Phosphatase activity of *Poa pratensis* seeds

II. Purification and characterization of acid phosphatase Ia₂ and Ia₃

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Abstract

Two acid phosphatases (Ia₂, Ia₃) have been isolated from *Poa pratensis* seeds and partially purified. Both enzymes showed maximal activity at pH 4.9. They exhibited high activity towards p-nitrophenyl phosphate, inorganic pyrophosphate and phenyl phosphate, much less activity towards glucose-6 phosphate, and mononucleotides. Phosphatases a₂ and a₃ differed in their activity towards ADP. Orthophosphate, fluoride and Zn²⁺ were effective inhibitors. EDTA, β-mercaptoethanol and Mg²⁺ activated phosphatase a₂ but had no effect on phosphatase a₃. Zn²⁺ inhibited the activity of phosphatase a₂ noncompetitively, whereas phosphatase a₃ showed inhibition of mixed type. Trypsin, chymotrypsin and pronase had no effect on the enzyme activities of both molecular forms.

INTRODUCTION

Acid phosphatases (orthophosphoric monoester phosphohydrolase EC 3.1.3.2) of higher plants are characterized by chromatographic, (Verjee 1969; Felenbok, 1970) and electrophoretic (Kubicz and Morawiecka, 1970; Lorenc-Kubis, Wieczorek, 1973) heterogeneity.

In *Poa pratensis* seeds three groups of acid phosphatases (I_a, II_b and V_c) were demonstrated. Of these phosphatase II has been described in a previous paper (Lorenc-Kubis, Morawiecka, 1973). The present study is devoted to the purification and some enzymatic properties of phosphatases Ia₂ and Ia₃ from *Poa pratensis* seeds.

MATERIAL AND METHODS

The investigations were performed on *Poa pratensis* seeds from the 1973 harvest. The seeds were received from the Cracow Plant Cultivation Station in Polanowice.

Chemical reagents: sodium p-nitrophenylphosphate, sodium α -naphthylphosphate, sodium β -glycerophosphate, sodium phenylphosphate, bovine trypsin, once crystalized, acrylamide, N,N'-methylenebisacrylamide (Koch Light Lab. Ltd. — Colnbrook, Bucks, England); sodium pyrophosphate, acrylamide (British Drug Houses — Poole, Dorset, England); 2-mercaptoethanol, tris-(hydroxymethyl)-aminomethane (Fluka, Buchs, Switzerland); bovine chymotrypsin 3 times crystallized, glucose-6-phosphate (Reanal, Budapest, Hungary); amide black 10B, Fast Blue B (Merck A. G., Darmstadt, G. F. R); N,N,N',N'-tetramethylethylenediamine (Temed, Eastman Kodak, New York, U.S.A.); pepsin 3 times crystalized (Nutritional Biochem. Corp. Cleveland, Ohio, U.S.A.); sodium bis (p-nitrophenylphosphate), 5'-UMP, lyophilized pure pronase P (Serva, Heidelberg, G. F. R); 5'-AMP, 3'(+2') AMP, ADP (Calbiochem, San Diego, U.S.A.); carboxymethylcellulose Whatman CM-11, diethylaminoethylcellulose Whatman DE-11 (Balston Ltd., Maidstone, Kent, England); tannin was a French product packed in Poland (POCH).

The other chemicals used were of analytical grade, and were supplied by the Polish Chemical Reagents Laboratories (Gliwice). Preparation of acid phosphatases from *Poa pratensis* seeds: Protein was extracted from the seeds with 0.1 M acetate buffer, pH 5.1 and subjected to preliminary purification by precipitation with alcohol and chromatography on DEAE cellulose after Lorenc-Kubis and Morawiecka (1973). The Ia protein fraction not adsorbing on DEAE cellulose and flowing out in 0.005 M acetate buffer, pH 5.1 was dialysed against water for 24h and lyophilized. The lyophilized protein preparations (100—150 mg) were dissolved in 0.005 M acetate buffer, pH 5.1 and chromatographed on a CM cellulose (2.0×30 cm) column equilibrated with the same buffer. Protein was eluted with increasing concentrations (0.005—0.2 M) of acetate buffer, pH 5.1 (in a noncontinuous system). The end fractions were eluted from the column with 1 M sodium chloride in 0.2 M buffer.

