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Phosphatase activity of *Poa pratensis* seeds

I. Preliminary studies on acid phosphatase II

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Abstract:

Acid phosphatase (EC 3.1.3.2) was extracted with 0.1 M sodium acetate buffer, pH 5.1 from *Poa pratensis* seeds, and separated into three fractions by chromatography on DEAE cellulose. The highest activity was found in fraction II-b (acid phosphatase II). The activity of the enzyme was optimal at pH 4.9. It hydrolyzed p-nitrophenyl phosphate most readily among the various phosphomonoesters examined. Acid phosphatase II showed also a high activity toward β-naphtyl phosphate and phenyl phosphate, very low activity towards β-glycero phosphate, 5'-GMP and no acitivity with glucose-1 phosphate. The enzyme was inhibited by Ca^{2+} and fluoride, but activated by Mg^{2+}. EDTA had no influence on the activity of the enzyme.

INTRODUCTION

Acid phosphatases (ortophosphoric monoester phosphohydrolase EC 3.1.3.2) exhibit low substrate specificity, and many of them are heterogenous in chromatography and electrophoresis. The genetic basis of heterogeneity of acid phosphatases has been elucidated only for a small group of these enzymes (Ogita 1968; Allen 1968). Chromatographic heterogeneity of acid phosphatase from bean sprouts was demonstrated by Felenbok (1970). Verjee (1969) isolated from wheat germs three chromatographically different phosphatases which differed in substrate specificity, pH optimum and sensitivity to inhibitors. Kubicz and Morawiecka (1970) found three electrophoretically different molecular forms of acid phosphatase in potatoes.

The present study was undertaken to investigate the proteins of *Poa pratensis* seeds and their phosphatase activity.

MATERIAL AND METHODS

The studies were performed on *Poa pratensis* seeds harvested in 1970 and 1971 in the Experimental Station in Polanowice. The proteins were

extracted from the seeds with water, 0.9 and 2.0 per cent sodium chloride solution and 0.1 M acetate buffer, pH 5.1.

The seeds (5—10 g) were ground in a cutting mill for 3—4 min and 2 g samples of the ground seeds were extracted with 20 ml of an appropriate solution. After 1 h of shaking the samples were centrifuged at 15 000 g for 20 min. Protein in the extracts was determined turbidimetrically by the tannin method after Mejbaum-Katzenellenbogen (1955). Acid phosphatase activity was determined by measuring inorganic phosphate liberated from sodium p-nitrophenylphosphate.

The incubation mixture contained 0.5 ml of 0.2 per cent sodium p-nitrophenylphosphate, 1 ml 0.1 M acetate buffer, pH 5.1 and 0.5 ml enzyme (10 or 20 μg protein). The mixture was incubated at 37° for 10 min. The reaction was stopped by the addition of 2 ml of 5 per cent trichloroacetic acid. Liberated inorganic phosphate was determined by the method of Fiske and SubbaRow. One unit of acid phosphatase activity was defined as the amount of enzyme which liberates 1 μmole of inorganic phosphate per minute at 37°. Specific enzyme activity was expressed as units of enzyme activity per 1 mg of protein.

Ion exchange chromatography was performed on a 1.7×20 cm DEAE cellulose column equilibrated with 0.005 M acetate buffer, pH 5.1. Protein were collected by stepwise elution with increasing concentrations of acetate buffer (from 0.005 to 0.2 M), pH 5.1. Finally the column was eluted with 1 M sodium chloride in 0.2 M acetate buffer.

Electrophoresis in polyacrylamide gel was performed after Ornstein (1964) and Davis (1964) at pH 8.4 on 7.5 per cent gels. Glass tubes 0.6×6.5 cm, were filled to within 2 cm of the open end with the small pore gel solution containing 0.0005 per cent riboflavin as initiator. The solution was photopolymerized under water and 0.1 ml of the large pore solution containing 20 per cent sucrose was layered over it and photopolymerized. Protein samples in 50—100 μl of 20 per cent sucrose were layered on the upper gel. Electrophoresis was performed at 4° for 1.5 h at 2.5 mA/tube for the first 15 min, and continued at 4 mA/tube. The protein was stained for 1 h with 1 per cent amido black solution in 7 per cent acetic acid. The excess of the dye was washed out with 7 per cent acetic acid.

