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PARTIAL CHARACTERIZATION OF 5′(3′)-RIBONUCLEOTIDE AND 3′-RIBONUCLEOTIDE PHOSPHOHYDROLASES FROM *TRADESCANTIA ALBIFLORA* LEAVES

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Key Word Index—*Tradescantia albiflora*; Commelinaceae; phosphohydrolases; 5′(3′)-ribonucleotide; 3′-ribonucleotide; purification; properties.

Abstract—Two acid phosphomonoesterases, 5′(3′)-ribonucleotide phosphohydrolase and 3′-ribonucleotide phosphohydrolase, were isolated from *Tradescantia albiflora* leaf tissue and purified by ammonium sulphate precipitation, gel filtration on Sephadex G-200 and repeated chromatography on DEAE-cellulose. The enzymes differed in their sensitivity to dialysis against 1 mM EDTA; the activity of 5′(3′)-ribonucleotide phosphohydrolase was unaffected, while 3′-ribonucleotide phosphohydrolase showed an increase of 60–90%. Both enzymes were rapidly inactivated above 50°. Their ion sensitivity was identical: 1 mM Zn^{2+} and Fe^{2+} were inhibitors for both by 20–80%; while Mg^{2+}, Ca^{2+}, Co^{2+}, K^+, Na^+ at 1–10 mM had no significant effect on the activity of either enzyme. Inorganic phosphate inhibited both enzymes almost completely. EDTA (1 mM) did not inhibit either enzyme; none of the divalent cations tested were enzyme activators. 3′-Ribonucleotide phosphohydrolase hydrolysed both 3′- and 5′-nucleoside monophosphates (3′-AMP, 3′-CMP, 3′-GMP, 3′-UMP, 5′-AMP, 5′-CMP, 5′-GMP, 5′-UMP). 5′(3′)-Ribonucleotide phosphohydrolase showed a preference for the 3′-nucleoside monophosphates. Adenosine 3′,5′-cyclic monophosphate, purine and pyrimidine 2′,3′-cyclic mononucleotides at 0.1–1.0 mM did not inhibit the enzymes.

INTRODUCTION

The elucidation of key steps and the characterization of major components of nucleic acid metabolism are of considerable importance in biological systems [1–3]. A number of hydrolytic enzymes, mostly non-specific for various phosphate esters, have been detected in plant tissues. Phosphatases (orthophosphoric monoester phosphohydrolase EC 3.1.3.2) have been isolated from wheat leaves [4], wheat germ [5], soybean [6], pea leaves [7], dwarf bean seedlings [8], mung bean sprouts [9], tobacco leaves [10], *Phaseolus mungo* [11], spinach leaves [12], sweet potato [13, 14], *Phaseolus vulgaris* [15], cultured tobacco cells [16] and cultured cells of rice plant [17]. All these enzymes proved to be non-specific for the cleavage of various phosphate ester bonds. Phosphatase isoenzymes have been reported to occur in plants as revealed by disc electrophoresis and column chromatography, respectively [18–23]. Only a few of these isoenzymes were characterized to any great extent. The present work describes the partial purification and characterization of two acid phosphatases from *Tradescantia albiflora* leaf tissue.

RESULTS

Enzyme purification

Only 5% of phosphomonoesterase activity remained in the 40 000 g sediment under the conditions used. Upon gel filtration on Sephadex G-200, three distinct phosphomonoesterase peaks were obtained (Fig. 1). Regarding the low MWs of phosphomonoesterases found in higher plant tissues [24], peak 1 is probably an enzyme aggregate, and has not been further characterized. The fractions corresponding to the second and third peaks were pooled and the combined fractions were resolved into three separate peaks of phosphomonoesterase activity on a DEAE-cellulose column. The phospho-

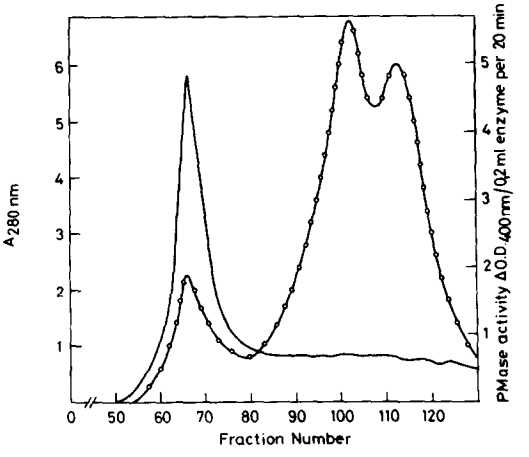

Fig. 1. Chromatography on Sephadex G-200 of phosphomonoesterases isolated from *Tradescantia albiflora* leaves. ——, protein; ○——○, phosphomonoesterase activity.

