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PHOSPHATASES IN SPINACH LEAVES

Subcellular Localization and The Stress Effect[1]

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Abstract

The nonspecific distribution of acid phosphatase in the subcellular fraction was found in the spinach leaves. Water or salt stress imposed on the spinach leaf discs inhibited the activity of acid phosphatase. Seven isoenzymes of acid phosphatase in spinach leaves were observed in a polyacrylamide gel electrophoresis. One of the DEAE-cellulose-adsorbed isoenzymes tends to be increased by salt or water stress in the spinach leaf discs.

Key words: Stress; phosphatases; spinach.

Introduction

Phosphatase is widely distributed in both lower and higher organisms, suggesting that phosphatase might be involved in fundamental reactions of organisms. Many biochemical changes are associated with the exposure of plants to unfavourable conditions. Acid phosphatase activity was increased by water stress in leaves of higher plants, e.g., cowpeas (Takaoki, 1968), cotton (Vieira-de-Silva, 1969) and wheat (Barrett-Lennard *et al.*, 1982). However, the physiological role of acid phosphatase in the stressed tissue is still unknown. Kiichiro *et al.* (1966) reported the hydrolysis of phosphorylcholine by a nonspecific acid phosphatase to release choline, which is one of potential precursor of glycinebetaine accumulated in some stressed plant tissues (Wyn Jones and Storey, 1978; Wyn Jones, 1979). However, this acid phosphatase which releases choline from phosphorylcholine has not yet been discussed in the role of glycinebetaine accumulation.

Several isoenzymes of acid phosphatase was reported (Mizuta and Suda, 1980; Yamagata *et al.*, 1979). Yamaja reported one specific increase in phosphatase

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isoenzymes in cucumber roots caused by calcium deficiency (Yamaya *et al.*, 1982). The present paper is to describe the subcellular distribution of acid phosphatases in spinach, and discuss the role of phosphorylcholine phosphatase in glycinebetaine accumulation. Studies on the effect of stress on phosphatases activity and its isoenzymes are also presented.

Materials and Methods

Plant Material

Spinach leaves were obtained from a local market. For water or salt stress studies, leaf discs 2.0 cm in diameter was cut with a cork borer, and allowed to float on a mannitol solution (−20 bars) or 150 mM NaCl solution in Petri dishes. These solutions were made with half strength Hoagland's solution. The Petri dishes were placed in a culture room with 10 h photoperiod at 30°C. After one or two days, the leaf discs were removed, rinsed with distillated water, blotted dry, and frozen in liquid nitrogen.

Subcellular Fraction Preparation

All operations were performed at 4°C. The fresh spinach leaves were chopped into small pieces and homogenized with a mortar and pestle in a grinding medium (2 ml/g) containing 0.15 M Tris-HCl (pH 7.5), 2 mM DTT, and 0.5 M sucrose. This procedure caused minimal damage to the organgelles (Huang and Beevers, 1971). The homogenate was filtered through four layers of cheese-cloth and centrifuged at 500 g for 10 min. The supernatant fraction was recentrifuged at 2,000 g for 10 min, and the resulting supernatant fraction was centrifuged at 10,000 g for 30 min. Each pellet fraction was resuspended in 0.15 M Tris-HCl (pH 7.5), 2 mM DTT. The integrity of chloroplasts and mitochondria was determined by the recovery of total chlorophyll content and fumarase activity in each fraction (Quail, 1979).

Enzyme Preparation

The liquid-nitrogen-frozen leaf discs were ground with the grinding the medium (2 ml/g). The homogenate of the leaf discs 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.

Column Chromatography

The soluble protein of spinach leaf extract was fractionated with 50–70% saturated $(NH_4)_2SO_4$. The resulting precipitate was dissolved in a small amount of 10 mM potassium phosphate buffer (pH 7.5) containing 10 mM β-mercaptoethanol, and 5% glycerol, and desalted with a Sephadex G-25, which is preequilibrated with

the same buffer. The desalted solution was then applied to a DEAE—cellulose column (2.6×30 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.5) containing 10 mM β-mercaptoethanol, and 5% glycerol.

Phosphatase activity was eluted with a 0 to 0.5 N linear gradient of NaCl in the same buffer, the active fractions were collected. Phosphatase activity assayed in the fraction which did not adsorb to the DEAE—cellulose was also collected.

Polyacrylamide Disc Gel Electrophoresis

Analytical disc gel slab electrophoresis on 7.5% acrylamide gel was carried out by the procedure (Gabriel, 1971). Slab gel of 0.75 mm thick was run at 200 V for 4 h 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 5.0), 0.1% α-naphthyl phosphate, 0.1% Fast red TR salt, and 5 mM $MgCl_2$ for 3 h at 30°C (Cullis and Kolodynska, 1975).