Acid phosphatase activity was localized in the gel by the diazo coupling technique. After electrophoresis the gels were incubated in 0.2 M acetate buffer, pH 5.1 at 4° for 30 min. Then they were placed in 5 ml of 0.2 M acetate buffer, pH 5.1 containing 5 cm of sodium α-naphthylphosphate and 3 mg of Fast Blue B and incubated at 37° until red bands appeared at the sites of enzyme activity.

RESULTS

In table 1 the results of preliminary analysis of the proteins from *Poa pratensis* seeds extracted with water and other solvents are presented. The highest specific activity of acid phosphatase was found in proteins extractable with acetate buffer, pH 5.1, and the lowest in the water-soluble proteins.

Table 1

Phosphatase activity of proteins from *Poa pratensis* seeds extracted with water, sodium chloride and 0.1 M acetate buffer, pH 5.1

Extraction with	Protein g/100 g of seeds	Phosphatase activity	
		Specific u./mg protein	Total u./100 g of seeds
Water	0.273	0.51	139.2
Sodium chloride 0.9%	0.293	0.70	205.1
Sodium chloride 2%	0.311	0.73	227.0
Acetate buffer 0.1 M pH 5.1 (sodium salt)	0.042	1.20	50.4

Phosphatase activity towards sodium p-nitrophenylphosphate was determined at pH 5.1. For procedure see Methods

Table 2

Preparation of acid phosphatase from *Poa pratensis* seeds

Procedure	Protein		Phosphatase activity		
	g/100 g of seeds	%	specific activity u./mg of protein	total activity u./100 g of seeds	yield %
Extraction of proteins from seeds with 0.1 M acetate buffer, pH 5.1	0.042	100	1.2	50.4	100
Dialysis of extract into water	0.030	73	1.6	49.2	97.6
Protein precipitation with alcohol and dialysis into water	0.012	30	3.3	41.5	82.3

Protein was determined by tannin micromethod, phosphatase activity by measuring the amount of inorganic phosphate liberated from p-nitrophenylphosphate. For reaction condition see Methods

Further investigations were performed on proteins extracted from seeds with 0.1 M acetate buffer, pH 5.1. The preparation of acid phosphatase from *Poa pratensis* seeds is shown in table 2. The ground seeds in 250—300 g portion were extracted with cooled 0.1 M acetate buffer, pH 5.1 in a 1 : 10 ratio (w/v). After 1 h stirring on a shaker the extract

