata

The 29 k Da acid phosphatase from seedling had optimum pH at 5.5 (Fig. 6) which was consistent with other pH optima 5.0-6.0 [20,28,29]. The pH stability was found between pH 4 and 7 (Fig. 7). The enzyme had optimum temperature of 50°C (Fig. 8) and had temperature stability around 50°C (Fig. 9). At 50°C, the activity loss was 3-5% after preincubation of 1 h and 95-97% of the activity was retained. But at 60, 70 and 80°C, the t½ values were 55 min, 3.5 min and ≤ 3 min., respectively (Fig. 10). This attitude seems to be a general character of plant acid phosphatases. The K_m obtained was 0.3 mM and V_{max} was 1336 nmol.sec^{-1}.mg^{-1} of protein.

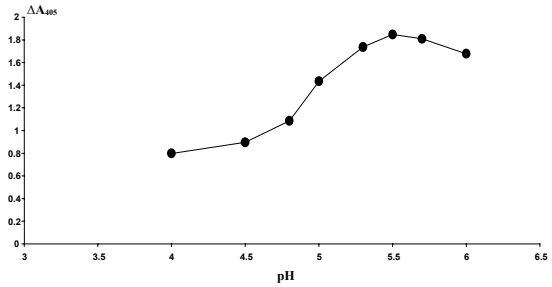


Fig. 6: Effect of pH on the acid phosphatase activity.

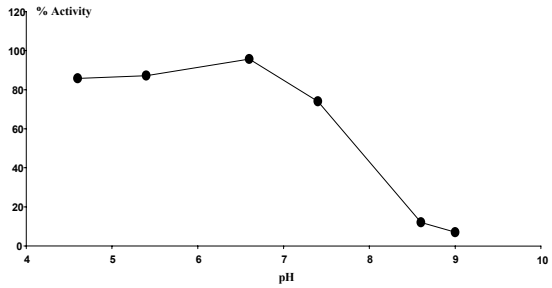


Fig. 7: pH stability of acid phosphatase.

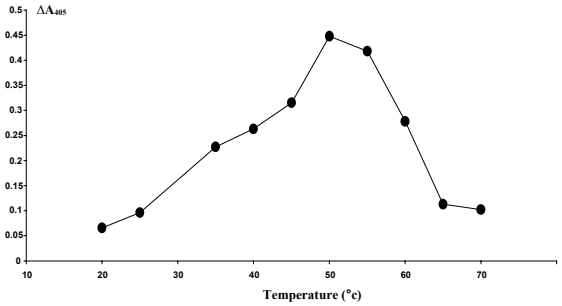


Fig. 8: Optimum temperature of acid phosphatase**.**

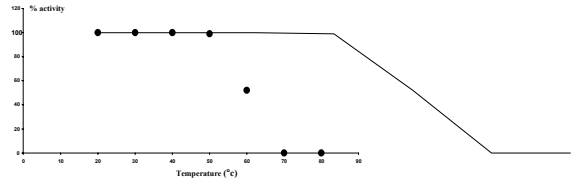


Fig. 9: Temperature stability of acid phosphatase.

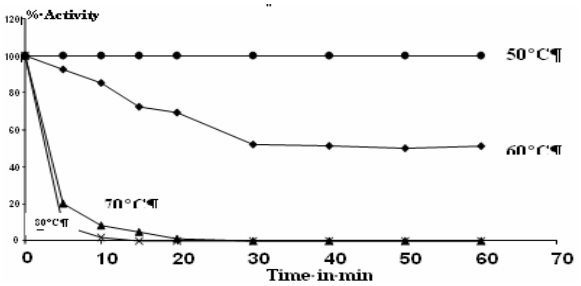


Fig. 10: Thermal inactivation of acid phosphatase.

The hydrolysis rate of some synthetic and natural substrate is reported in Table-3. The enzyme hydrolyzed all phosphate monoesters to various extents indicating that *v. radiata* acid phosphatase is non-specific enzyme. However pNPP, phenylphosphate, α-naphthyl phosphate and pyrophosphate seemed to be good substrates. Marked activity was observed with phosphotyrosine, ATP, α and β glycerophosphate and glucose-6-phosphate. Other substrates e.g phospho-amino acids and sugar phosphates were also hydrolyzed but at a slower rate. No activity towards ribulose-5-phosphate and FMN was detected. Thus the enzyme showed broad substrate specificity and it hydrolyzed aryl phosphates much more efficiently than the aliphatic phosphates. This non- specificity of enzyme is similar to those of other plant acid phosphatases [20, 30].