The flow rate was 4.0 ml per 10 min.

Acid phosphatase activity was determined by measuring the release of p-nitrophenol from sodium p-nitrophenylphosphate. The incubation mixture (2 ml) contained 0.01 M sodium p-nitrophenylphosphate in 0.1 M acetate buffer, pH 5.1 and 0.04—0.08 units of enzyme. After incubation at 37°C for 10 and 30 min, the reaction was stopped by the addition of 10 ml of 0.1 N NaOH, and the absorbance of the solution was measured at 410 nm.

One unit of phosphatase activity was defined as the amount of enzyme which liberates 1 μ mole of p-nitrophenol or inorganic phosphate per minute under the condition of the method.

The substrate-specificity of acid phosphatases was determined in terms of inorganic phosphate released from the substrate by the method of Fiske and Subbarow. As 100 per cent activity was considered the amount of inorganic phosphate released from sodium p-nitrophenylphosphate.

Digestion of phosphatases with trypsin, chymotrypsin and pronase was performed at pH 7.1. To 5–10 μ g of protein with phosphatase activity (phosphatase a_2 and a_3), contained in 0.9 ml of 0.025 M Tris-HCl buffer, 0.1 ml protease (1–2 μ g) dissolved in 0.001 N HCl was added. The mixture was incubated at 30°C for 2h and then 1 ml of sodium p-nitrophenylphosphate (10 mM) in 0.2 M acetate buffer, pH 5.1, was added to the samples and phosphatase activity was estimated.

Protein was determined by the tannin turbidimetric micromethod of Mejbaum-Katzenellenbogen (1955) with gelatin as standard. Electrophoresis in 7.5 per cent gels was performed after Ornstein (1964) and Davis (1964) in Tris-glycine buffer, pH 8.4. As polymerization initiator 0.0005 per cent riboflavin was used. For detection of protein, 70–140 μ g of protein was applied to electrophoresis, and 10–30 μ g for detection phosphatase activity.

Protein was stained with 1 per cent amide black 10B solution in 7 per cent acetic acid. Phosphatase activity in the gels was localized with 0.1 per cent 1-naphthylphosphate and 0.1 per cent Fast Blue B, both dissolved in 0.2 M acetate buffer, pH 5.1. The mixture was incubated at 37°C until red bands appeared corresponding to phosphatase activity.

RESULTS AND DISCUSSION

Chromatography on DEAE-cellulose showed 3 groups of nonspecific acid phosphatases in *Poa pratensis* seeds (Lorenc-Kubis, Morawiecka, 1973). Table 1 presents the results of purification. Specific activity of fraction Ia (not adsorbing on DEAE-cellulose) was 5 units/mg. Fraction Ia involved about 28 per cent of protein subjected to separation.

This fraction was further separated on CM-cellulose in acetate buffer, pH 5.1 with increasing ionic strength. Under these conditions about 30 per cent of protein is not adsorbed on CM-cellulose, and the remaining proteins are separated into 4 fractions with phosphatase activities — a_1 , a_2 , a_3 and a_4 . (Fig. 1).

The highest specific activity — 10 units/mg showed phosphatases a_2 and a_3 . These enzymes were analyzed by disc electrophoresis in 7.5 per cent polyacrylamide gel.

Table 1
Purification of acid phosphatases from *Poa pratensis* seeds

Procedure	Acid phosphatase activity		
	Specific activity (units/mg)	Total activity (units)	Yield (%)
Extraction of proteins from seeds with 0.1 M acetate buffer, pH 5.1.	1.2	50.4	100
Protein precipitation with alcohol and dialysis into water	3.3	41.5	82.3
Chromatography on DEAE-cellulose			
Fraction Ia	5.0	18.2	36.2
Fraction IIb	16.0	9.0	17.8
Fraction Vc	2.4	5.5	10.9
Chromatography of fraction Ia on CM-cellulose			
phosphatase a ₁	3.0	2.0	3.9
phosphatase a ₂	9.8	2.6	5.1
phosphatase a ₃	10.0	3.8	7.5
phosphatase a ₄	3.2	1.9	3.7

Specific enzyme activity is expressed as units of enzyme activity per mg of protein.

