CHARACTERIZATION AND LOCALIZATION OF ACID PHOSPHATASES IN THE SUSPENSION-CULTURED CELLS OF *NICOTIANA OTOPHORA* L.

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Abstract: Active cells were havested from the tobacco cell suspension culture. The acid phosphatase of tobacco cells was mostly located in the cell wall and vacuole. Similar zymogram of acid phosphatase revealed in the gradient polyacrylamide gel was observed between the tobacco leaves and their induced-callus. Multiple acid phosphatases of tobacco suspension cells separated by chromatography showed similar substrate specificties to most compounds tested. Zn^{+2} has differential inhibition on the multiple forms of acid phosphatase of tobacco cells.

INTRODUCTION

Acid phosphatase (EC 3. 1. 3. 2.) distributed in plant tissues, and the level present in the tissue varies in response to physiological status. In the previous reports, a high molecular weight acid phosphatase was significantly in the stressed tissues (Pan, 1985; Pan, 1987). In wheat, water stress caused an increase in only one of several phosphatases (Barrett-Lannard *et al.*, 1982); in cultured tobacco cells, phosphorus deficiency caused an increase in one of the extracted phosphatases (Katsuyi and Sato, 1977). Accordingly, not all isoenzymes are affected by the same environmental changes.

Besford (1979) has suggested that the soluble acid phosphatase activity may be useful as a biochemical index of phosphorus deficiency. We are looking for the specific isoenzyme of acid phosphatase having the potential as a biochemical index of developmental stage in tobacco cells. The cellular location of soluble acid phosphatase are also unclear in the tobacco cells. In this study, the subcellular localization of acid phosphatase of tobacco suspension cells by the methods of electron microscopic cytochemistry and differential centrifugation was examined; the multiple forms of acid phosphatases were resolved by column chromatography and gel electrophoresis. Substrate specificity and some metal ions effect on the multiple forms of acid phosphatases were investigated.

MATERIALS AND METHODS

Cells and culture conditions

The suspension cells of *Nicotiana otophora* L. were cultured in B5 medium (Gamborg *et al.*, 1968). Cultures were incubated in the light at 26°C on a reciprocal shaker operating at 120 rpm. The cells were harvested from the culture flasks at intervals, the cell volume (Street, 1977) and triphenyl tetrazolium chloride reduction (Wu and Wallner, 1983) of each sample were measured respectively. Then frozen at liquid nitrogen, and stored at −20°C until enzyme extraction and protein determination.

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Electron microscopic study

Fresh suspension cells were prefixed with cacodylate buffered 2.5% glutaraldehyde for 90 min and washed in 0.05 M cacodylate buffer overnight. They were then transferred to the incubating media mixtured with 1.5 mM *p*-nitrophenol phosphate (*p*NPP), 2.0 mM $Pb(NO_3)_2$ and 0.05 mM acetate buffer (pH 5.8) for 60 min at 37°C. The incubated material were washed with cold vernol buffer, fixed with veronal buffered OsO_4 for one hr, dehydrated in a series of graded ethanol solutions and then embedded in the spurr resin (Miyayama *et al.*, 1975). Ultrathin sections were obtained by the MT 5000 Ultramicrotome, sections were double-stained with uranyl acetate and lead citrate and examined with the Hitach H 600 electron microscope.

Subcellular fraction preparation

All operations were performed at 4°C. The freshly harvested tobacco suspension cells were homogenized with a motar and pestle in a grinding medium (2 ml/g) containing 0.10 M Tris-HCl (pH 7.5), 10 mM 2-mercaptoethanol and 0.5 M sucrose. This procedure caused minimal damage to the organelles (Huang and Beevers, 1971). The homogenate was filtered through four layers of cheese-cloth and centrifuged at 1000 g for 10 min. The supernatant fraction was recentrifuged at 10,000 g for 30 min. Each pellet fraction was resuspended in 10 mM Tris-HCl (pH 7.2), 10 mM 2-mercaptoethanol (buffer A).

Enzyme preparation

The liquid-nitrogen-frozen tobacco suspension cells, tobacco leaves or the induced-callus were ground with the grinding medium (2 ml/g). The homogenate was filtered through two layers of cheesecloth and centrifuged at 10,000 g for 30 min. The supernatant was taken for measurement of enzyme activity and protein content. For electrophoresis, 5-10 g of tobacco leaves or the induced-callus was ground, and the soluble fraction was precipitated with 70% saturation ammonium sulfate. The precipitate was dissolved in a minimal amount of buffer A, and dialyzed against buffer A overnight. The dialyzate was taken for analytical electrophoresis.

Column chromatography

The soluble protein of tobacco suspension cells was fractionated with 70% saturation ammonium sulfate. The resulting precipitate was dissolved in a small amount of buffer A, and dialyzed against buffer A overnight, changed buffer three times. The dialyzate was applied to a DEAE-Sepharose CL 6B column (2.2×16 cm), which was preequilibrated with the buffer A. After sample application, 200 ml of buffer A was used to elute first, the 500 ml of a 0 to 0.5 N linear gradient of NaCl in the buffer A as the eluant. The active fractions were collected.