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monoesterases corresponding to the first and second peaks were further purified by rechromatography on DEAE-cellulose. A summary of the purification procedure is given in Table 1.

Stability

In crude extracts the enzyme was rapidly inactivated, probably due to the high polyphenol content of the extract. Stability could not be maintained by adding 2 mM mercaptoethanol, 0.1 % cysteine, 2 mM potassium metabisulphite or 2 mM EDTA, respectively, to the extraction medium. Ascorbic acid (0.5 %) proved to be effective and was used in routine work. Partially purified enzymes, however, kept at −18° were stable for several months.

pH *optima*

The two enzymes were characterized with respect to their optimum pH for the hydrolysis of *p*-nitrophenyl phosphate. The pH optimum for peak 1 was 5.5 and that for peak 2 was *ca* 5.

Thermal inactivation

The two enzymes showed the same degree of heat stability. Both were rapidly inactivated at temperatures higher than 50°.

Effect of various ions and EDTA

The cations Mg^{2+}, Ca^{2+}, Co^{2+}, K^+ and Na^+ in the concentration range of 1–10 mM, had no significant effect on the enzymes (Table 2). Zn^{2+}, Cu^{2+} and Fe^{2+} were inhibitors by 20–80 % at 1 mM. Inorganic phosphate almost totally inhibited both enzymes. EDTA (1–10 mM) remained ineffective. Dialysis of the proteins of peak 1 against 100 volumes of 1 mM EDTA at 4° for 24 hr did not affect the enzyme activity. With the same treatment, however, the enzyme belonging to peak 2 was activated by 60–90 % (Table 3).

Effect of other substances

Iodoacetate, sodium azide, mercaptoethanol and ascorbic acid at 1 mM inhibited the enzyme activity belonging to peak 1 by 20–35 %. The same chemicals had

Table 1. Purification of acid phosphomonoesterases from *Tradescantia albiflora* leaf tissue

Fraction	Volume (ml)	PMase activity (units*)	Total protein (mg)	Specific activity (units/mg protein)	Degree of purification	Yield (%)
Crude extract†	135	4480	660	6.8	1	100
2nd $(NH_4)_2SO_4$ fraction, 35–80 % saturation	8	4180	276	15	2.23	93
Sephadex G-200						
Fractions 85–127	126	350	25.1	140.8	20.6	78
1st DEAE-cellulose						
Peak 1 fr. 89–97	24	942	1.0	942	140	21
Peak 2 fr. 105–125	60	797	2.5	319	47	17.8
Peak 3 fr. 133–145	36	56	1.5	37	5.5	1.2
2nd DEAE-cellulose						
Peak 1 fr. 52–62	30	471	0.4	1180	174	10.5
Peak 2 fr. 62–73	33	400	1.1	364	53.6	8.9

* 1 enzyme unit is defined as A 400 nm/ml of enzyme per 20 min under the assay conditions.
† Prepared from 50 g of *Tradescantia albiflora* leaf tissues.

Table 2. Summary of inhibiting activity of various chemical agents for acid phosphomonoesterases from *Tradescantia albiflora* leaf tissue*

Chemical agent	Final conc. (mM)	Peak 1 $A_{400\,nm}$ (0.03 ml E) 20′	Peak 1 %	Peak 2 $A_{400\,nm}$ (0.2 ml E) 20′	Peak 2 %
Control	–	0.200	100	0.271	100
$ZnSO_4$	1.0	0.159	79	0.221	81.2
$CuSO_4$	1.0	0.134	67	0.075	27.5
$FeCl_2$	10.0	0.007	3.5	0.014	5.5
	1.0	0.036	18	0.067	24.6
NaH_2PO_4	10.0	0.036	11.5	0.022	8
EDTA	10.0	0.219	109	0.275	101
	1.0	0.239	119	0.337	124

* The assay system described in the Experimental was used.

Table 3. Effect of dialysis against EDTA on the acid phosphomonoesterases from *Tradescantia albiflora**

Peak	Enzyme (ml)	Before dialysis $A_{400\,nm/20\,min}$	%	After dialysis $A_{400\,nm/20\,min}$	%
1	0.03	0.850	100	0.801	94
	0.06	1.660	100	1.620	98
2	0.05	0.444	100	0.711	160
	0.10	0.690	100	1.300	188

*Peaks 1 and 2 of phosphatase activity were dialysed against 0.01 M Tris–HCl buffer (pH 7.5) containing 1 mM EDTA overnight. Before and after dialysis samples were withdrawn and assayed for enzyme activity as described in the Experimental.

no effect on the enzyme activity in peak 2. Sodium fluoride (1 mM) and 3M urea inhibited both enzymes to the same extent (70–80%).