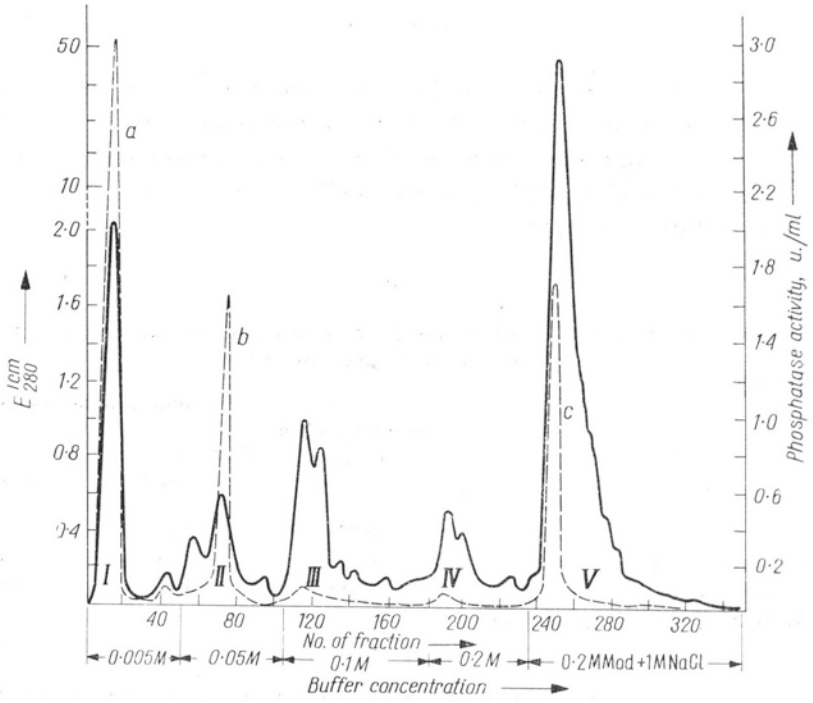

Fig. 1. Chromatography of proteins from *Poa pratensis* seeds on DEAE cellulose, pH 5.1

Protein was eluted from the column with increasing acetate buffer concentrations at pH 5.1. 5-ml fractions were collected. Protein determined by absorption measurement at 280 nm (1). Phosphatase activity determined towards p-nitrophenyphosphate (2). I, II, III, IV, V — protein peaks; a, b, c — enzymatic activity peaks

was filtered through cheesecloth and centrifuged at 2500 g for 30 min. After 24-h dialysis into water the extract was centrifuged and to the clear liquid 2 volumes of cold alcohol were added. After 3 h the mixture was centrifuged, the precipitate was suspended in water, dialysed and centrifuged. The supernatant was withdrawn and the precipitate was washed 4—5 times with water. The first supernatant and the washings were combined and lyophylized.

Precipitation of proteins with alcohol leads to an about threefold increase in enzyme activity as compared with that of the crude extract.

The lyophylized material was dissolved in 0.005 M acetate buffer, pH 5.1 and chromatographed on DEAE-cellulose equilibrated with the same buffer. Protein was eluted from the column with increasing concentrations of acetate buffer. The elution pattern is shown in Fig. 1.

Peak I correspondes to proteins unadsorbed on the column and eluted with 0.005 M acetate buffer pH 5.1. Peaks II, III and IV were eluted with increasing acetate buffer concentration, and peak V with 1 M sodium chloride in 0.2 M acetatet buffer pH 5.1.

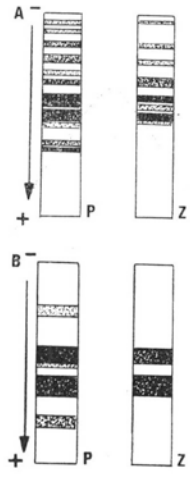

Fig. 2. Separation of proteins from *Poa pratensis* seeds on polyacrylamide gel Electrophoresis was run in tris-glycine buffer, pH 8.4 for 1.5 h at 4°. Staining for protein and phosphatase activity described in Methods. 40—80 μg of protein were subjected to electrophoresis when gels were stained for enzymatic activity. P — scheme of proteinogram, Z — scheme of phosphatase activity zymogram. A — proteins of crude seed extract, B — proteins of II-b peak

Table 3

Substrate specificity of acid phosphatase II from *Poa pratensis* seeds

Substrate	Relative activity %
sodium p-nitrophenylphosphate	100
sodium phenylphosphate	73
sodium β-naphthylphosphate	92
sodium α-naphthylphosphate	16
sodium β-glycerophosphate	6
sodium pyrophosphate	60
glucose-1-phosphate	0
ATP	40
AMP (3′)	50
CMP (2′+3′)	60
UMP (2′+3′)	40
GMP (5′)	4

Incubation mixture contained: 0.5 ml substrate (4 or 8 μmoles), 1 ml 0.1 M acetate buffer, pH 5.1 and 0.5 ml of enzyme (5 or 10 μg). Reaction was stopped by the addition of 2 ml 5% trichloroacetic acid. The liberation inorganic phosphate was determined after Fiske and Subbarow. Activity expressed as per cent of μmoles of Pi liberated from various substrates in reference to Pi liberated from sodium p-nitrophenylphosphate during 10° min at 37° (100%)