Table-3: Substrate specificity of *V. radiata* acid phosphatase

Substrates	% activity
pNPP	100
Phenyl phopsphate	90
α-glycerophosphate	44
β-glycerophosphate	45
α-naphthyl phosphate	103
β-naphthyl phosphate	19
FMN	0
Ribulose-5-phosphate	0
Glucose-1-phosphate	25
Glucose-6-phosphate	44
Fructose-1-phosphate	22
Phosphothreonine	30
Phosphoserine	18
Phosphotyrosine	74
AMP	37
ATP	72
Sodium pyrophosphate	108

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

Effect of metal ions, inhibitors and modifier substances on the enzyme activity.

Large number of metal ions was tested for their effects on the acid phosphatase activity (Table 4). The enzyme was not affected by Ca^{+2}, Mg^{+2}, Ba^{+2}, Mn^{+2} and Co^{+2} in the reaction mixture, indicating that divalent cations are not required for the catalytic activity. A similar effect of these ions was reported for acid phosphatases isolated from axes and cotyledons of germinating soybeans [2]. The Zn^{+2}, Hg^{+2}, Cu^{+2}, Al^{+2}, + and Pb^{+2} strongly inhibited the enzyme. The Cu^{+2} and Hg^{+2} were found the most effective metal cation inhibitors, while Zn^{+2} was lesser effective. Zn^{+2} ion inhibited the enzyme non-competitively and K_i value was 16 mM, while Hg^{+2} was found a competitive inhibitor of acid phosphatase with K_i value of 0.03 mM (Fig. 11).

Table-4: Effect of metal ions on acid phosphatase of germinating seeds of *v. radiata*

Metal ions	Concentration	Acid phosphatase of seedlings (% Activity)
H_2O		100
NaCl	5 mM	99
KCl	5 mM	110
$CaCl_2$	5 mM	107
$MgCl_2$	5mM	95
$BaCl_2$	5 mM	92
$MnCl_2$	5m M	100
$CoCl_2$	5 mM	101
$CdCl_2$	5 mM	82
$ZnCl_2$	5 mM	18
$HgCl_2$	5 mM	0
Cu_2SO_4	5 mM	0
$AlCl_3$	5 mM	30
$Pb(CH_3COO)_2$	5 mM	56

The results were expressed as relative percentage of activity respect to the control
reaction without ion added.

The most notable anion inhibitors were phosphate, fluoride, vanadate and molybdate (Table-5 and Fig. 11). The acid phosphatase was inhibited competitively by inorganic phosphate, the reaction product with a K_i value of 3.5 mM. The enzyme was also inhibited competitively by orthvanadate (K_i =11.5μM). Fluoride inhibited non-competitively (K_i = 0.6mM) wheras molybdate ion showed a very strong inhibition of mixed type (K_i =2.9μM). These values are with the range typical for other plant acid phosphatases. Two known acid phosphatase inhibitors, tartrate and citrate did not inhibit this enzyme. There was no change in the enzyme activity in the presence of EDTA. However, the enzyme was slightly activated by non-ionic detergents like Triton X-100 and Tween-20 (see table-5). Sheker *et al*. [31] have also reported the same effect of Triton X-100 on the lysophosphatidic acid phosphatase activity from developing peanut cotyledons. The *v. radiata* acid phosphatase did not catalyse the transfer of phosphate group to phosphate acceptors such as glycerol,

methanol and ethanol, during the hydrolytic reaction of pNPP. Hence no activation of acid phosphatase enzyme was observed with these modifiers, reflecting a no phosphotransferase activity . The effect of some reducing agents on the activity of acid phosphatase is shown in Table-6. The was no actvation by β-mercaptoethanol. It is suggested that this acid phosphatase is not a thiol enzyme [30]. This effect was supprted by determining the activity with DTT. The phosphatase activity was unaffected with DTT which indicates that histidine residue is not the part of the active site and free –SH groups are not necessary for catalysis. Moreover, sulfhydryl reagents such as *p*-chloro-mercuribenzoate and iodoacetate produced no loss of enzyme. This also suggests that a free –SH group is not required for the enzyme function.