Polyacrylamide disc gel electrophoresis (PAGE)

Analytical disc gel electrophoresis on 7.5% acrylamide gel by the procedure (Gabriel, 1971) or a 7 to 15% linear gradient gel according to the method of Lambin, and Fine (1979) was carried out. Slab gel 0.75 mm thick was run at 200 V for 6 hr at room temperature. For staining acid phosphatase, the slab gel was incubated in the 100 ml of reaction solution containing 0.2 M sodium acetate buffer (pH 5.4), 0.1% α-naphthyl phosphate, 0.1% Fast red TR salt, and 5 mM $MgCl_2$ overnight (Cullis and Kolodynska, 1975).

Assay of acid phosphatase activity

The assay medium consisted of 100 mM sodium acetate buffer (pH 5.4), appropriate amount of enzyme preparation and 5 mM *p*NPP in a final volume of 1.0 ml. The reaction was initiated by adding 0.1 ml of substrate in the medium at 37°C and after 20 min incubation, it was terminated by the addition of 4.0 ml of 0.3 M Na_2CO_3. The unit of enzyme activity was expressed as micromoles of *p*NPP hydrolyzed per hr under the conditions described above. The amount of *p*-nitrophenol liberated in the assay medium was determined spectrophotometrically at 400 nm, taking $4.0\times10^3\ M^{-1}\ cm^{-1}$ as the molar extinction coefficient for *p*-nitrophenol. When substrate other than *p*NPP were used in the assay, the reaction was terminated by heating at the boiling water for 20 sec. After the precipitate was removed by centrifugation, an aliquot was taken for determination of inorganic phosphorus (Taussky and Shorr, 1953).

Determination of protein content

Protein content was determined either by Bradford's method (1976), using bovine serum albumin as a reference protein, or by measuring the samples' absorbance at 280 nm in each fraction of column chromatography.

RESULTS

Fig. 1 showed that the growth of tobacco suspension cells reached to the optimum on the day 6, which was measured by the cell density or by packed cell volume per ml of cell suspension. The capacity of reducing TTC by tobacco cells started to decline on