Phosphatase activity was localized in peaks, I, II and V. In the remaining ones only minor amounts of the activity was found. The highest activity of enzyme was demonstrated in peak II-b. The specific

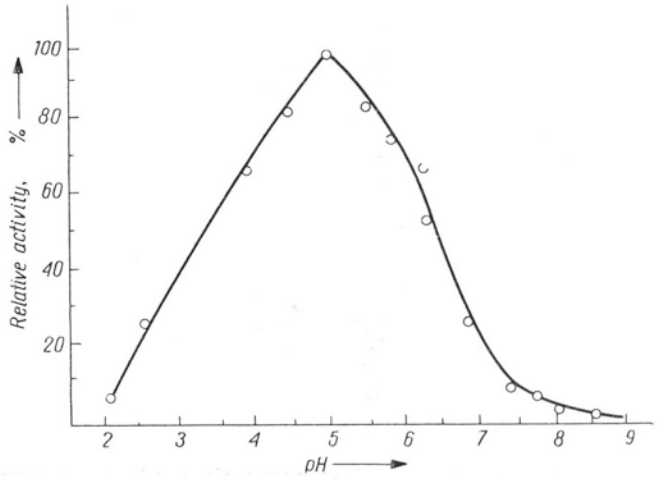

Fig. 3. Influence of pH on phosphatase II activity

Phosphatase activity was determined towards p-nitrophenylphosphate in 0.14 M veronal buffer. Enzyme concentration 10 μg. Reaction run at 37° for 10 min. Activity expressed as per cent in reference to highest activity obtained in given experiment

activity of these proteins was 16 units/mg, thus a 15-fold increase in enzyme activity has been achieved as compared with the crude seeds extract. In peaks I-a and V-c, a 5- and 2-fold increase in enzyme activity was observed respectively.

Since phosphatase activity was the highest in peak II-b these proteins were the object of further studies. This material was compared by disc electrophoresis with proteins from the crude seed extract.

Fig. 2 shows proteinograms (P) and zymograms (Z) of the crude extract and peak II-b (fractions 66—78, phosphatase II). Proteins from *Poa pratensis* seeds soluble in 0.1 M acetate buffer pH 5.1 (crude seed extract) were separated into 11 distinct bands whereas in proteinograms of peak II-b only 5 protein bands were visible. In zymograms of the crude extract 8 bands of acid phosphatase activity can be distinguished, but in those of the peak II-b only two such bands with anodic mobility were found. Table 3 shows result of investigation on substrate specificity phosphatase II (peak II-b). The enzyme activity towards p-nitrophenylphosphate was taken as 100 per cent. The highest activity was found in the presence of p-nitrophenylphosphate, and β-naphthylphosphate, phenylphosphate and pyrophosphate were hydrolized much less intensively. Relative activity of phosphatase II towards monophosphates of pyrimidine and purine nucleosides, with the exception of guanosine-5′phosphate, was 40—50 per cent. Guanosine-5′phosphate like β-glycerophosphate was hydrolized only in a slight degree. Glucose-1-phosphate proved resistant to the action of acid phosphatase II.

Table 4

The effect of various ions on the activity of phosphatase II from *Poa pratensis* seeds

Substance	Relative activity (%)
No addition	100
KCl	100
$CaCl_2$	77
$MgCl_2$	149
NaF	55
EDTA	100

Ion concentration in incubation mixture 2.5×10^{-2}M, enzyme concentration 5 or 10 μg. Reaction run at 37° for 10 min. Enzyme activity in the presence of ions expressed as per cent of activity without ion addition (100%)

The influence of pH on phosphatase II activity toward sodium p-nitrophenylphosphate is shown in Fig. 3. Optimum pH for acid phosphatase II is about 4.9.

Table 4 presents the effect of various ions on phosphatase activity. It was found that potassium chloride and EDTA in concentration 2.5×10^{-2} M did not affect the activity of acid phosphatase II. Mg^{2+} ions had an activating influence, whereas calcium and fluoride ions inhibited acid phosphatase activity.

