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PURIFICATION AND PROPERTIES OF ACID PHOSPHATASE FROM CULTURED TOBACCO CELLS

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Abstract—One component of acid phosphatase was purified from cultured tobacco cells. The purified enzyme was homogeneous on polyacrylamide gel electrophoresis with or without sodium dodecyl sulfate. The enzyme possesses high activity toward nucleoside di- and triphosphate, much less activity toward nucleoside monophosphates and sugar esters. The MWs of the phosphatase determined by Sephadex G-100 gel filtration and dodecyl sulfate gel electrophoresis were 74000 and 76000, respectively. The phosphatase showed high affinity for concanavalin A-Sepharose and single superimposed bands of protein and carbohydrate on gel electrophoresis, suggesting that it is a glycoprotein.

INTRODUCTION

Previously we have reported purification and properties of phosphodiesterase from cultured tobacco cells [1, 2]. The phosphodiesterase also shows pyrophosphatase activity and hydrolyses NAD^+, ATP and inorganic pyrophosphate. During the course of the study, it was found that the tobacco cells possess acid phosphatase activity that also hydrolyses ATP, ADP and inorganic pyrophosphate, and the cultured cells contain higher activity of acid phosphatase than tissues of the intact plant. In order to clarify its properties further, the acid phosphatase was purified. Acid phosphatase from a number of plant tissues have been reported [3–8]. However, only a few enzymes have been purified to a homogeneous state [7, 8]. The present paper describes the purification and properties of acid phosphatase from cultured tobacco cells.

RESULTS

Purification of acid phosphatase

The results of a typical purification of acid phosphatase are summarized in Table 1. At the final step, the enzyme was purified 55-fold over the crude extract with an overall yield of 4%. The purified enzyme preparation migrated as a single protein band on 7.5% polyacrylamide gel electrophoresis at pH 8.3. All the phosphatase activity was found in the region corresponding to the protein band. A single protein band was also detected on electrophoresis at pH 8.3 in 5% acrylamide gel, and in SDS polyacrylamide gel electrophoresis.

Properties of acid phosphatase

The purified phosphatase migrated as a single protein band on polyacrylamide gel electrophoresis, and an identical gel stained with periodic acid-Schiff reagent showed a single band of carbohydrate in exactly the same position as the protein band.

Concanavalin A is known to bind to glycoproteins with a terminal α-D-mannose or α-D-glucose residues [9]. When the purified enzyme was applied to concanavalin A-Sepharose column equilibrated with 10 mM Tris–HCl buffer (pH 7.5), the enzyme was adsorbed to the column and eluted with 0.5 M α-methyl-D-mannopyranoside in the same buffer. These results indicated that the acid phosphatase is a glycoprotein.

The MW of the phosphatase was determined by gel filtration on a Sephadex G-100 column by the method of Andrews [10] using the following proteins as markers; yeast alcohol dehydrogenase (MW 150000); BSA (MW 68000); ovalbumin (MW 45000); cytochrome *c* (MW 12500). The column was eluted with 10 mM Tris–HCl buffer (pH 7.5) containing 0.2 M NaCl. The MW was estimated to be 74000.

It has been shown that some glycoproteins show anomalous migration on SDS gel electrophoresis [11, 12]. In order to detect anomalous migration, the phosphatase and reference proteins were examined at different acrylamide gel concentrations to determine free electrophoretic mobilities. When the logarithms of the mobilities of the proteins were plotted against the gel concentrations

Table 1. Purification of acid phosphatase from cultured tobacco cells

Fraction	Volume (ml)	Total activity (units)	Specific activity (units/mg)
I Crude extract	1000	970	3.2
II Ammonium sulfate	100	681	3.4
III DEAE-cellulose	30	248	9.5
IV Phosphocellulose	50	127	33.9
V Hydroxylapatite	5	42	100
VI 2nd hydroxylapatite	2.5	38	177