Substrate specificity

ATP, ADP and inorganic pyrophosphate were hydrolysed at *ca* equal rates as *p*-NPP by the enzyme present in peak 1. The enzyme of peak 2 cleaved these substrates by 50–70% less. Both enzymes exhibited broad specificities for various types of phosphoester bonds (Table 4).

Intrinsic differences in their specificities, however, were also found. The enzyme in peak 2 cleaved all the 3′- and 5′-nucleoside monophosphates at about the same rate. The enzyme of peak 1, however, had a preference for attacking 3′-mononucleotides (Table 5). The question as to whether the activities tested in the pooled fractions of peak 1 belong to the same enzyme was tested by loading the combined fractions onto a DEAE-cellulose column and assaying all the fractions eluted for their activities on various monophosphate esters. All the activities tested had a maximum at the same fraction number.

Effect of cyclic mononucleotides

The effect on the enzyme activity of cAMP (1.0–0.1 mM) was tested on peaks 1 and 2, using 3′- and 5′-adenosine monophosphates and *p*-nitrophenylphosphate as substrates. The effect of 2′,3′-cyclic mononucleotides was also tested. None of these compounds had any significant effect on enzyme activity.

DISCUSSION

This paper describes the partial purification of two phosphatases from *Tradescantia albiflora* leaf tissue. Extraction of the leaf tissue in 0.05 M Tris–HCl containing 0.5% ascorbic acid (pH 7.5) resulted in almost complete solubilization of the enzymes. During the preparation of the crude extract, a loss of enzyme activity was observed. In a number of properties, the enzymes proved to be similar to other acid phosphatases of plant origin. The enzymes studied are not true orthophosphoric monoester phosphohydrolases because, at different rates, they also catalyse the cleavage of inorganic and nucleotide pyrophosphate bonds. Their specificities towards the substrates tested are similar to those of the typical non-specific phosphatases in plants which hydrolyse most carbohydrate esters at about equal rates and to a lesser extent than the artificial substrate *p*-NPP [10–12, 18–21].

Significant differences were, however, found in the ability of the enzymes to split 3′-, and 5′-mononucleotides. The enzyme belonging to peak 2 hydrolysed both 3′- and 5′-nucleotides at about equal rates. In this respect the enzyme resembles the phosphatases from the cotyledons of germinating peanut seeds [19], those isolated from wheat seedlings [25] and the marine alga *Enteromorpha* [26]. The enzyme belonging to peak 1, however, preferentially attacked various nucleoside 3′-monophosphates. In this respect the peak 1 enzyme is similar to the acid phosphatase purified from tobacco leaves [10], cultured tobacco cells [16] and the 3′-nucleotidases from mung bean sprout [9, 27], potato tubers [28] and ryegrass [29].

Table 4. Rate of hydrolysis of several phosphate esters by phosphomonoesterases from *Tradescantia albiflora* leaf tissue*

Substrate	μmol Pi liberated/20 min Peak 1	Peak 2	Relative % Peak 1	Peak 2
p-NPP	0.555	0.896	100.0	100.0
bis-*p*-NPP	0.0005	0.025	0.09	2.7
NADP	0.309	0.451	55.6	50.3
ATP	0.658	0.464	118.5	51.7
ADP	0.632	0.348	113.8	38.8
Gl-1-P	0.154	0.180	27.7	20.0
Gl-6-P	0.206	0.451	37.1	50.3
Fr-1, 6-diP	0.219	0.503	39.4	56.1
2-PG acid	0.322	0.452	58.0	60.4
ThiaminepyroP	0.180	0.150	32.4	16.7
Inorganic pyroP	0.464	0.232	83.6	25.8

* The standard assay described in the Experimental was used. Reaction mixtures contained 0.03 and 0.10 ml enzyme fraction, respectively.