Table-5: Effect of anions on acid phosphatase of germinating seeds of *v. radiata*

Anions	Concentration	Acid phosphatase of seedlings (% Activity)
H_2O		100
Na_3PO_4	5 mM	93
	10mM	85
	20 mM	73
NaF	5 mM	8
	10 mM	0
Na_2VO_3	0.3 mM	20
Na_3MoO_4	0.1mM	12
	0.5 mM	0
Tartrate	5 mM	100
	10 mM	99
Citrate	5 m M	100
EDTA	10 mM	100
Triton X-100	0.5%	114
Tween-20	0.5%	100
Ascorbic acid	5 mM	99
Na NO_3	0.5 mM	94
Na_2CO_3	5 mM	92
Na_2SO_4		
$NaHCO_3$	5 mM	104
Methyl	5 mM	103
alcohol	0.5%	102
Ethyl	0.5%	104
alcohol	0.5%	100
Glycerol		

As expressed in table 4.

Table-6: Effect of some reducing agents on acid phosphatase of germinating seeds of *v. radiata*

Reducing agents	Concentration	Acid phosphatase of seedlings (% Activity)
H_2O		100
β-mercaptoethanol	5 mM	100
	10mM	100
Dithiothreitol	5ul	103
	10ul	103
p-Hydroxymercuri-benzoate	0.05 mM	96
Iodoacetate	0.5 mM	98
Phenyl-methyl sulphonyl fluoride	5 mM	93

Percentage expressed as above tables

During the germination, synthesis or activation of enzymes takes place which normally break down macromolecular organic reserves in seeds into simpler molecules. Acid phosphatase is one of the enzymes which plays an important role in the germination of seeds. Acid phosphatases are constitutively expressed in seeds during germination and hence their activities increased with germination to release phosphates for the growing embryo [32]. In the present study, the specific acid phosphatase activity increased and reached the maximum on the fourth day of *v. radiata* seed germination at temperature between 28 -35°C. Gonnety *et al*. [25] reported that maximum specific acid phosphatase activity of peanut seedlings was detected on the 5th day of germination at temperature below 25°C. Similarly, Prazeres *et al*. [33] showed that the maximum activity of soybean seedlings was appeared on the 6th day of germination at temperature of 28°C. Hegeman and Grabau [34] also determined that 3-9 days were the mean time for germination of peanut seedlings regarding acid phosphatase activity. In our case, seedlings of 2-5 days of age could be the reasonable time considered for rapid growth in relation to acid phosphatase activity (Table-1) but we used 3-4 days of seedlings regarding their size for the purification. The enzyme was purified by ammonium sulphate fractionation, ion-exchange chromatography and affinity chromatography from *v. radiata* seedlings. A 222 - folds purification was resulted with specific activity of 1291 nkat.mg^{-1} of protein. SDS-PAGE exhibited two bands of protein with molecular weight of 29 kDa and 18 kDa and by gel filtration, the native enzyme had 29 kDa and 18 kDa, indicating the existence of two forms of acid phosphatase in *v. radiata* seedlings as reported for multiple forms of acid phosphatases from many other sources [32,35,-36]. Reverse phase HPLC was used in the last stage of purification. Since acetonitrile caused some denaturation of the enzyme, the only 20 % of the activity was lost in case of of 29 kDa enzyme while more than 70 % activity was decreased in 18 kDa enzyme, therefore, HPLC step was not included in the purification scheme. However, the major protein peak (A) obtained from HPLC was found to be homogeneous on SDS- PAGE and moved as a single protein band of molecular weight of 29 k Da. Molecular weight of 29 k Da of major acid phosphatase isoenzyme secreted by maize endosperm culture have also been reported [37]. Similarly one isoenzyme of 18 kDa was also purified from *hypericum perforatum* [38]. Gonnety *et al.*[25)] also isolated three isoenzymes with molecular weights of 22, 24 and 25 k Da from peanut seedling. Generally the molecular weight found for acid phosphatases from other sources were between 13 kDa and 100 kDa [39] but most of the enzymes that had molecular weight ranging from 50 k Da to 60 k Da, were found glycoprotein [1,15,35].

