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Characterization of the phosphatase from spinach thylakoids[1]

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Abstract. The protein kinase activity of spinach thylakoid membrane has been identified and widely studied. However, its reverse reaction, catalyzed by the protein phosphatase, still requires more detailed

tein phosphatase is supposed to play a role in dephosphorylation of thylakoid membrane proteins (Bennett, 1980b). Recently, the thylakoid protein phosphatase has been shown to be a peripheral rather than an integral membrane protein (Sun *et al.*, 1989). It was also revealed that there exists a NaF-sensitive protein phosphatase activity on thylakoid membrane (Guitton and Mach, 1987). Sun and Markwell demonstrated that the thylakoid protein phosphatase does not belong to types 1 or 2A protein phosphatases of mammals (Sun and Markwell, 1992). Furthermore, Kieleczawa *et al.*, claimed successful isolation of an alkaline phosphatase from pea thylakoids (Kieleczawa *et al.*, 1992). However, properties, identity, function and structure of thylakoid protein phosphatase still require more intensive studies.

In this paper, we demonstrate that the thylakoid contains a phosphatase which dephosphorylates phosphoserine, phosphothreonine, p-nitrophenol phosphate and phospho-histone V-S. The characterization of the thylakoid phosphatase was investigated. We also determined the functional size of the thylakoid phosphatase by radiation inactivation.

Materials and Methods

Preparation of Thylakoid Membrane

Fresh spinach leaves (*Spinacia oleracea* L.), 100 g, were deveined and ground for 15~20 sec in 250 ml Buffer I containing 0.3 M sucrose, 30 mM Tris-HCl (pH 7.8), 10 mM NaCl, 3 mM $MgCl_2$, and 0.5 mM EDTA. The homogenate was filtered through 4 layers of cheesecloth. The filtrate was subjected to centrifugation at 11,000 xg for 15 min and the pellet was resuspended in Buffer I. The resuspended solution was centrifuged at 500 xg for 3 min. The pellet was discarded and the supernatant was centrifuged again at 2,300 xg for 5 min. The pellet was resuspended in Buffer II consisting of 0.2 M sucrose, 20 mM Tris-HCl (pH 7.6), 10 mM NaCl, and 5 mM $MgCl_2$. After washing twice with Buffer II at 13,300 xg for 1 min, thylakoid membranes were redissolved in a medium containing equal amount of Buffer II and storage buffer which consisted of 10% glycerol, 10 mM NaCl, and 25 mM Tris-HCl (pH 7.5).

Activity Assay and Protein Determination

The phosphatase activity of thylakoid membrane was assayed at 30°C for 15 min, or for the time period indicated. The reaction mixture contained 50 mM Tris -Mes, 0.1 M sorbitol, and 1 mM $MgCl_2$, thylakoid membrane (400 μg Chl/ml), and substrates. The concentrations of the substrates were 8 mM phosphothreonine and phosphoserine, and 10 mM p-nitrophenol phosphate, respectively. The pH of the reaction medium was adjusted to optimal values for individual substrates as indicated. Pi release, using phosphothreonine and phosphoserine as substrates, was measured as described by LeBel *et al.* (LeBel *et al.*, 1978). The phosphatase activity using p-nitrophenol phosphate as substrate was determined spectrophotometrically as the rate of absorbance decrease at 405 nm with extinction coefficient of 18,300 $mM^{-1} \cdot cm^{-1}$.

The Chl concentration of isolated chloroplasts was measured using the equations developed by Arnon (Arnon, 1949). Protein concentration was determined according to the modified method of Lowry (Larson *et al.*, 1986).

Irradiation Procedure and Calculation of Functional Size

Irradiation was performed with a ^{60}Co irradiator (~1000 Ci) at our institute. The dose rate was 1.14 ± 0.08 Mrad/h which was determined by the method of Hart and Fricke (Hart and Fricke, 1967) using Fe^{2+}/Fe^{3+} or Ce^{4+}/Ce^{3+} couple. Thylakoid membranes (1 mg/ml) were irradiated at -18 to -25°C maintained by a cryothermostat. Control samples were run concurrently under the same condition but without irradiation.

Molecular mass (functional size) was calculated from the equation of Beauregard and Potier (Beauregard and Potier, 1985):

$$log\ m = 5.89 - log\ D_{37,t} - 0.0028t$$

where m is the functional size in daltons, $D_{37,t}$ is the dose of radiation in megarads required to reduce the activity to 37% of that found in the unexposed control at temperature t (°C).

Chemicals

Phosphoserine, phosphothreonine, and p-nitrophenol phosphate, were purchased from Sigma. All chemicals were of highest analytic grade and used without further purification.