Table 5. Rate of hydrolysis of various mononucleotides by phosphomonoesterases from *Tradescantia albiflora* leaf tissue*

Substrate	μmol Pi liberated/20 min		Relative %	
	Peak 1	Peak 2	Peak 1	Peak 2
p-NPP	0.387	0.671	100.0	100.0
5′-AMP	0.039	0.284	10.1	42.3
3′-AMP	0.245	0.361	63.3	53.8
5′-GMP	0.026	0.297	6.7	44.3
3′-GMP	0.200	0.374	51.7	55.7
5′-CMP	0.026	0.258	6.7	38.4
3′-CMP	0.310	0.400	80.1	59.6
5′-UMP	0.021	0.258	5.4	38.4
3′-UMP	0.245	0.313	63.3	46.6

* The standard assay described in the Experimental was used. Reaction mixtures contained 0.03 and 0.10 ml enzyme fraction, respectively.

Repeated rechromatography on DEAE-cellulose did not resolve the activity of peak 2. In agreement with Polya and Ashton [25], the denomination 5′(3′)-ribonucleotide phosphohydrolase and 3′-ribonucleotide phosphohydrolase may be a better definition for the enzyme fractions described in our work.

EXPERIMENTAL

Plant material. Tradescantia albiflora leaves were supplied by the Arboretum of the József Attila University, Szeged; prior to use the leaves were rinsed with H_2O and kept at −16° until use.

Preparation of crude extract. Leaf tissue was homogenized at 4° with quartz sand in a 3-fold amount of 0.05 M Tris–HCl buffer containing 0.5% ascorbic acid (pH 7.5). The homogenate was passed through 4 layers of cheese-cloth. The slurry was centrifuged at 40000 *g* for 30 min at 4° and the resulting supernatant was used for further work.

Determination of enzyme activities. Phosphomonoesterases and phosphodiesterases were assayed as previously described [30] using *p*-nitrophenyl phosphate (*p*-NPP) and bis-*p*-nitrophenyl phosphate (bis-*p*-NPP) as substrates, respectively. Activity for natural substrates was determined as described in ref. [9]. The release of Pi was measured by the micromethod of ref. [31]. One enzyme unit was defined as ΔA 400 nm/ml enzyme per 20 min. Sp. act. was expressed as enzyme units per mg of protein.

Estimation of protein. Protein content in crude extracts was determined by the method of ref. [32]. In the later stages of purification, the protein concn was calculated from the *A* of the soln at 280 nm.

Enzyme purification. Extraction (step 1): The 40000 *g* supernatant obtained from 50 g leaf tissue served as starting material. To the crude extract solid $(NH_4)_2SO_4$ was added with stirring to a final concn of 35% and the soln was kept for 3 hr in the cold room. The ppt. was removed by centrifugation at 8000 *g* for 30 min. Then, solid $(NH_4)_2SO_4$ was again added to the supernatant to 80% satn and the soln was allowed to stand overnight. The ppt. was collected by centrifugation, dissolved in 3 ml 0.05 M Tris–HCl (pH 7.5) and dialysed overnight against 5 l. 0.01 M Tris–HCl (pH 7.5). The insoluble material was discarded after centrifugation.

Chromatography on Sephadex G-200 (step 2): The soln obtained in step 1 was loaded on a 2.4 × 100 cm Sephadex G-200 column equilibrated with 0.01 M Tris–HCl buffer (pH 7.5). Elution was carried out with the same buffer at a flow rate of 18 ml/hr. Fractions (3 ml) were collected and analysed for protein content and enzyme activity. Three separate peaks of phosphomonoesterase activity were found (Fig. 1).

Chromatography on DEAE-cellulose (step 3): The fractions corresponding to peaks 2 and 3 were pooled and applied to a DEAE-cellulose column (2 × 20 cm). Elution was carried out by a linear NaCl gradient, 0–0.5 M NaCl (pH 7.5). Elution was continued with 100 ml M NaCl. A flow rate of 15 ml/hr was maintained and 3 ml fractions were collected. Three peaks of enzyme activity emerged. Samples from peaks 1 and 2 were further purified separately.

Rechromatography of first phosphomonoesterase fraction on DEAE-cellulose (step 4): Combined fractions of peak 1 from the previous step were dialysed against 0.01 M Tris–HCl (pH 7.5) overnight. The soln was then applied to the same DEAE-cellulose column used earlier and the elution was repeated using a linear NaCl gradient, 0–0.5 M (pH 7.5). As a result, a single peak was obtained.

Rechromatography of second phosphomonoesterase fraction on DEAE-cellulose (step 5): Combined fractions of the second phosphomonoesterase peak from step 3 were also pooled, dialysed and rechromatographed as in step 4. Two peaks of enzyme activity emerged. The main peak was studied in detail. Deep frozen enzyme prepns were used for all later investigations.

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