Table 2. Substrate specificity of tobacco acid phosphatase

Substrate	Relative activity* (%)
p-Nitrophenylphosphate	130
2′-AMP	1.9
3′-AMP	12.1
5′-AMP	0.8
5′-ADP	80.3
5′-ATP	100
Glucose 6-phosphate	5.7
Fructose 1-phosphate	5.7
Inorganic pyrophosphate	64.4
NAD^+	0
Adenosine 3′,5′-cyclic phosphate	0
Adenosine 2′,3′-cyclic phosphate	0

* Activities are expressed relative to that obtained with ATP.

the plots for all the proteins were linear. The free electrophoretic mobilities for the phosphatase is identical with those of standard proteins subjected to the electrophoresis. Therefore it is possible to determine the MW of the enzyme by electrophoresis. The MW of the phosphatase is estimated to be 76000 using following standard proteins; phospholylase a (subunit MW 94000); BSA (MW 68000); ovalbumin (MW 45000); chymotrypsinogen (MW 25000).

The activity of acid phosphatase toward various substrates is shown in Table 2. *p*-Nitrophenylphosphate gives the highest activity, and ATP, ADP and inorganic pyrophosphate are also significantly hydrolysed. The enzyme is only weakly active toward nucleoside monophosphates and sugar phosphates. It showed much less activity toward 2′- and 5′-AMP than toward 3′-AMP. The enzyme shows no activity toward NAD^+, adenosine 2′,3′-cyclic phosphate and adenosine 3′,5′-cyclic phosphate when they were incubated with 20 times the amount of enzyme required to obtain marked activity toward 5′-AMP.

The enzyme had a pH optimum of 4.7 in 0.2 M acetate buffer with *p*-nitrophenylphosphate as substrate.

Table 3. Effect of various compounds on the activity of tobacco acid phosphatase

Addition	Concentration (mM)	Relative Activity* (%)
$ZnCl_2$	1	35
$FeCl_2$	1	49
$CuSO_4$	1	14
$MnCl_2$	1	73
$CaCl_2$	1	91
$MgCl_2$	1	79
EDTA	10	89
$(NH_4)_6Mo_7O_{24}$	0.1	0
KH_2PO_4	10	24
NaF	10	12
2-Mercaptoethanol	10	100
Ascorbate	10	100

* Enzyme activity with no addition was taken as 100%.

The influence of *p*-nitrophenylphosphate concentration on the reaction velocity was examined. Lineweaver and Burk plots [13] were linear, and the *Km* value for *p*-nitrophenylphosphate is calculated to be 5.6×10^{-4} M.

The effect of various substances on activity is shown in Table 3. Various divalent metal ions examined inhibited the enzyme activity. Addition of EDTA to the reaction mixture caused 10% inhibition at 10 mM. Fluoride, phosphate and particularly molybdate inhibited the activity.

DISCUSSION

The cultured tobacco cells produce several forms of acid phosphatase which can be separated by ion-exchange column chromatography. Among them, one major component of acid phosphatase was purified to a state which appeared homogeneous on polyacrylamide gel electrophoresis with or without SDS. The isolated acid phosphatase is much less active toward some phosphomonoesters such as nucleoside monophosphates and sugar phosphates than the other plant acid phosphatases previously reported [3–5, 7, 8]. The tobacco phosphatase hydrolyses 3′-AMP most rapidly among the 3 isomers of adenylic acid and has very little activity on 2′- and 5′-AMP. As with the other plant acid phosphatases, the enzyme readily hydrolyses terminal pyrophosphate bonds of ATP and ADP, but it is not active toward NAD^+, adenosine 3′,5′-cyclic phosphate and adenosine 2′,3′-cyclic phosphate. A phosphodiesterase from the cultured tobacco cells that hydrolyses *p*-nitrophenyl thymidine 5′-phosphate, *p*-nitrophenyl thymidine 3′-phosphate and cyclic nucleotides, is also shown to have pyrophosphatase activity, and it hydrolyses ATP, ADP and NAD^+ [1]. Although the phosphodiesterase also hydrolyses *p*-nitrophenylphosphate, it shows no activity toward the other phosphomonoesters. Therefore, in higher plants, activity toward *p*-nitrophenylphosphate cannot be regarded as proof of existence of phosphatase.