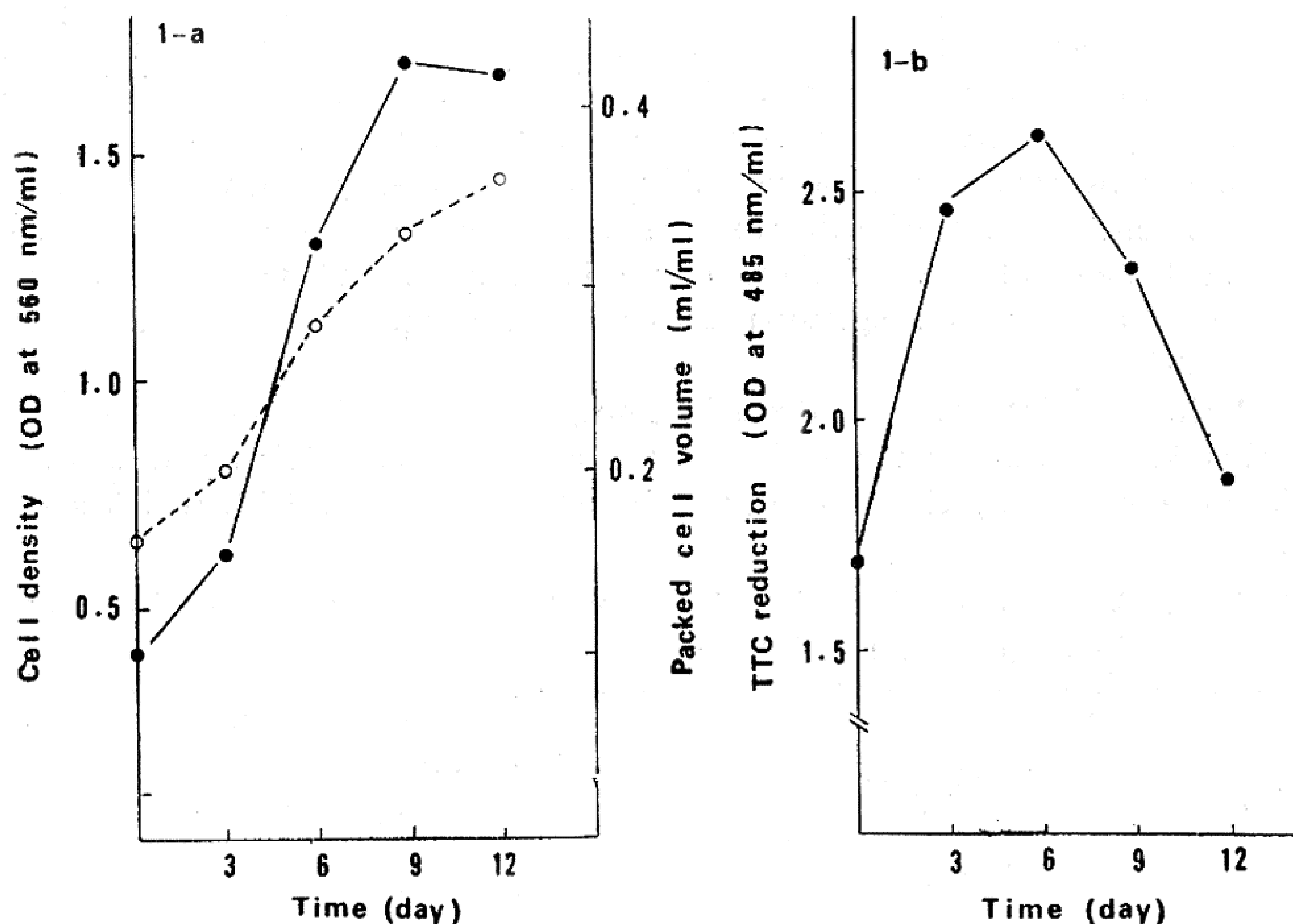

Fig. 1. The growth of tobacco cells in suspension culture
1-a: Cell density (●—●—●) or packed cell volume (○---○---○) vs. time
1-b: Capacity of TTC reduction vs. time