Results

Time Course of the Thylakoid Phosphatase

The time-course for the thylakoid phosphatase using phosphothreonine (PT), phosphoserine (PS), and p-nitrophenol phosphate (PNPP) as substrates are shown in Figure 1. The release of Pi from the exogenous substrates increased gradually as the incubation time prolonged. The reaction rates of phosphatase using different substrates are linear within 50 min. The specific activities for the substrate PT, PS and PNPP were thus calculated as approximately 9.6, 6.0, and 12.0 μmol Pi released/mg Chl. h, respectively. As reported, the protein kinase activity could be activated by light (Bennett, 1979). However, we demonstrated that the phosphatase activity was not stimulated by illumination indicating the absence of light regulation (data not shown). Among the substrates scrutinized, p-nitrophenol phosphate (PNPP) was more effective than phosphothreonine (PT) and phosphoserine (PS) for the phosphatase reaction. However, PT was preferentially used as substrate in this study except where otherwise indicated.

pH Profile of Thylakoid Phosphatase

The pH profile for phosphatase was observed (Fig. 2). For phosphatase using PT and PS as substrates, the optimal pH values were around 6.5 while that for PNPP was obtained at 8.0. It is possible that the thylakoid phosphatase has a broad pH specificity for various substrates. Whether the thylakoid contains different, distinct enzyme complexes for phosphatase reactions still requires further investigation.

Effects of Ions on Thylakoid Phosphatase

Fig. 3 showed the concentration curves of Mg^{2+} and Mn^{2+} for the thylakoid phosphatase. At lower concentrations (< 2 mM) of Mg^{2+} and Mn^{2+}, the phosphatse activities was slightly stimulated. However, as the concentrations of Mg^{2+} and Mn^{2+} increased, the phos-

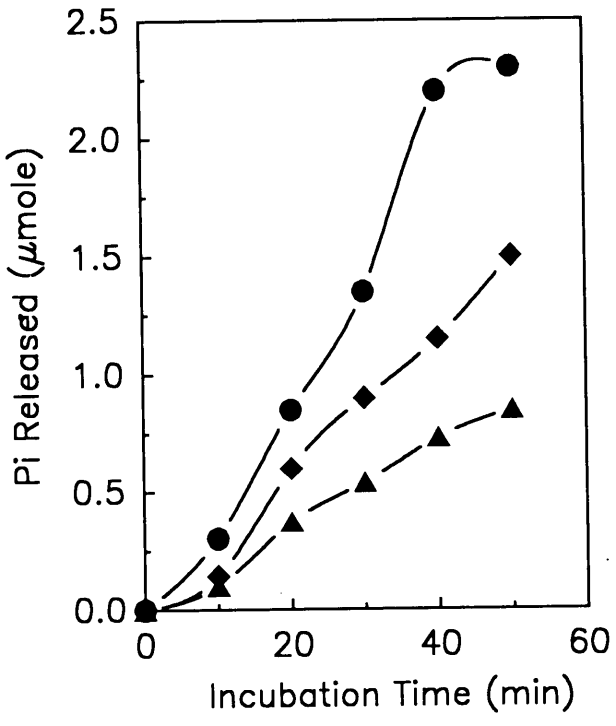


Fig. 1. Time course of thylakoid phosphatase activity. Reaction conditions were as described under "Materials and Methods". (-◆-), phosphothreonine; (-▲-), phosphoserine; (-●-), p-nitrophenol phosphate.

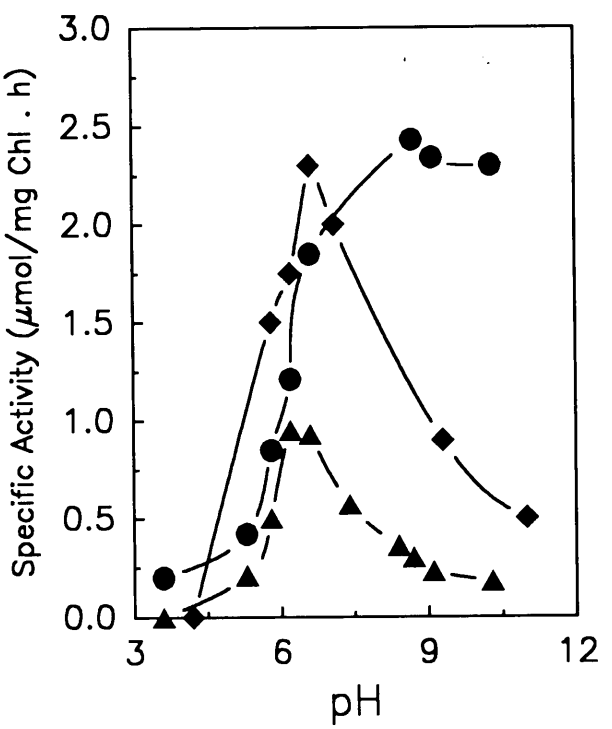


Fig. 2. pH profile of thylakoid phosphatase activity. Reaction conditions were as described under "Materials and Methods". The pH was maintained by 50 mM Tris-Mes. (-◆-), PT; (-▲-), PS; (-●-),PNPP.