The tobacco acid phosphatase is a glycoprotein as shown by periodic acid-Schiff staining after gel electrophoresis and affinity toward concanavalin A-Sepharose. Acid phosphatase from rice cultured cells was also reported to be a glycoprotein [8].

The MWs of the tobacco phosphatase determined by gel filtration and SDS gel electrophoresis were 74000 and 76000, respectively. The results indicated that the enzyme consists of a single polypeptide chain.

EXPERIMENTAL

Cells of tobacco (*Nicotiana tabacum*, cv. Bright yellow) were cultured in suspension as described [1].

Enzyme assay. Enzyme activity was routinely estimated by using *p*-nitrophenylphosphate as substrate. The standard reaction mixture contained 0.1 M NaOAc buffer (pH 5), 0.5 mM *p*-nitrophenylphosphate and enzyme in a total vol. of 1 ml. After incubation at 30° for 5 min, the reaction was stopped by the addition of 2 ml of N NaOH and the *A* at 400 nm measured. One unit of enzyme activity was defined as the amount which liberated 1 μmol of *p*-nitrophenol per min. A molar extinction coefficient of 18000 was used in the calculation [1]. When phosphate esters other than *p*-nitrophenylphosphate were used as substrate, phosphatase activity was assayed by measuring the amount of Pi liberated by the procedure of ref. [14]. Enzyme activity toward NAD^+ and cyclic nucleotides was also estimated as described in [1].

Protein determination. Protein was determined by the method of ref. [15] or [16] as previously described in [1].

Polyacrylamide gel electrophoresis was carried out at pH 8.3 according to ref. [17]. SDS gel electrophoresis was performed as described in ref. [18]. Protein samples subjected to the electrophoresis in SDS were denatured by heating at 100° for 3 min in the presence of 1% SDS and 5% 2-mercaptoethanol. The gels were stained for protein with Coomassie blue. Periodic acid-Schiff staining followed the method of ref. [19]. The gels were stained for enzyme activity as described in [20].

Purification of acid phosphatase. All operations were carried out between 0 to 4°.

Step 1. *Crude extract.* Frozen tobacco cells (1 kg fr. wt) were thawed and suspended in 400 ml of 0.1 M NaOAc buffer (pH 5) containing 0.2 M NaCl and 14 mM 2-mercaptoethanol. The cell suspension was homogenized in a Waring blendor for 4 min. The homogenate was centrifuged at 6700 *g* for 20 min and the supernatant collected (fraction 1).

Step 2. $(NH_4)_2SO_4$ *precipitation.* To fraction 1 solid $(NH_4)_2SO_4$ was added with stirring to give 80% satn. After stirring for 30 min, the ppt. formed was collected by centrifugation at 6700 *g* for 1 hr and dissolved in 90 ml of 10 mM Tris–HCl buffer (pH 7.5) containing 14 mM 2-mercaptoethanol (Buffer A). This prepn was dialysed for 18 hr against Buffer A. The dialysate was clarified by centrifugation at 20000 *g* for 15 min to yield fraction 2.

Step 3. *DEAE-cellulose column chromatography.* Fraction 2 was applied to a DEAE-cellulose column (3 × 8 cm) previously equilibrated with Buffer A. The column was washed with the buffer and then eluted with 200 ml of a linear gradient of 0.01–0.25 M Tris–HCl buffer (pH 7.5) containing 14 mM 2-mercaptoethanol. The phosphatase activity was resolved into 2 components by this chromatography. Fractions from the first major peak, 60% of the acid phosphatase activity, eluted with 0.08 to 0.12 M Tris–HCl buffer were pooled and dialysed against Buffer A (fraction 3). The second peak, 40% of the activity, was not studied further.