The proteinograms (P) and zymograms (Z) are schematically shown in Fig. 2. The fraction Ia was separated into 7 protein bands staining with amide black and 6 bands with acid phosphatase activity. In the proteinograms of fraction a₂ and a₃ one dominating and two trace bands are seen, whereas in the zymograms single bands appear corresponding in mobility to bands 4 and 5 in the zymogram of fraction Ia.

Phosphatases a₂ and a₃ like most plant phosphatases are characterized by thermostability (Verjee, 1969). When heated for 10 min at 50° and pH 5.1, they preserve about 95 per cent of their total activity and are inactivated at 80°C. No loss of activity was observed after treatment with trypsin, chymotrypsin and pronase. Optimum activity of both the phosphatases (with p-nitrophenyl phosphate as substrate) like that of phosphatase II of *Poa pratensis* seeds (Lorenc-Kubis, Morawiecka, 1973) was at pH 4.9.

Table 2 presents the substrate-specificity of phosphatases a₂ and a₃. These enzymes show the highest activity toward inorganic pyrophosphate, were the activity of phosphatase a₂ is by 86 per cent and that of phosphatase a₃ by 10 per cent higher than that when compared with the activity towards p-nitrophenylphosphate as a substrate. Activity of the same order is shown by the both acid phosphatases when phenylphosphate was used as substrate. On the other hand their activity is low in the case of α -naphthylphosphate, glucose-6 phosphate and

Fig. 1 Chromatography on CM-cellulose of Ia fraction (nonadsorbing on DEAE-cellulose) at pH 5.1.

Elution conditions as described in Methods

1 — protein- A_{280nm} , 2 — acid phosphatase activity

Fig. 2. Electrophoresis in polyacrylamide gel:

A — fraction Ia, B — acid phosphatase a_2 , C — acid phosphatase a_3 ; P — proteinograms, Z — zymograms of phosphatase activity. Electrophoresis was run in 7.5 per cent gel. Electrophoresis conditions given in Methods.

Table 2

Activity of acid phosphatases a_2 and a_3 toward various substrates

Substrate	Relative activity (%)	
	Phosphatase a_2	Phosphatase a_3
p-Nitrophenyl phosphate	100	100
Phenylphosphate	92	84
α -Naphthylphosphate	11	10
β -Glycerophosphate	22	18
Glucose 6-phosphate	8	6
Inorganic pyrophosphate	186	110
Bis-p-nitrophenyl phosphate	0	4
5'-AMP	0	6
3' (+2') AMP	14	16
5'-UMP	8	8
ADP	51	8

Reaction mixtures contained in a total volume of 2.0 ml: 0.04 unit of enzyme, 0.01 M of substrate. Enzyme and substrate were in 0.1 M acetate buffer, pH 5.1. Incubation was for 10 and 30 min at 37°. The reaction was stopped by the addition of 2 ml 5% trichloroacetic acid. The liberation inorganic phosphate was determined after Fiske and Subbarow.

5'-UMP. Adenosine diphosphate was hydrolysed in 50 per cent by phosphatase a_2 , whereas phosphatase a_3 hydrolysed it in as little as 8 per cent as compared with the hydrolysis of p-nitrophenylphosphate. Phosphatase a_3 exhibits also a trace activity towards 5'-AMP. The effect of activators and inhibitors on phosphatase a_2 and a_3 is presented in table 3. It was found that beta mercaptoethanol, EDTA and Mg^{2+} ions

Table 3

Effect of various substances on acid phosphatases a_2 and a_3 activity

Substance added	Relative activity (%)	
	Phosphatase a_2	Phosphatase a_3
None	100	100
$CaCl_2$	86	95
$ZnCl_2$	14	17
$MgCl_2$	108	100
NaF	27	31
KH_2PO_4	52	40
EDTA	112	96
β -Mercaptoethanol	114	100

The reaction mixture contained in a total volume of 2.0 ml: 0.04 – 0.08 units of enzyme, 1×10^{-2} M of various substances and 0.01 M of p-nitrophenyl phosphate. Acetate buffer – 0.1 M, pH 5.1. Incubation was for 10 and 30 min at 37°C.