phatase activities were then restored to control level. Table 1 depicts the effects of other divalent cations on the phosphatase activity of the thylakoid. At lower con-

Table 1. *Effects of divalent cations on thylakoid phosphatase activity*

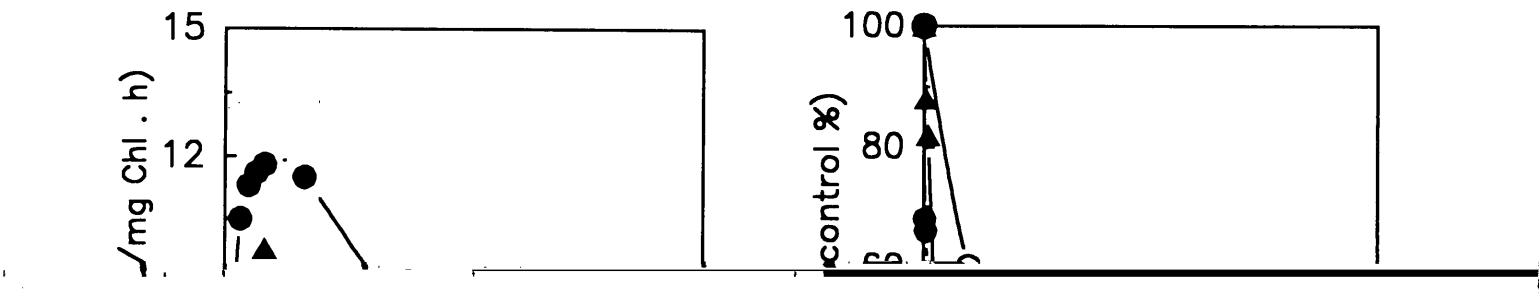
Reaction conditions were as described in "Materials and Methods" except that the concentration of divalent cations was 1 mM or 10 mM as indicated.

Divalent cations	1 mM		10 mM	
	μmol Pi released mg Chl. h	%	μmol Pi released mg Chl. h	%
None	9.24	100	9.25	100
Mn^{2+}	11.82	128	8.34	90
Mg^{2+}	10.44	113	6.48	70
Ca^{2+}	10.32	112	5.82	63
Ni^{2+}	8.40	91	0.54	6
Cd^{2+}	9.30	101	3.48	38
Zn^{2+}	6.54	71	0.10	0
Cu^{2+}	7.38	80	0.20	2

Table 2. *Effects of monovalent ions on thylakoid phosphatase activity*

Reaction conditions were as described in "Materials and Methods" except that the concentration of monovalent ions was 25 mM or 100 mM as indicated.

Ions	25 mM		100 mM	
	μmol Pi released mg Chl. h	%	μmol Pi released mg Chl. h	%
None	9.22	100	9.23	100
Li^+	6.00	65	4.92	53
Na^+	6.48	70	7.74	84
K^+	7.86	85	6.18	67
Cs^+	7.50	81	5.82	63
NH_4^+	7.68	83	6.00	65
F^-	4.50	49	0.10	0
Cl^-	6.48	70	7.74	84
Br^-	7.32	79	7.00	75
NO_3^-	8.94	97	7.00	75
SO_4^{2-}	7.74	84	3.60	39



centration (1 mM), divalent cations had little effects on the phosphatase activity. It is obvious that phosphatase may not require exogenous divalent cations for its enzymatic activity. However, we can not exclude the possibility that the tightly bound cations are still essential to the activity of the enzyme. Nevertheless, at higher concentration (10 mM), the phosphatase activities were severely inhibited by most divalent cations scrutinized, except Mg^{2+} and Mn^{2+}.

The enzymatic activity of thylakoid phosphatase is sensitive to vanadate, tartrate, and F^- (Fig. 4). The I_{50} values of 1.5, 2.5, 25 mM were obtained for vanadate, tartaric acid and NaF, respectively. The inhibition effect by vanadate and tartaric acid was dramatically higher than that of NaF. Table 2 further lists the effects of other monovalent ions on the phosphatase activity of the thylakoid. At lower concentration (10 mM), most monovalent ions except Li^+ and F^-, do not show any significant effects on phosphatase activity. However, phosphatase activity could be inhibited at higher concentrations (100 mM) of monovalent ions by at least 20~40%.