Assay of Phosphatase

The activities of phosphatase were assayed with either β-glycerophosphate (GP), phosphorylcholine (PC) or disodium *p*-nitrophenol phosphate (PNPP) as the substrate. PNPP phosphatase activity was measured at either pH 5.0 or pH 8.5 as the measurement of acid or alkaline phosphatase (Pan, 1983). The unit of enzyme activity was expressed as micromoles of PNPP hydrolyzed per h under the conditions described above, taking $4.0\times10^{3}\ M^{-1}cm^{-1}$ as the molar extinction coefficient for *p*-nitrophenol. When GP or PC was used as substrate, the activity was assayed in a medium containing 0.1 M sodium acetate buffer (pH 5.0) and 10 mM substrate in a total volume of 2.0 ml. The reaction with GP as the substrate was started by adding enzyme and incubated for 30 min at 35°C, but when PC was used as the substrate, the incubation time was 20 h. Enzyme reaction was stopped by adding 2.0 ml of 25% trichloroacetic acid, the precipitate was removed by centrifugation, and an aliquiot was taken for inorganic phosphorus determination (Taussky and Shorr, 1953). One unit of enzyme activity is defined as the amount of enzyme which liberates one μmole of inorganic phosphorus per h under the assay condition described.

For each sample, a blank was run under identical conditions but with β-glycerophosphate or phosphorylcholine added after trichloroacetic acid to correct Pi present in the sample. The sodium salt of phosphorylcholine was obtained by Amberlite IR-120 treatment of the calcium phosphorylcholine chloride.

Protein Determination

The protein content was determined by the method of Bradford's method (Bradford, 1976). BSA was used as standard protein.

Results

Table 1 showed the distribution of acid phosphatase activity in subcellular fraction of spinach leaves. The integrity of chloroplasts and mitochondria located in the 2,000 g pellet fraction and 10,000 g pellet fraction respectively was determined by the recovery of total chlorophyll content and fumarase activity in each fraction. The result showed that 70% of total chlorophyll content and 65% of total fumarase activity were located in the resuspended 2,000 g pellet fraction and 10,000 g pellet fraction, respectively (data not shown). Table 1 indicated that most of acid phosphatase activity was found in the soluble fraction (10,000 g supernatant), and the highest specific activity was found in 2,000 g pellet fraction representing the chloroplasts fraction for GP phosphatase, but in the soluble fraction for PNPP phosphatase or PC phosphatase. The activity of PC phosphatase under the assay condition was calculated to be 63 μmole per day per g fresh weight, which was 1% of the apparent acid phosphatase using PNPP as substrate.

After two days treatment, the PC phosphatase activities in the control, the water-stressed and the salt-stressed leaf discs were respectively reduced to 70%, 67% or 38% of that in the fresh tissue (Table 2). This indicated the inhibitory effect of fresh stress on the PC phosphatase of spinach leaf discs. Table 3 shows that both the weight and protein content in the stressed tissue were decreased to some extent.

Table 1. *Subcellular localization of acid phosphatases in spinach leaves*

Fraction	PC phosphatase		GP phosphatase		PNPP phosphatase	
	Total activity (unit)	Specific activity (unit/mg protein)	Total activity (unit)	Specific activity (unit/mg protein)	Total activity (unit)	Specific activity (unit/mg protein)
Crude	263.2	1.24	1656	7.84	27608	69.78
500 g pellet	5.8	0.34	182.8	10.88	940	45.86
2,000 g pellet	4.3	0.39	207.4	18.84	627	57.20
10,000 g pellet	6.1	0.66	137.5	15.10	6020	31.42
10,000 g supernatant	241.4	1.24	1456.0	7.702	11400	108.28

Table 2. *The effect of stress on PG phosphatase activity of spinach leaf discs*

Spinach leaf discs were stressed for two days

Treatment	Enzyme activity	
	unit/g f. wt.	% of initial
Fresh leaf discs	8.05	100
Control leaf discs	5.67	70
Water stressed leaf discs (−20 bars of mannitol)	5.37	67
Salt stressed leaf discs (300 mM NaCl)	3.06	38

Table 3. *The effect of stress on the growth of spinach leaf discs*

Treatment	Time of treatment (day)			
	1		2	
	Fresh weight (g/30 leaf discs)	Protein (mg/30 leaf discs)	Fresh weight (g/30 leaf discs)	Protein (mg/30 leaf discs)
Control	3.26	15.6	3.16	13.4
Water stress (−20 bars of mannitol)	2.54	12.0	3.03	10.1
Salt stress (150 mM NaCl)	3.64	15.9	2.48	7.4

Table 4. *The effect of stress on the acid phosphatase of spinach leaf discs*
The concentration of PNPP was 10 mM.