Activities were expressed as per cent of that without added substance.

slightly activated. phosphatase a_2 , but have no effect on phosphatase a_3 activity. Fluoride and phosphate ions, as in the case of acid phosphatase III from wheat (Verjee, 1969) and phosphatase II from *Fusarium*

Fig 3. Inhibition by Zn^{2+} of the hydrolysis of p-nitrophenyl phosphate by acid phosphatase a_2

The reaction mixture contained in a total volume of 2.0 ml: 0.04 unit of enzyme 0.1 M acetate buffer, pH 5.1 and indicated amount of p-nitrophenyl phosphate. Incubation was made for 10 min at 37°C. Substrate concentration [S] is expressed in mM, and initial velocity v expressed in μ moles of p-nitrophenyl

1 — with no inhibitor; 2 — with $1 \times 10^{-3}M$ Zn^{2+} ions

Fig 4. Inhibition by Zn^{2+} of hydrolysis of p-nitrophenyl phosphate by acid phosphatase a_3

1 — with no inhibitor, 2 — with $1 \times 10^{-3}M$ Zn^{2+} ions. For description see Fig. 3

moniliforme (Yoshida and Tamiya, 1971), inhibited the activity of both the acid phosphatases. Zn^{2+} was also a strong inhibitor of these enzymes. At concentrations $1 \times 10^{-2}M$ these ions inhibited about 85 per cent, and at $1 \times 10^{-3}M$ about 60 per cent of the total activity of both

the acid phosphatases. At concentration 1×10^{-4} , Zn^{2+} inhibited acid phosphatase a_3 in 30 per cent and a_2 in as little as 15 per cent. At lower concentrations these ions had no influence on the activity of both phosphatases.

The course of the curves in the Lineweaver-Burke system demonstrated that zinc ions exhibit uncompetitive inhibition in the case of phosphatase a_2 (Fig. 3), and inhibition of mixed type in relation to phosphatase a_3 (Fig. 4). The Michaelis constants determined for phosphatase a_2 and a_3 are 1.66×10^{-3} and 6.66×10^{-4} , respectively. The K_m values for other phosphatases of plant origin vary within the limits 2.5×10^{-3} — 6.8×10^{-5} (Hsu et al., 1966, Yoshida and Tamiya, 1971; Baker and Takao, 1973; Ushara et al., 1974).

In the preceding paper we have demonstrated that acid phosphatase of *Poa pratensis* seeds occurs in several molecular forms after separation by chromatography and electrophoresis. The present study showed that two of these enzyme forms (a_2 and a_3) differ significantly by their Michaelis constants and their activity toward certain substrates and inhibitors.

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*Aktywność fosfatazowa nasion wiechliny łąkowej (Poa pratensis)**II. Oczyszczanie i właściwości kwaśnej fosfatazy Ia₂ i Ia₃*

Streszczenie

1. Z nasion wiechliny łąkowej wydzielono i częściowo scharakteryzowano 2 kwaśne fosfatazy a₂ i a₃.

2. Enzymy te o optimum działania w pH 4,9 wykazywały duże powinowactwo do p-nitrofenylofosforanu, pirofosforanu nieorganicznego i fenylofosforanu. Śladową aktywność przejawiały względem glukozy-6-fosforanu oraz mononukleotydów. Fosfatazy a₂ i a₃ wykazują znaczne różnice w hydrolizie ADP.

3. Kwaśna fosfataza a₂ i a₃ hamowana jest przez jony fluorkowe, fosforanowe i cynkowe. EDTA, beta-merkaptioetanol oraz jony Mg⁺⁺ w stężeniu 10 mM aktywują nieznacznie fosfatazę a₂, pozostają natomiast bez wpływu na aktywność fosfatazy a₃.

4. Jony Zn⁺⁺ są inhibitorami niekompetycyjnymi w stosunku do kwaśnej fosfatazy a₂, podczas gdy hamowanie aktywności fosfatazy a₃ ma charakter inhibicji mieszanej.

5. Enzymy nie tracą aktywności pod wpływem działania trypsyny, chymotrypsyny i pronazy.