Effects of Inhibitors on Thylakoid Phosphatase

Several specific site-directed inhibitors were used to investigate their effects on thylakoid phosphatase. NBD-Cl exerted little inhibition on thylakoid phosphatase. Further, it is shown that the sulfhydryl modifier NPM displayed approximately 20% inhibition while its derivative NEM had little effect on the phosphatase activity (Table 3). It is possible that the active site of phosphatase may not contain essential sulfhydryl groups. However, the phosphatase activity was decreased by 50% in the presence of 0.2 mM DCCD. Interestingly, the hydrophilic derivative of carbodiimide, EDC, is slightly inhibitory to phosphatase. The active site may be embedded on the hydrophobic domain of membrane preventing access by the more hydrophilic derivative, EDC.

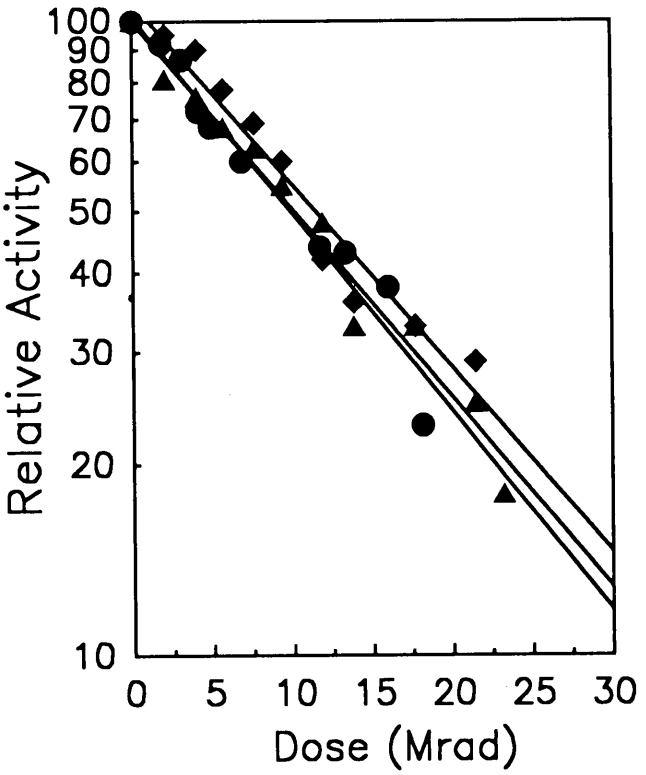


Fig 5. Radiation inactivation of thylakoid phosphatase activity. Irradiation of samples and reaction conditions were as described under "Materials and methods". All data points are means of 4 assays with lines fitted by regression analysis ($r > 0.98$). The intersections of regression lines at 37 % control activity give the D_{37} dose values. The functional size was calculated using the Beauregard and Potier equation (Beauregard and Potier, 1985). (-◆-), PT; (-▲-), PS; (-●-), PNPP.

Table 3. *Effects of inhibitors on thylakoid phosphatase activity*

Reaction conditions were as described in "Materials and Methods". Thylakoid membranes were preincubated with inhibitors for 60 min at 30°C. The concentrations of the inhibitors are indicated in parentheses.

Inhibitor		Specific activity	
		μmol Pi released	
	(mM)	mg Chl. h	%
None	(0.0)	10.40	100
NEM	(0.5)	10.32	98
NPM	(1.0)	4.20	80
DCCD	(0.2)	6.24	62
EDC	(5.0)	8.82	84
NBD-Cl	(1.0)	8.82	84

Kinetic Studies of Thylakoid Phosphatase

The kinetic parameters of thylakoid phosphatase activity are tabulated in Table 4. Apparently, the K_m and V_{max} values are very similar for phosphatase activity using PT and PS as substrates. On the other hand, the V_{max} was higher and K_m lower for phosphatase activity using PNPP, indicating that PNPP is a better

Table 4. *Kinetic parameters of thylakoid phosphatase activity*
Reaction conditions were as described under "Materials and Methods". The V_{max} and K_m values were obtained by Lineweaver-Burke plots. The concentrations of Mg^{2+} and Mn^{2+} were 1 mM, while that of F^- was 25mM

	Addition	Substrate		
		PT	PS	PNPP
V_{max} (μmol Pi released) mg Chl. h	Control	3.17	2.71	5.00
	Mg^{2+}	5.50	5.50	6.37
	Mn^{2+}	6.82	6.70	7.57
	F^-	2.87	2.61	2.00
K_m (mM)	Control	5.53	5.15	4.73
	Mg^{2+}	10.28	11.43	5.53
	Mn^{2+}	14.46	15.10	1.78
	F^-	7.57	7.12	2.99

substrate than PT and PS.