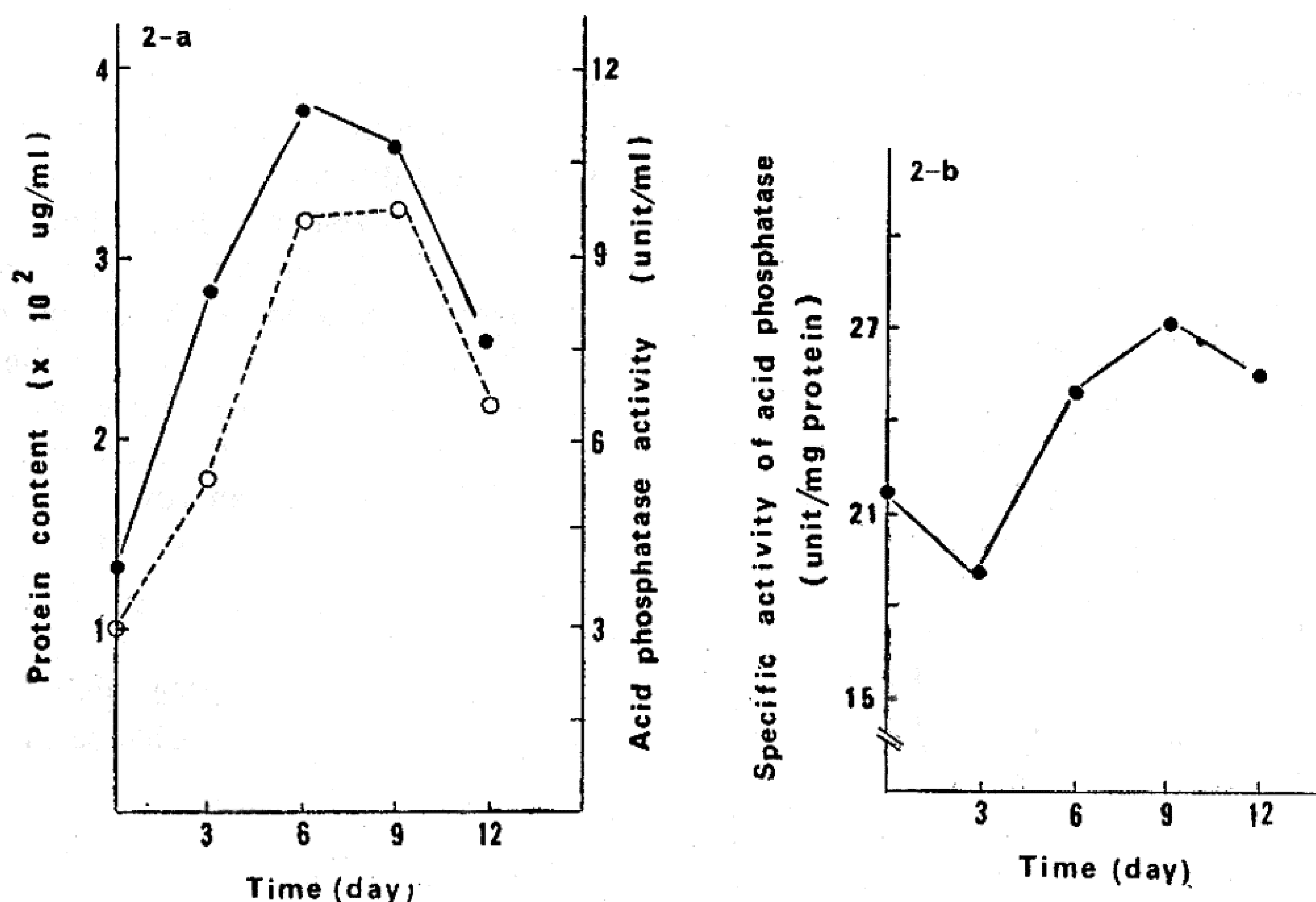

Fig. 2. Acid phosphatase of tobacco cells in suspension culture
2-a: Protein (●—●—●) or acid phosphatase (○---○---○) vs. time
2-b: Specific activity of acid phosphatase vs. time

the day 6 of growth under the experimental conditions (Fig. 1-b). This indicated that the cells started to senescence gradually. Therefore, the tobacco suspension cells collected on the day 6 of growth were taken to carry out the experiments followed. The activity profile of acid phosphatase of tobacco cells during the 12-day growth paralleled to that of protein content per ml of cell suspension culture (Fig. 2-a). The lowest specific activity of acid phosphatase was on the day 3 of growth, and the specific activity of acid phosphatase of tobacco suspension cells did not change greatly after 6 days of cell growth (Fig. 2-b).

Table 1 showed that most of the acid phosphatase of tobacco cells was located in the 10 Kg soluble fraction by the differential centrifugation. This result was quite consistent to that obtained by electron microscopic study (Fig. 3-a, 3-b), which showed that

Table 1. The distribution of acid phosphatase of tobacco suspension cells in the different cellular fractions

Fraction	Total protein (mg)	Acid phosphatase		
		Total activity		Specific activity
		unit	% of total	unit/mg protein
Crude extract	165.76	3938.40	100	23.91
1000 g pellet	25.06	369.42	0.93	14.73
10000 g pellet	15.22	148.05	0.37	9.72
10000 g soluble	98.58	3490.50	87.98	35.40

* 91.74 g fresh weight of tobacco suspension cells was used.