Step 4. *Phosphocellulose column chromatography.* Fraction 3 (30 ml) was applied to a phosphocellulose column (2 × 5 cm) previously equilibrated with Buffer A. The column was washed with the buffer and eluted with a linear gradient of 0–0.3 M NaCl in Buffer A. About 30% of the activity was not adsorbed to the column and the other 70% of the activity was eluted with 0.2–0.25 M NaCl. The latter fractions eluted from the column with NaCl were pooled to yield fraction 4.

Step 5. *Hydroxylapatite column chromatography.* Fraction 4 (40 ml) was directly applied to a hydroxylapatite column (2 × 4 cm) previously equilibrated with Buffer A. The column was washed with 10 mM Pi buffer (pH 7.6) and then eluted with 200 ml of a linear gradient of 0–0.2 M Pi buffer (pH 7.6). The enzyme was eluted with 0.08–0.12 M Pi buffer. The fractions with the activity were combined and dialysed against Buffer A (fraction 5).

Step 6. *Rechromatography on hydroxylapatite column.* Fraction 5 was applied to a hydroxylapatite column previously equilibrated with Buffer A. The column was washed with the buffer and elution carried out with a linear gradient of 0–0.4 M $(NH_4)_2SO_4$ in Buffer A. The enzyme was eluted with 0.2–0.25 M $(NH_4)_2SO_4$, and the fractions with activity were pooled and concd by ultrafiltration on a PM-10 Diaflo membrane and used for enzyme characterization.

REFERENCES

1. Shinshi, H., Miwa, M., Katō, K., Noguchi, M., Matsushima, T. and Sugimura, T. (1976) *Biochemistry* **15**, 2185.
2. Shinshi, H., Katō, K., Miwa, M., Matsushima, T., Noguchi, M. and Sugimura, T. (1977) *Biochim. Biophys. Acta* **495**, 71.
3. Newmark, M. Z. and Wenger, D. S. (1960) *Arch. Biochem. Biophys.* **89**, 110.
4. Roussos, G. G. (1962) *Biochim. Biophys. Acta* **64**, 187.
5. Shaw, J. G. (1966) *Arch. Biochem. Biophys.* **117**, 1.
6. Bieleski, R. L. and Johnson, P. N. (1972) *Aust. J. Biol. Sci.* **25**, 707.
7. Uehara, K., Fujimoto, S. and Taniguchi, T. (1974) *J. Biochem.* **75**, 627.
8. Igaue, I., Watabe, H., Takahashi, K., Takekoshi, M. and Morota, A. (1976) *Agric. Biol. Chem.* **40**, 823.
9. Goldstein, I. J., Hollerman, C. E. and Merrick, J. M. (1965) *Biochim. Biophys. Acta* **97**, 68.
10. Andrews, P. (1964) *Biochem. J.* **91**, 222.
11. Segrest, J. P., Jackson, R. L., Andrews, E. P. and Morchesi, V. T. (1971) *Biochem. Biophys. Res. Commun.* **44**, 390.
12. Frank, R. N. and Rodbard, D. (1975) *Arch. Biochem. Biophys.* **171**, 1.
13. Lineweaver, H. and Burk, D. (1934) *J. Am. Chem. Soc.* **56**, 658.
14. Josse, J. (1966) *J. Biol. Chem.* **241**, 1938.
15. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. (1951) *J. Biol. Chem.* **193**, 265.
16. Kalckar, H. M. (1947) *J. Biol. Chem.* **167**, 461.
17. Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404.
18. Weber, K. and Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406.
19. Zacharius, R. M., Zell, T. F., Morrison, J. H. and Woodlock, J. J. (1969) *Analyt. Biochem.* **30**, 148.
20. Polya, G. M. (1975) *Biochim. Biophys. Acta* **384**, 443.