Treatment	Time of treatment (day)			
	1		2	
	Acid phosphatase		Acid phosphatase	
	unit/g f. wt.	unit/mg protein	unit/g f. wt.	unit/mg protein
Control	73.34	17.0	116.9	27.6
Water stress (−20 bars of mannitol)	71.40	15.1	58.0	17.4
Salt stress (150 mM NaCl)	50.70	11.6	52.7	17.6

Table 5. *The effect of stress on the alkaline phosphatase of spinach leaf discs*
The concentration of PNPP was 10 mM.

Treatment	Treatment of time (day)			
	1		2	
	Alkaline phosphatase		Alkaline phosphatase	
	unit/g f. wt.	unit/mg protein	unit/g f. wt.	unit/mg protein
Control	8.2	1.9	13.60	3.2
Water stress (−20 bars of mannitol)	15.9	3.4	6.86	2.1
Salt stress (150 mM NaCl)	10.5	2.4	5.20	1.7

Abrupt drop in protein content was found in the 2-day-salt-stressed leaf discs.

Acid phosphatase (acid PNPP phosphatase) activity in the spinach leaf discs was decreased by the imposed stress (Table 4). After one day of the stress treatment, the specific activity of acid phosphatase was 89%, and 68% of the control in the water-stressed and salt-stressed leaf discs, respectively. After two days of the stress treatment, the activity was gradually dropped to 63% of the control (Table 4). However, a slight increase of alkaline phosphatase (alkaline PNPP phosphatase) was observed in the stressed leaf discs after one day of treatment. But,

after two days of treatment, the alkaline phosphatase activity in both water and salt stressed leaf discs was reduced to 50% or lower of that in the control (Table 5). Under the assay condition, alkaline phosphatase in spinach leaf discs was 11% of acid phosphatase.

Figure 1 showed multiplicity of acid phosphatase in different fraction of enzyme preparation. There were divided into two groups of acid phosphatase isoenzymes identified in disc polyacrylamide gel. These two groups of isoenzymes can be separated with a DEAE-cellulose column chromatography at pH 7.5 (data not shown). The fast-moving isoenzymes were adsorbed to DEAE-cellulose (called DEAE-cellulose-bound forms), the slow-moving ones were not adsorbed to DEAE-cellulose (called DEAE-cellulose-bound forms) under the experimental condition. There were three diffuse bands in the DEAE-cellulose-bound isoenzymes and possibly four distinct bands in the DEAE-cellulose-unbound isoenzymes (Fig. 1).

Fig. 1. The isoenzymes of acid phosphatase of spinach leaves.
Lane 1: Proteins adsorbed to DEAE-cellulose
Lane 2: Proteins unadsorbed to DEAE-cellulose
Lane 3: Proteins fractionated with 40-70% ammonium sulfate
Lane 4: Total soluble proteins

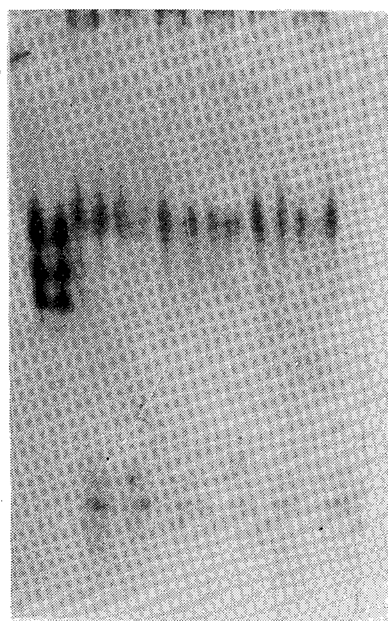

Fig. 2. The effect of stress on the isoenzymes of acid phosphatase of spinach leaf discs.
Lane 1: Proteins unadsorbed to DEAE-cellulose
Lane 2: Control after 24 h incubation
Lane 3: Water stress for 24 h
Lane 4: Salt stress for 24 h
Lane 5: Control after 48 h incubation
Lane 6: Water stress for 48 h
Lane 7: Salt stress for 48 h

The effect of water or salt stress on the isoenzymes of acid phosphatase in spinach leaf discs did not affect the pattern of acid phosphatase zymogram. One of DEAE-cellulose-bound forms (1st band of the anodic ones) was specifically increased, but the others were slightly decreased in the stressed leaf discs (Fig. 2).