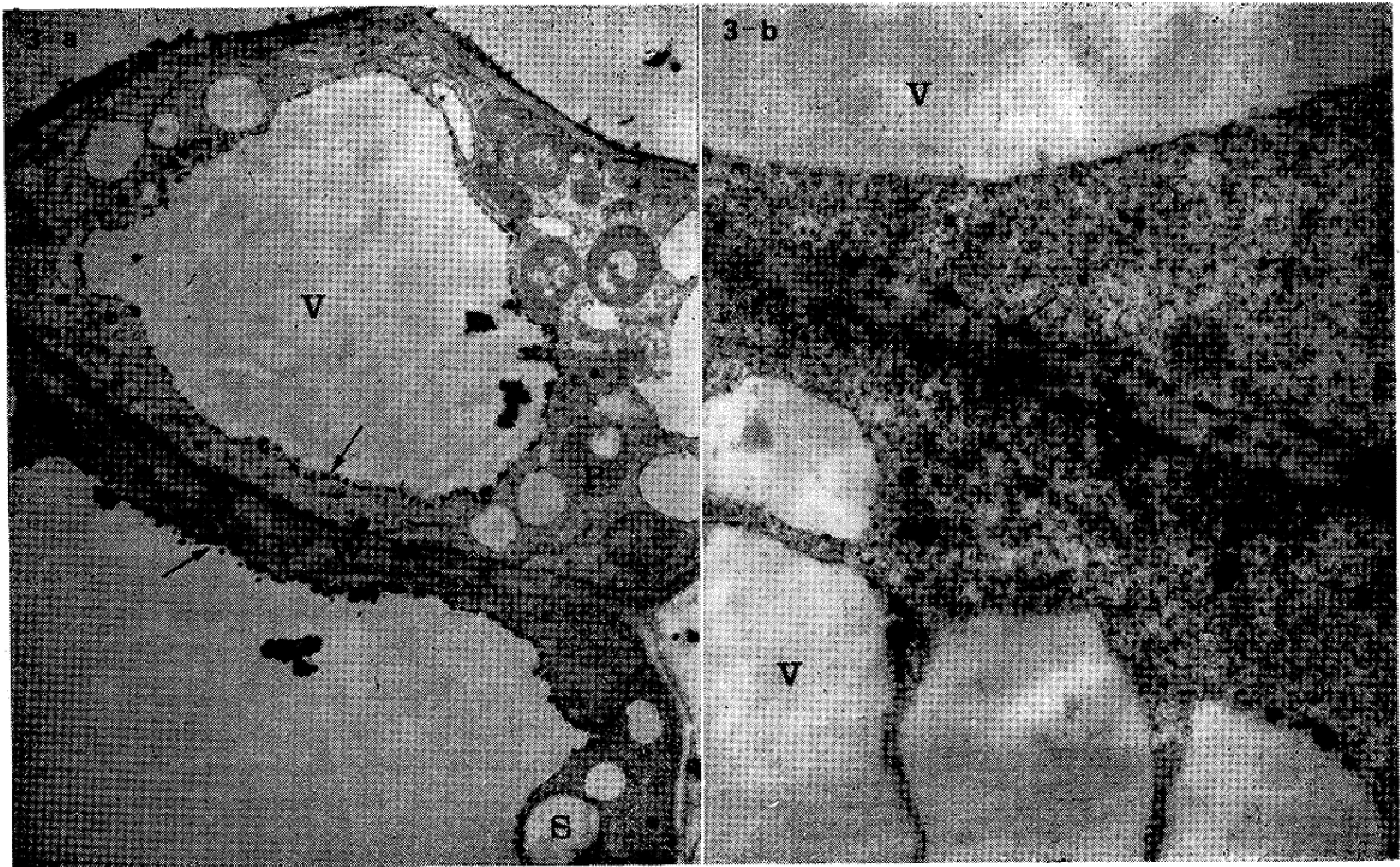

Fig. 3. Parts of a tobacco suspension cell. Acid phosphatase localization by the use of the substrate pNPP; P, plastid; S, starch granule; V, vacuole; W, cell wall; the arrowhead denotes the localization of acid phosphatase.
3-a: The localization of acid phosphatase in vacuole. ×3,400
3-b: The localization of acid phosphatase in cell wall. ×20,400

