Solubilization, Purification, and Characterization of the ATPase from Spinach Leaves Plasma Membrane Fraction and its Sensitivity to Herbicides

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ABSTRACT

The plasma membrane ATPase of spinach leaves (*Spinacia oleracea*) had been solubilized with a two-step procedure using the anionic detergent, deoxycholate (DOC) and the non ionic detergent, aminoxid WS35 as follows: 1) loosely bound membrane proteins are removed by treatment with 0.1% DOC; 2) The ATPase is solubilized with 0.1% aminoxid WS35 in the presence of 1% DOC; 3) the solubilized material is further purified by centrifugation through a glycerol gradient (45-70%). Typically, about 10% of the ATPase activity is recovered, and the specific activity increases about 11-fold. The properties of the purified ATPase are essentially the same as those of membrane-bound ATPase, with respect to substrate specificity, inhibitor sensitivity, and ion stimulation.

Acifluorfen-sodium from trifluoromethyl; diphenyl ether group of herbicides, fomesafen from bridged diphenyl; trifluoromethyl; nitro compound group, and butachlor from acetamide have been tested on the purified ATPase of plasma membrane. The three herbicides showed that all of them were inhibitory to the enzyme. The inhibition was in the following order: Acifluorfen-sodium > fomesafen > butachlor. These results indicate that the plasma membrane ATPase of plant cell was suggested to be a target for the inhibitory action of these herbicides.

INTRODUCTION

Many papers have described the effect of herbicides from different groups on plant mitochondria and chloroplasts (Macherel, *et al.*, 1982; Ravanel, *et al.*, 1990). In comparison little is known about the effects of these groups of chemicals on plasma membrane associated ATPase activity. This is probably could be due to the inability to obtain vesicle fractions containing only one type of membrane.

The existence of an electrogenic H^+-translocating ATPase in plant plasma membranes has been postulated on the basis of electrophysiological and biochemical evidence (Spanswick, 1981). Despite rapid advances in the purification and characterization of

transport proteins from biological membranes (Helenius and Simons, 1975; Tanner, 1979), however, there are few examples of purified, well characterized transport proteins from plant plasma membranes and reconstitution of H^+-ATPase (O'neill and Spanswick, 1984; Vara and Serrano, 1982). In this study we solubilize the H^+-translocating ATPase in two steps using deoxycholate (DOC) and aminoxid WS35 detergents in the presence of glycerol, and purified by glycerol-gradient centrifugation. The characteristics of the purified enzyme and its sensitivity to herbicides were also investigated.

MATERIALS AND METHODS

Plant Material. Spinach leaves (*Spinacia oleracea*) were purchased in a local market, and leaves were cleaned by washing in tap then, cold distilled water.

Chemicals. Acifluorfen-sodium (sodium 5-(2-chloro-α, α, α-trifluoro-*p*-tolyloxy)-2- nitrobenzoate); fomesafen (5-(2-chloro-α, α, α-trifluoro-*p*-tolyloxy)-N-methylsulphonyl-2-nitrobenzamide); and butachlor (N-butoxymethyl-2-chloro-2', 6'-diethylacetanilide) pure highly purified samples (99%) obtained from Zeneca Agrochemicals Co. UK. Solutions of these compounds were prepared in absolute
ethanol. For all chemicals used appropriate controls were run. All biochemicals were purchased from Sigma Chemical Company, St. Louis, Missouri.

Isolation of Plasma Membrane Fraction. Plasma membrane enriched fractions from a 10,000 to 80,000g pellet were isolated by the method described by kasamo, 1986. To increase the yield, microsomes were layered on a 20/30/40 %(w/w) discontinuous sucrose gradient and centrifuged at 80,000g for 2h. Membrane fractions were collected from 30/40 interface.

Detergent Treatment of Plasma Membrane Fractions. A two step solubilization procedure DOC and Aminoxid WS35 was employed. The plasma membrane ATPase was purified from spinach leaves as described by Bowman *et al.*, 1981.

ATPase Assay. ATPase activity was measured at 38°C for 30 min. with 5 to 10 μg protein per assay as described by Kasamo, 1986. The reaction was carried out in a volume of 0.5 ml containing 3mM Tris-ATP, 3mM $MgSO_4$, 50mM KCl, 30mM Mes-Tris (pH 6.5) in the presence of 0.03% asolectin (plant phospholipids). Phosphate was
determined by the method of Kasamo, 1979.

Protein Assay. Protein was determined by the method of Bradford (1976) with BSA as a standard.