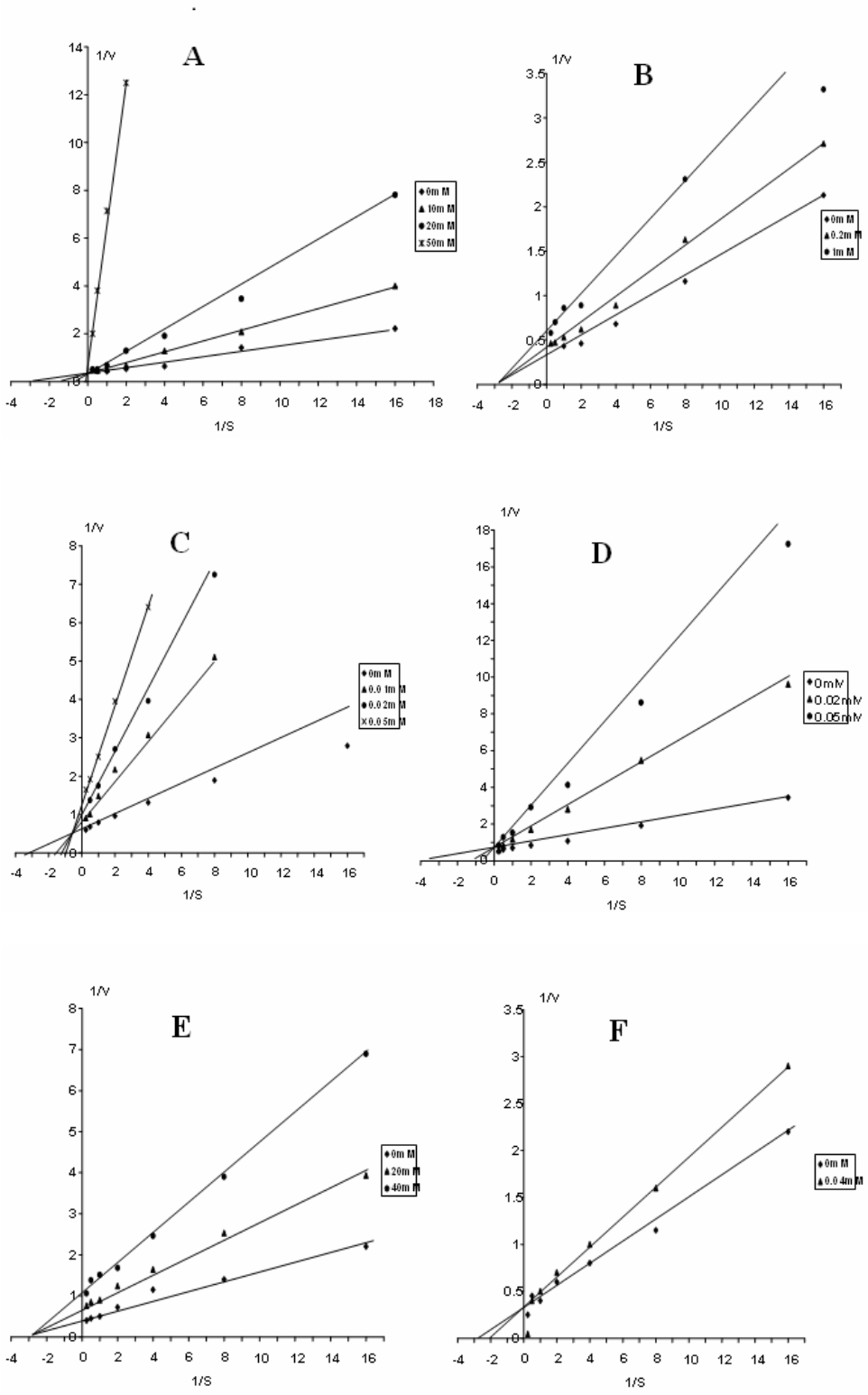


Fig. 11: Competitive inhibition of 29 kDa acid phosphatase from germinating *v.radiata* seeds. Lineweaver-Burk plots of 1/v versus 1/S; A. phosphate; B. sodium Fluoride; C. molybdate; D. orthovanadate; E. $ZnCl_2$; F. $HgCl_2$.