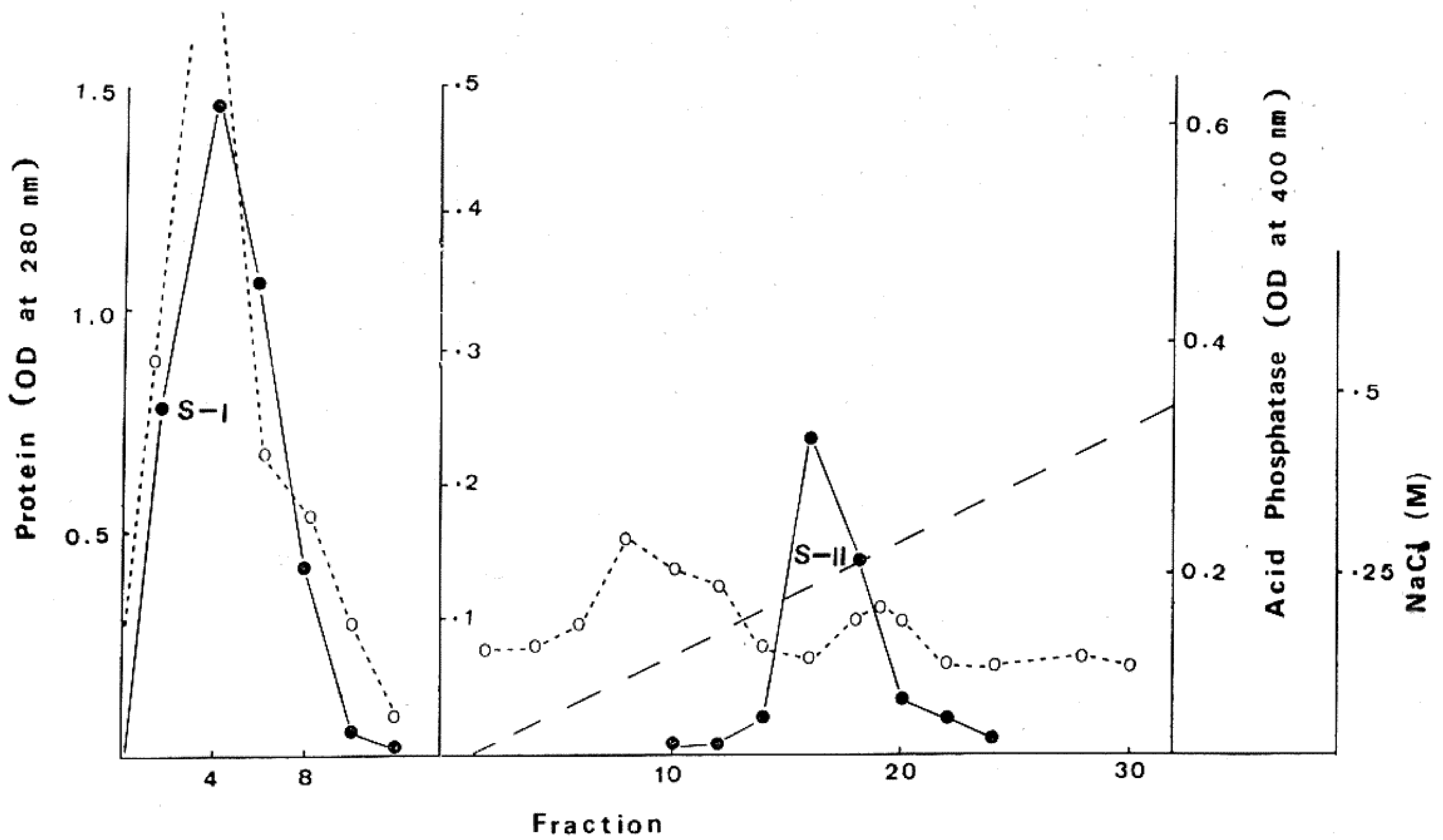

Fig. 4. DEAE-Sepharose CL 6B chromatogram of tobacco acid phosphatase from suspension cells. A 0.0 to 0.5 N NaCl gradient in 10 mM Tris-HCl, pH 7.2 as eluant, protein (○---○---○) and acid phosphatase (●—●—●), gradient concentration (— — —).

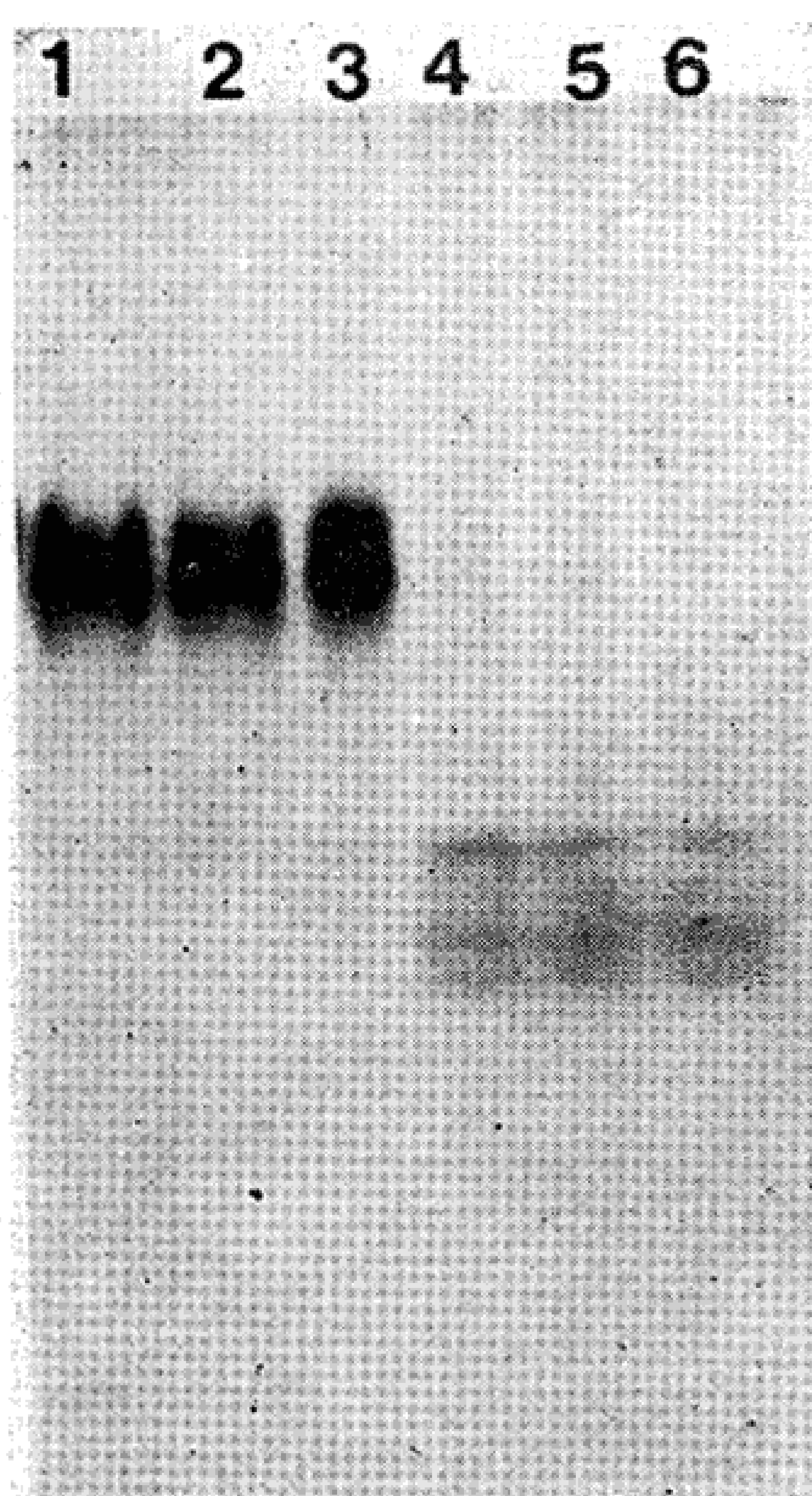

Fig. 5. Zymogram of disc 7.5% PAGE of tobacco acid phosphatase.
Lane 1-3: DEAE-unadsorbed fraction having acid phosphatase activity (S-I), containing 50, 60 and 100 μg protein
Lane 4-6: DEAE-adsorbed fractions having acid phosphatase activity (S-II), containing 30, 25 and 20 μg protein