Discussion

Acid phosphatase were found in each subcellular fraction, indicating that the acid phosphatase was distributed nonspecifically in the cell. This result is consistent with that reported by Mizuta and Suda (1980). The highest specific activity of GP phosphatase in the 2,000 g pellet fraction, which comprised mostly intact chloroplasts, suggested that GP phosphatase might be involved in the regulation of photosynthetic carbon metabolism (Schwenn *et al.*, 1973; Baier and Latzko, 1975; Woodrow and Walker, 1980). However, the highest specific activity of PC phosphatase was found in the soluble fraction. This suggests that the release of chloine from phosphorylcholine is possibly located in the cytosol. Choline is one of potential precursor of glycinebetaine which is accumulated in stressed tissue (Wyn Jones, 1979). If chloine is limiting factor for glycinebetaine accumulation, and the major source of choline is from phosphorylcholine, then promotion of PC phosphatase by stress treatments would be expected. However, the results showed that PC phosphatase was decreased by stress treatments. It, therefore, seems unlikely that glycinebetaine accumulation is mediated through the regulation of PC phosphatase. Choline released from phosphorylcholine via PC phosphatase in the stressed tissue seems to be sufficiently enough for glycinebetaine accumulation in stressed spinach leaf discs (Pan *et al.*, 1981) if the amount of phosphorylcholine is not limited in the stressed tissue.

The fresh weight or protein content of the spinach leaf discs was reduced to 30% or more by the stress treatment for two days. This is consistent with the inhibitory effect of stress on the growth of most glycophytes (Poljakoff-Mayber, 1982). The growth inhibition is often explained as being due to (i) the utilization of the photosynthate for osmoregulation but not for growth; (ii) division of part of the energy derived by respiration to damage repair needs instead of the usual cellular events. The inhibitory effect of water or salt stress on the acid phosphatase of spinach leaf discs was observed in this experiment. This is consistent with the effect of incremental salt stress imposed on the spinach seedling leaves (Pan, 1983), and the promotion of acid phosphatase activity by water stress in the leaves of cowpeas (Takaoki, 1968), cotton (Vieira-De-Silva, 1969), and wheat (Barrett-Lennard *et al.*, 1982) has been reported. On the contrary, the inhibitory alkaline phosphatase in the salinized spinach plant (Pan, 1983) did not occur in the 1-day-stressed spinach leaf discs. No explanation for this discrepancy can be

offered at this time. Acid phosphatase activity was much higher than alkaline phosphatase activity in the spinach leaf discs. The unpublished data also showed that the salinized spinach plants and the seeds of sorghum, corn, soybean and mungbean had higher acid phosphatase activity than alkaline phosphatase activity. This suggests that acid phosphatase rather than alkaline phosphatase in plant tissues are highly involved in the every aspect of cellular biochemical reaction. Thus, acid phosphatase in plant tissue has been extensively studied (Baker and Takeo, 1973; Besford, 1979; Bhargava and Sachar, 1983), as well as in animal tissues (Hara *et al.*, 1983; Fujimoto *et al.*, 1984), but very little report regarding the alkaline phosphatase in plant tissue (Pan, 1983). In sharp contrast, the alkaline phoaphatase was intensively studied in animal system (Li and Chan, 1981) and its activity in the human sera has long been known to be helpful in the clinical dignosis (Rider and Taylor, 1980).

Several isoenzymes of acid phosphatase were identified in many plants, e.g., bean hypocotyl (Mizuta and Suda, 1980), aleurone particles of rice grains (Yamagata *et al.*, 1979), and *Avena fatua* (Hooley, 1984). However, not all isoenzymes can be affected to the same extent by the environmental changes. In wheat, water stress specifically increased one of acid phosphatases (Barrett-Lennard *et al.*, 1982). One of the extracted acid phosphatase in cultured tobacco cells was increased by phosphate deficiency (Katsuyi and Sato, 1977). In this present work, water or salt stress imposed on spinach leaf discs did not affect the acid phosphatase zymogram of disc gel electrophoresis. Qualitatively, the similar seven isoenzymes were found in each sample. Interestingly, one of the DEAE-cellulose bound acid phosphatase was specifically stimulated, but the other isoenzymes were inhibited slightly by water or salt stress. The increase in this specific isoenzyme in spinach leaf discs caused by stressed treatments is also observed in the intact spinach plants grown hydroponically in the incremental salt solution (Pan, 1985). The physiological significance of this increased isoenzyme in the stressed tissue is obscure. The biochemical characterization of this specific isoenzyme is under investigation.

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菠菜葉片去磷酵素的研究

── 在細胞內之分佈及逆境處理的效應 ──

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本研究探討酸性去磷酵素在菠菜葉片細胞內之分佈及逆境處理的效應。酸性去磷酵素在細胞中之分佈並非只限於某個細胞胞器。大部分活性存在細胞質內。以膠體電泳法分析得到七個同功酵素。大部分都受到逆境：缺水或高鹽分的處理而抑制。能被 DEAE-cellulose (pH 7.5) 所吸附的同功酵素之一，却受到逆境處理而活性提高。