Radiation Inactivation Analysis of Thylakoid Phosphatase

When thylakoid membranes were exposed to high energy γ-ray irradiation, the phosphatase activities were reduced with increasing radiation dosage. The activity decayed as a simple exponential function of dosage, allowing a straight-forward application of target theory for determination of the functional size involved in phosphatase activity (Fig. 5). The D_{37} values for inactivation of phosphatase activity using PT, PS and PNPP are 16.2 $\pm$ 1.5, 14.1 $\pm$ 1.5 and 14.3 $\pm$ 1.5 Mrads, respectively. These gave the functional sizes of that phospho-amino acids such as PT, and PS, and PNPP could mimic the phosphoprotein as substrates for thylakoid phosphatase. The dephosphorylation catalyzed by the thylakoid phosphatase is summarized as follows:

$$\text{Phospho-amino acid} + H_2O \xrightarrow[\text{phosphatase}]{Mg^{2+}} \text{amino acid} + Pi$$

The characteristics of the dephosphorylation using phosphoamino acids and PNPP by thylakoid phosphatase are in a good agreement with that using phosphoproteins as reported by other workers (Bennett, 1980b; Sun *et al.*, 1989) in several aspects: [1] the pH optimum value is approximately 6.5; [2]the phosphatase could be stimulated by Mg^{2+}, Mn^{2+}, and Ca^{2+}, whereas Cu^{2+}, Zn^{2+} inhibit the enzymatic activity; [3] the enzyme is sensitive to a common plant phosphatase inhibitor, NaF, but not to NaCl; [4] the enzyme is light-independent (data not shown); [5] the enzyme is inhibited by Triton X-100 (data not shown). The results strongly indicated that phosphatase activities using PT, PS and PNPP as substrates were carried out by the same enzyme described by Bennett and Sun *et al.* (Bennett, 1980b; Sun *et al.*, 1989).

It has been shown that the thylakoid surface showed a pI of 4.3 and would be negatively charged at physiological pH (Barber, 1982). The negative charges on the thylakoid surface will result in different ionic composition in the proximity of the membrane from that of the bulk solution. However, evidence revealed that the protein phosphatase contains a net positive charge (Sun *et al.*, 1989), attributing to the inhibition by

phosphatase activity. We believed that the modified carboxylate residue could probably be embedded in the hydrophobic pocket in the active site. This possibility was further verified by kinetics studies of the dephosphorylation under various conditions. The kinetic characteristics of phosphatase using different substrates were almost identical (Table 4). However, the K_m values for dephosphorylation of PNPP are lower than that for PT and PS. The higher binding affinity of PNPP phosphatase indicates that it could more easily access the hydrophobic pocket in the active site than could PT and PS.

The functional sizes of phosphatase using PT, PS and PNPP as substrates are very similar (50~60 kD), indicating that the enzymes involved might be identical. Compared to the functional masses of the protein kinase for LHC II and 9 kD polypeptide (620 and 420 kD), (Pan, Chen, Hong, and Huang, unpublished data) the functional size of phosphatases was far smaller. It is suggested that phosphatase may not involved a complicated regulatory mechanism as in the case of the protein kinase. The structure and function of protein phosphatase require further study.

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菠菜葉綠餅囊膜上去磷脂酵素之特性研究

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菠菜葉綠餅上蛋白激酵素已被確認且廣泛研究了。然而，司其逆反應的蛋白去磷脂酵素仍有待更詳盡的研究。在本篇論文裡，我們利用了不同外加受質，如磷化絲氨酸，磷化蘇氨酸及對硝基苯環磷酸來探討蛋白去磷脂酵素的生化特性。葉綠餅上的去磷脂酵素是不受光所調節，而對氟化鈉相當敏感。用這三種外加受質得其最適合的酸鹼值，分別是 6.5,6.5 和 8.0。低濃度的鎂、錳和鈣離子可促進此去磷脂酵素的活性，在高濃度下則可抑制之。酒石酸及氧化鎂亦可抑制此酵素的活性。專一標示在硫化藥物有零到百分之二十的抑制，而作用此酵素對厭水性羥基藥物較親水性藥劑敏感。此去磷酯酵素在不同環境下具有不同的 K_m和 V_{max}值。利用輻射純化法得知其功能大小爲 54 到 61 仟道爾吞。