The kinetic study showed a linear Lineweaver-Burk plot with a K_m of 0.3 mM for pNPP. This value was very similar to that of acid phosphatases of yellow lipin seeds[1)], group III isoenzyme of rice plant [21] and soyabean seeds [2]. It was also consistent with the K_m value of the barley root acid phosphatases which was reported to be pH dependent and at pH 5.5, it was about 0.3 mM [40].

Acid phosphatase from seedlings of *v. radiata* like other plant acid phosphatases was competitively inhibited by inorganic phosphate [1,2,13,41]. It indicates that feed back inhibition of acid phosphatases by inorganic phosphate may represent a general form of cellular regulation of these enzymes [1,3]. The enzyme was also inhibited non-competitively by fluoride while molybdate showed mixed type inhibition. Similar results have been reported in acid phosphatases isolated from axes and cotyledons of germinating of soybeans [2]. Orthovanadate, an inhibitor of acid phosphatases [26] also inhibited this enzyme competitively (K_i =11.5μM). The molybdate was four times more powerful inhibitor (K_i =2.9μM) than vanadate. The same results was obtained in purple acid phosphatase from tobacco plants [42]. Similarly, Zn^{+2} was found non-competitive inhibitor of this enzyme. Non-competitive inhibition was also observed in acid phosphatses purified from seedlings of soybeans [2], but the enzyme was found insensitive to tartrate inhibition. This tartrate resistance was also found in many other plant acid phosphatases[1,41,43-45].

Experimental

Chemicals

Sephadex G-100, p-nitrophenyl phosphate, α-naphthyl phosphate, β-glycerol phosphate, β-naphthyl phosphate, flavinmononucleotide phosphate, phenyl phosphate, bovine serum albumin, Concanavalin A-Sepharose 4B, SDS molecular weight protein markers were purchased from Merck, Sigma Chemical Co. & Fluka Chemical Co., DEAE-Cellulose was obtained from Whatman Biosystem., The chemicals for polyacrylamide gel-electrophoresis were supplied by Acros Chemical Co., while all other chemicals were of highest purity analytical grade.

Plant Material

Seeds of *vigna radiata* were sterilized with 1% (v/v) of sodium hypochloride solution for 10 min, washed with water three times and soaked in water for 3-4 h. After hydration, germination of seeds was performed on moist sand trays during at least 8 days in a room at ambient temperature of 28-35°C. The germinating seeds were daily watered.

Extraction of the Enzyme

Seedlings (whole plants) were collected every day after germination during the period of 8 days. It was then ground with a mortar and pestle in 0.1M acetate buffer (pH 5.5) at the rate of 1g whole plant /5ml buffer and the homogenate was centrifuged at 45,000 x g for 5-10 min. The supernatant collected, was used for enzyme assay and protein concentration.

Enzyme Assay

The acid phosphatase activity was determined at 37°C in 1 ml of 0.01M acetate buffer (pH 5.5) and 4 mM pNPP as described by Panara *et al*. [9].

The reaction was started by the addition of small amount of enzyme solution and was terminated after 5 min by the addition of 4 ml of 0.1N KOH. The p-nitrophenol ions, yellow colour produced was measured spectrophotometrically at 405 nm (ε18000 $M^{-1}cm^{-1}$). The control was prepared in the same way but enzyme solution was added to the mixture after KOH. One kat of acid phosphatase activity was defined as the amount of enzyme required to produce 1 mol of p-nitrophenol /sec. The specific activity was expressed as $nkat.mg^{-1}$ of protein.

The enzyme activity for number of substrates was determined under above conditions by estimation of librated inorganic phosphate as the result of hydrolysis. For inorganic phosphate determination, the enzyme reaction was stopped by the addition of 0.2 ml of trichloroacetic acid (10%) and the colour reaction was carried out by following Black and Jones method [46].