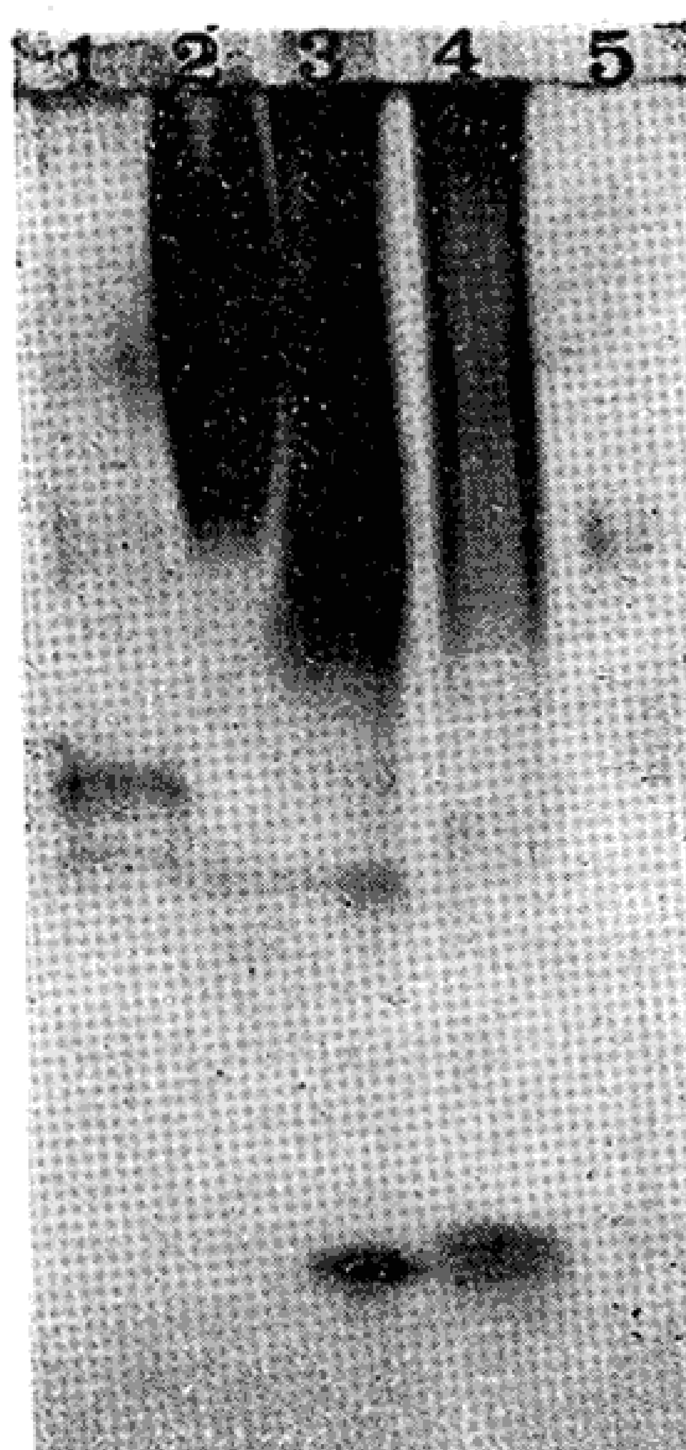

Fig. 6. Zymogram of 7 to 15% linear gradient PAGE of tobacco acid phosphatase.
Lane 1: DEAE-adsorbed fractions showing acid phosphatase activity (S-II)
Lane 2: partial purified acid phosphatase of rice milky grains
Lane 3: tobacco leaves extract
Lane 4: tobacco callus extract
Lane 5: DEAE-unadsorbed fraction showing acid phosphatase activity (S-I)

the small black particles in the vacuole (Fig. 3-a) and cell wall (Fig. 3-b) were the reaction product of the acid phosphatase localization.

Two fractions with phosphatase activity were obtained by DEAE-Sepharose CL 6B chromatography (Fig. 4), the non-adsorbed fraction (S-I) contained the major portion of the acid phosphatase. This fraction was further separated into two isoenzymes, which were the slow-moving ones in the disc 7.5% PAGE (Fig. 5). There were possibly three isoenzymes in the DEAE-adsorbed fraction (S-II), which were eluted starting at 0.2 N NaCl-containing buffer A (Fig. 4). And there were the fast-moving ones in the disc PAGE (Fig. 5). Similar multiple acid phosphatases of tobacco leaves and the induced-callus were observed in the 7 to 15% gradient PAGE (Fig. 6). There were three groups of acid phosphatase separated on the basis of molecular weight, since linear polyacrylamide gels offered the possibility to determine the molecular weight of a native protein (Lambin and Fine, 1979). Partially purified acid phosphatase from rice milky grains seemed apparently different from those of tobacco's.