DISCUSSION

Caryopses of *Poa pratensis* belong to seeds of low protein concentration (Mejbaum-Katzenellenbogen, Lorenc-Kubis 1967)). Proteins soluble in water and sodium chloride solution constitute about 0.3 per cent of the dry mass of these seeds, whereas proteins extracted with 0.1 M acetate buffer, pH 5.1 constitute about 0.042 per cent.

The seed proteins soluble in water, sodium chloride solution and O.1 M acetate buffer, pH 5.1 exhibit acid phosphatase activity. The highest specific enzymatic activity was noted in the case of proteins soluble in acetate buffer. Separation of these proteins on DEAE cellulose column revealed the presence of three chromatographically different groups of acid phosphatases which are eluted with the proteins of the I, II and V peaks. Among phosphatases localized in peaks I-a, II-b and V-c the highest specific activity was shown in peak II-b (phosphatase II).

Three chromatographically different acid phosphatases have been isolated from wheat germs (Verjee, 1969). These phosphatases showed differences in optimum pH, substrate specificity and sensitivity to some

metal ions. Investigations of the substrate specificity of acid phosphatase II from *Poa pratensis* seeds demonstrated that this enzyme like acid phosphatase from *Fusarium moniliforme* (Yoshida, Tamiya, 1971) and acid phosphatase from *Staphylococcus aureus* (Malveaux, San Clemente, 1969) are most active towards p-nitrophenylphosphate. Sodium phenylphosphate and pyrophosphate as well as monophosphates of purine and pirimidine nucleosides were hydrolysed to a lesser extent. Nucleotidase activities were also found in acid phosphatase II from *Fusarium moniliforme* (Yoshida, Tamiya, 1971) which hydrolysed AMP, CMP and UMP.

In studies of acid phosphatases of plant origin and from microorganisms it has been repeatedly demonstrated that fluoride ions are strong inhibitors of these enzyme (Yoshida, Tamiya, 1971; Hagiwara, Masuda, Sakakibara, 1969; Malveaux, San Clemente, 1969; Verjee, 1969). EDTA, however, which acts as a metal chelating agent very offten showed no effect on the activity of acid phosphatases (Yoshida, Tamiya, 1971; Hagiwara, Masuda, Sakakibara, 1969; Verjee, 1969), or had an activating influence (Verjee, 1969). In the present investigations EDTA was found to exert no influence on acid phosphatase II activity, whereas fluoride ions had a strong inhibitory effect.

After electrophoretic separation of proteins of the peak II-b two bands of phosphatase activity were demonstrated on polyacrylamide gels. This might be evidence of electrophoretic heterogeneity of acid phosphatase II localized in this peak.

The results presented in this paper indicate that the partly purified acid phosphatase II from *Poa pratensis* seeds has some properties which are typical for plant acid phosphatases, as for instance highest activity towards p-nitrophenylphosphate, lack of activation by EDTA, and strong inhibition by fluoride ions.

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Aktywność fosfatazowa białek nasion wiechliny łąkowej (Poa pratensis) *

I. Wstępna charakterystyka kwaśnej fosfatazy II.

Streszczenie

Białka nasion wiechliny łąkowejj (*Poa pratensis*) ekstrahujące się do wody, 0,9% i 2% roztworu chlorku sodu oraz 0,1 M moderatora octanowego o pH 5,1 wykazują aktywność kwaśnej fosfatazy. Rozdział białek rozpuszczalnych w moderatorze octanowym na kolumnie z DEAE celulozy w pH 5,1 wykazał obecność trzech grup fosfataz, z których fosfataza szczytu II-b (fosfataza II) wykazywała najwyższą aktywność właściwą. Optimum pH tego enzymu wobec p-nitrofenylofosforanu sodu wynosi około 4,9. Aktywność fosfatazy II hamowana była przez jony wapniowe i fluorkowe. EDTA oraz chlorek potasu nie wywierał wpływu na aktywność tego enzymu, podczas gdy znaczny wzrost aktywności stwierdzono w obecności jonów magnezowych.

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