The effect of metal ions and some other substances on the enzyme activity was determined as described by Panara [47].

pH and Temperature Optima

The effect of pH on the enzyme activity was determined by measuring the hydrolysis of pNPP at 37°C in a series of buffers at various pH values ranging from 3.6 to 9.0. Buffers used were 0.1 M sodium acetate buffer from pH 3.6 to 5.6, cacodylate buffer (0.1M) from pH 5.0 to 7.4 and Tris-HCl buffer (0.1M) from pH 7.5 to 9.0.

The effect of temperature on acid phosphatase activity was performed in 0.1M acetate

buffer (pH 5.5) over a temperature range of 25 to 80°C at intervals of 10°C.

pH and Temperature Stability

The pH stability was assessed in pH range from 3.6 to 9.0 in 0.1M buffers. The buffer were the same as in the determination of pH optima. After 24h pre-incubation of the enzyme at 37°C, aliquots were taken and residual activity was measured under standard assay conditions. Temperature stability was assessed by measuring the residual activity after pre-incubating the enzyme in 0.1M acetate buffer (pH 5.5) at temperatures ranging from 0 to 80°C for 1h while thermal inactivation of the enzyme was studied by measuring the residual activity after exposure the enzyme in buffer described above to 50 - 80°C for various intervals of time.

Kinetic Parameters

K_m values were determined by measuring the p-nitrophenol produced at different concentrations of substrate ranging from 0.06 to 4 mM in the absence and presence of two or three fixed concentrations of inhibitors. Lineweaver-Burk plots were used to arrive at the values of K_m and inhibition constants (K_i). Straight lines were drawn by applying least square rule.

Protein Determination

Protein concentration was determination by the Lowry method [48]. For column effluents, the relative protein concentration was estimated from absorbance at 280 nm.

Electrophoresis

SDS-polyacrylamide gel electrophoresis was carried out by the method of Laemmli [49] under reduced conditions. The sample was prepared in sample buffer with β-mercaptoethanol and heated at 95°C for 2-3 min. The enzyme purity was checked in 12% acrylamide mini-slab gel. After the run, the proteins in gel were stained with coomassie blue and molecular weight estimates were made using standard size marker proteins as indicated in the respective figures.

Gel Filtration and Apparent Molecular Weight Determination

Apparent molecular weights of acid phosphatases were estimated on a calibrated Sephadex G-100 column (1.8 x 85 cm) by a comparison of their elution volumes to those of standard proteins. The proteins used were bovine serum albumin, carbonic anhydrase, cytochrome c and aprotinin. Plant extract was salted out with ammonium sulphate (30-60% saturation) and placed on Sephadex G-100 column which was previously equilibrated and eluted with 0.01 M acetate buffer (pH 5.5) containing 1mM β-mercaptoethanol and 0.1 M NaCl at flow rate of 30 ml/h. Fractions (about 3 ml each) were collected for assays of protein and enzymes activities.

Enzyme Purification

All operations were carried out at 4°C. The enzyme was purified by a procedure of Pasqualini *et al.* [35, 50-52] with slight modifications.

The seeds were grown as described above. Seedlings (whole plants) were collected after germination of 3-4 days and homogenized in 0.1M acetate buffer (pH 5.5) at the rate of 1g /5 ml buffer, with mortar and pestle. This was followed by stirring for Ih. The homogenate was centrifuged at 45,000 x g for 1 h. The supernatant was collected while pellet was discarded. To the supernatant solid ammonium sulfate was added to 30 % saturation with gradual addition and constant stirring. After ½ h, the mixture was centrifuged at 10,000 x g for 20 min. The supernatant was then brought to 70% saturation with ammonium sulfate. The resulting mixture was allowed further to stir for ½ h and then centrifuged at 45,000 x g for 20 min. The precipitate, thus obtained was dissolved in reasonable amount of 0.01M Tris-HCl buffer (pH 7.4) containing 1mM EDTA and dialysed against same buffer for 12h. The dialysate was applied to DEAE-Cellulose column (2.8 x 24 cm) previously equilibrated with dialyzing buffer. After washing the column with same buffer, the bound acid phosphatase activity was eluted with linear 0-0.5 M NaCl gradient (300 ml). Fractions (10 ml) were collected at flow rate of 27 ml /h. The most active fractions were pooled and concentrated to 10 ml by ultra-filtration using Amicon YM2 membrane at 45 psi pressure. The enzyme was then dialysed against 0.01 M acetate buffer (pH 5.5) containing 1mM Ca^{+2} and 1mM Mn^{+2}. The dialysed enzyme was loaded onto a Concanavalin A-Sepharose 4B column (1.8 x 14 cm), previously equilibrated with dialyzing buffer and washed with same buffer. The column was then eluted with 10% α (+)-D-methyl mannoside in buffer. Fractions (3 ml) were collected at flow rate of 15 ml /h. The most active fractions eluted with mannoside was pooled, concentrated by ultra-filtration and was used for biochemical and PAGE analysis.