Eight phosphoric esters were tested to determine the substrate specificities of the two forms of acid phosphatase, which were obtained from the DEAE-Sepharose CL 6B chromatography. The strong pyrophosphatase and ATP phosphatase were present in S-I. S-I and S-II showed the similar substrate specificities against most compounds tested (Table

Table 2. Substrate specificity of two forms of acid phosphatase of tobacco suspension cells

Substrate	Relative activity (%)	
	S-I	S-II
p-Nitrophenyl phosphate	100	100
β-Glycerophosphate	1	2
Glucose-1-phosphate	22	40
Glucose-6-phosphate	5	11
Fructose-6-phosphate	20	35
Fructose-1, 6-diphosphate	22	34
ATP	140	108
Pyrophosphate	143	102

S-I, S-II refer to acid phosphatase activity peaks of tobacco cell suspension, which are eluted in position corresponding to those shown in Fig. 4

Table 3. Effect of various compounds on the activity of two forms of acid phosphatase of tobacco suspension cells

Compound added (2.0 mM)	Relative activity (%)	
	S-I	S-II
None	100	100
$MgCl_2$	94	104
$CaCl_2$	88	100
$FeSO_4$	89	100
$ZnSO_4$	42	77
$CuCl_2$	50	57
Na_2MoO_4	29	36
KH_2PO_4	46	42
NaF	73	70

The activities are expressed as percent against the no-addition run.

2). Mg^{+2}, Ca^{+2}, and Fe^{+2} showed slight effect on the activity of S-I and S-II. The inhibitory effect of Zn^{+2} was much severe to S-I than to S-II. Mo^{+6}, Cu^{+2}, F^{-1} and Pi showed clear inhibition to both types of acid phosphatase (Table 3).

DISCUSSION

Cellular location of acid phosphatase was mostly in the vacuole of pea leaves (Noble and Dalling, 1982); acid phosphatase was found inside chloroplasts of stressed cotton leaves, but not in nonstressed plant (Vieira da silva *et al.*, 1974); acid phosphatase of cell wall, mitochondria and microsomal fractions of bean hypocotyl were partially purified (Mizuta and Suda, 1980). But, there was no report concerning the location of acid phosphatase in the tobacco cells. By using combinations of electron microscope cytochemistry and differential centrifugation, we concluded that vacuole and cell wall of the healthy suspension cells of *Nicotiana otophora* were the major location of acid phosphatase.

In answering the question of whether the specific isoenzyme of acid phosphatase for each developmental stage of tobacco exists, we compared the acid phosphatase of tobacco leaves, the induced-callus, and derived-suspension cells. The result obtained from the gradient PAGE showed that quite similar multiple acid phosphatases were present in each enzyme preparation which was collected from the 70% saturation ammonium sulfate precipitation. Unfortunately, the fraction precipitated with 70% saturation ammonium sulfate from tobacco suspension cells was not run in the above gel. However, the two portions containing acid phosphatase obtained from the DEAE-Sepharose CL 6B chromatography of the 70% ammonium sulfate fractionated from tobacco suspension cells can represent most or part of the above fraction. A low molecular weight (LMW) acid phosphatase observed in both the callus and leaves was not found in the tobacco suspension cells. Two possibilities can be proposed, one: this LMW acid phosphatase was not present in the suspension cells, the other alternative: this LMW acid phosphatase can not be eluted by 0.5 N NaCl-containing buffer A. Accordingly, it was not successful to find out the specific isoenzyme of acid phosphatase in tobacco different tissues at this moment.

Both types of acid phosphatase showed similar substrate specificities to the most compounds tested. It was interesting to observe the high pyrophosphatase and ATPase in S-I. This was observed from that of spinach leaves (Pan, 1987). The pH optimum of S-I and S-II was at pH 4.0, and above pH 5.0 the activity of both types of acid phosphatases was decreased sharply (data not shown). The cellular locations of each of multiple acid phosphatase are not clear at this moment. However, differences in substrate specificity and response of acid phosphatase to inhibitors could be of use in their histochemical localization.

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培養菸草細胞中酸性磷解酶的鑑定及細胞定位

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摘要

自菸草懸浮培養中收集之細胞，發現其酸性磷解酶大部分位於液泡及細胞壁。菸草葉片及其癒合組織經由聚丙烯醯胺梯度電泳分析，發現其酸性磷解酶之同功酵素圖譜類似。而且菸草懸浮培養之細胞中之酸性磷解酶以管柱色層析法可分出多種酸性磷解酶。它們具有相似之基質特異性，然而鋅離子對它們却表現不同程度之抑制。