Reverse-phase HPLC

The enzyme preparation obtained from a Concanavalin A-Sepharose 4B chromatography was loaded on Ultra pore RPSC Beckman (C18; 5µm, 4.6x 75 mm) column, previously equilibrated with 0.01M trifluoro-acetic acid (TFA). The enzyme was bound strongly and eluted by applying linear gradient from 20-70% acetonitrile in 0.01M TFA in 15 min.

Conclusions

In this study, two low molecular acd phosphatase isoenzymes of size 29 kDa and 18 kDa from *v. radiata* seedlings have been identified. The 29 kDa isoenzyme has been purified to homogeneity and biochemically characterized. Since the enzyme was found insensitive to tartrate inhibition, it may be recognized as a tartrate resistant acid phosphatase class and also would be useful for adapting organic phosphorus into available inorganic phosphate that involves in many different biological processes.

Acknowledgements

This research was carried out under M.Phil degree program in the department of Chemistry, University of Science and Technology, Bannu, Pakistan in collaboration with Gomal University, Dera Ismail Khan, Pakistan.

References

1. M. Olezak, W. Watorek and B. Morawieka, *Biochimica et Biophysica Acta.*, **134**, 114 (1997).
2. J. Kaneko, M. Kuroiwa, K. Aoki, S. Okuda, Y. Kamio and K. Izaki, *Agriculture Biological Chemistry*, **54**, 745 (1990).
3. S. M. G. Duff, G. Sarath and W. C. Plaxton, *Plant Physiology*, **90**, 791 (1994).
4. S. Pan, *Australian journal of Plant Physiology*, **14**, 117 (1987).
5. E. G. Barrett-Lennard, A. D. Robson and H. Greenway, *Journal of Experimental Botany*, **33**, 682 (1982).
6. P. Coello, *Plant Physiolology*, **116**, 293 (2002).
7. A. Goldstein, A. Danon, D. Baertlein and R. Mc Daniel, *Plant Physiol*ogy, **87**, 716 (1988).
8. M. C. Trull and J. Deikman, *Planta*, **206**, 544 (1998).
9. S. Panara, S. Pasqualini and M. Antonielli, *Biochimica et Biophysica Acta.*, **1037**, 73 (1990).
10. J. B. Vincent, M. W. Crowder and B. A. Averill, *Trends in Biochemical Sciences*. **17**, 105 (1992).
11. A. R. Penheiter, S. M. G. Duff and G. Sarath, *Plant Physiology*, **114**, 597 (1997).
12. P. E. Staswick, C. Papa, J. Huang and Y. Rhee, *Plant Physiology*, 104, 49 (1994).
13. A. H. Ullah and D. M. Gibson, *Archives of Biochemistry and Biophysics*, **260**, 514 (1988).
14. P. A. Granjeiro, C. V. Ferreira, E. M. Taga and H. Aoyama, *Plant Physiology*, **107**, 151 (1999).
15. K. S. Gellatly, G. B. G. Moorehead, S. M. G. Duff, D. D. Lefebvre and W. Plaxton, *Plant Physiology*, **106**, 223 (1994).
16. T. Kusudo,T. Sakaki and K. Inouye, *Biosciences Biotechnololgy and Biochemistry*, **67**, 1609 (2003).
17. H. C. Park and R. L. Van Etten, *Phytochemistry*, **25**, 351 (1986).
18. P. P. Waymark and R. L. Van Etten, *Archives of Biochemistry and Biophysics*, **288**, 621 (1991).
19. Y. Kawasaki, H. Nakano and T. Yamane, *Plant Sciences*, **119**, 67 (1996).
20. C. V. Ferreira, P. A. Granjeiro, E. M. Taga and H. Aoyama, *Plant Physiol*, *Biochem.*, **36**, 487 (1998).
21. S. C. Tso and Y. R. Chen, *Botanical Bulletin Academic Sinica*, **38**, 245 (1997).
22. R. Bhargava and R. C. Sachar, *Phytochemistry*, **26**, 1293 (1987).
23. B. Yenigun and Y. Guvenilir, *Applied Biochemistry and Biotechnology*, **105**, 677 (2003).
24. N. A. Tejera Garcia, M. Olivera, C. Iribarne and C. Lluch , *Plant Physiology and Biochemistry*, **42**, 585 (2004).
25. J. T. Gonnety, S. Niamke, B. M. Faulet, E. J. N. Kouadio and L. P. Kouame, *African Journal of Biotechnology*, **5**, 35 (2006).
26. L. A. Tabaldi, R. Ruppenthal, L. B. Pereira, D. Cargnelutti, J. F. Goncalves, V. M. Morsch and M. R. C. Schetinger, *Ciencia Rural. Santa Maria*, **38**, 650 (2008).
27. R. Senna, V. Simonin, M. A. C. Silva-Neto and E. Fialho, *Physiology and Biochemistry*, **44**, 467 (2006).
28. H. Hass, B. Redl, E. Leitner and E. Stoffler, *Biochimica et Biophysica Acta.*, **1074**, 392 (1991).
29. I. Jagiello, A. Donella-Deana, J. Szczcgielniak, L. A. Pinna and G. Muszynska, *Biochimica et Biophysica Acta.*, **1134**, 129 (1992).
30. S. M. Basha, *Canadian Journal of Botany*, **62**, 385 (1984).
31. S. Shekar, A. W. Tumaney, T. J. Rao and R. Rajasekharan, *Plant Physiology*, **128**, 988 (2002).
32. T. K. Biswas and C. Cundiff, *Phytochemistry*, **30**, 2119 (1991).
33. J. N. Prazeres, C. V. Ferreira and H. Aoyama, *Plant Physiology and Biochemistry*, **42**, 15 (2004).

34. C. E. Hegeman and E. A. Grabau, *Plant Physiol*ogy, **126**, 1598 (2001).
35. S. Pasqualini, F. Panara, L. Ederli, P. Batani and M. Antonielli, *Plant Physiology and Biochemistry*, **35**, 95 (1997).
36. T. Tamura, T. Minamikawa and T. Koshiba , *Journal of Experimental Botany*, **33**,1332 (1982).
37. J. A. Miernyk, *Phytochemistry*, **31**, 2613 (1992).
38. Y. Demir, A. Alayli, S. Yildirim and N, Demir, *International Journal of Agriculture Biolology*, **6**, 1089 (2004).
39. A. Felenbok, *European Journal of Biochemistry*, **17**, 165 (1970).
40. W. N. Arnold, K. H. Sakai and L. C. Mann, *Journal of Genetic Microbiology*, **133**, 1503 (1987).
41. T. D. Cirkovic, M. D. Gavroic-Jankulovic, M. N. Bukilica, L. Mandic, S. Petrovic and R. M. Jankov, *Journal of Serbian Chemical Society*, **67**, 567 (2002).
42. R. Kaida, T. Hayashi and T. S. Kaneko, *Phytochemistry*, **69**, 2546 (2008).
43. A. Rossi, M. S. Palma, F. A. Leone and M. A. Brigliador, *Phytochemistry*, **20**, 1823 (1981).
44. T. M. Ching, T. P. Lin and R. J. Metzger, *Plant physiology*, **84**, 789 (1987).
45. K. Doi, B.L. Antanaitis and P. Aisen, *Structur Bond*, **70**, 1 (1988).
46. M. J. Black and M. E. Jones, *Analytical Biochemistry*, **135**, 233 (1983).
47. F. Panara, *International journal of Biochemistry*, **17**, 1213 (1985).
48. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randal, *Journal of Biological Chemistry*, **193**, 265 (1951).
49. U. K. Laemmli, *Nature* **277**, 680 (1970).
50. S. Pasqualini, F. Panara, P. Bracardi and M. Antonielli, *Phytochemistry*, **31**, 3703 (1992).