The data are presented as mean values (±SD) of measurements made with purified ATPase preparations from five independent isolates.

RESULTS AND DISCUSSION
Solubilization of the Plasma Membrane Fraction.

Plasma membrane-bound ATPase was solubilized by a two-step procedure using DOC and aminoxid WS35. Table 1 is the summary of the recoveries of enzyme activity and protein during purification. In the first step, 0.1% DOC was employed to remove loosely bound membrane proteins. Typically, about 60% of the protein was removed but close to 100% of the ATPase activity remained in the pellet. In the next step, solubilization of the ATPase from extracted membranes was attempted with DOC and/or aminoxid WS35 in the presence of glycerol. When aminoxid WS35 (0.1%) or DOC (1%) was used singly, the final supernatant contained 18 to 21% of the ATPase, with a 2.8- to 3.3-fold increase in specific activity. By contrast, when aminoxid WS35 and DOC were used together, the final supernatant contained 31% of the starting ATPase and had a nearly 6.2-fold increase in specific activity. Thus, the ATPase in 0.1% DOC extracted membranes was solubilized with 0.1% aminoxid WS35 in the presence of 1% DOC. This finding is in agreement with the results reported by several researchers (Bowman *et al.,* 1981; Briskin and Poole, 1984; Imbrie and Murphy, 1984).

Purification of Glycerol Gradient Centrifugation.

The results of a representative purification are summarized in Table 1. In this experiment, the specific activity of the ATPase increased from 0.6 µmol Pi/mg protein . min in the starting plasma membranes to 1.4 µmol Pi/mg protein . min after treatment with 0.1% DOC, 3.6 µmol Pi/mg protein . min following solubilization with 0.1% aminoxid WS35 in the presence of 1% DOC, and finally 6.2 µmol Pi/mg protein . min in the pooled fractions from the glycerol gradient. The final recovery was about 10% although there was some loss of enzyme units at each step. Under these conditions, about 21% of total ATPase still remained in the glycerol gradient
pellet without inactivation of the enzyme. The purity obtained in this study is in agreement with the findings of Briskin and Poole, 1984.

Effect of Phospholipids on ATPase activity.

During ATPase assay, addition of phospholipids is absolutely required for activity of DOC-solubilized ATPase, but less important for aminoxid WS35-solubilized enzyme. Following purification, ATPase activity increased upon addition of asolectin to the assay mixture. In the case of enzyme solubilized with 0.1% aminoxid WS35 in the presence of 1%

DOC, there was more than 2-fold stimulation by asolectin (Table 1). Bennett and Spanswick, 1983 showed that the activation by added phospholipids is relatively nonspecific and results from hydrophobic interaction between the H^+-ATPase and lipid environment.

Yields were calculated on the basis of activity in the presence of asolectin. Plasma membrane (PM); DOC pellet (DP); DOC supernatant (DS); Aminoxid WS35 supernatant (AS); Glycerol gradient peak fractions (GGPF).

Effects of Various Inhibitors on Purified ATPase.

The effect of various phosphohydrolase inhibitors on the purified ATPase activity were examined (Table 2). The activity was insensitive to oligomycin and azide, the

mitochondrial ATPase inhibitors and ouabain, a specific inhibitor of Na^+-K^+-ATPase. Molybdate, an inhibitor of nonspecific phosphatase in plant cells, did not affect the activity. Nitrate, an inhibitor of tonoplast ATPase in plant cells, slightly affected the activity while N,N-dicyclohexylcarbodiimide (DCCD), diethylstilbestrol (DES), vanadate, and gramicidin S inhibitors of plasma membrane ATPase (Kasamo, 1986), strongly inhibited the enzyme. These results are consistent with those previously reported by Gallagher and Leonard, 1982; Serrano, 1984; Kasamo, 1986.

Other Characteristics of the Purified Enzyme.

The enzyme properties of the purified ATPase were examined so that they could be compared with the previously reported properties of the membrane-bound enzyme (Kasamo, 1986). Table 3 shows that the purified ATPase was activated by divalent cations in the presence of 50 mM KCl in the following order: $Mg^{2+}>Mn^{2+}>Co^{2+}>Cu^{2+}$. The lower part of Table 3 shows the effect of monovalent cations. Stimulation exhibited the following order: $K^+>NH_4^+>Na^+>Li^+$. Table 4 illustrates the substrate specificity of the purified ATPase. The enzyme actively hydrolyzed ATP, and other nucleotides were hydrolyzed to a lesser degree. These results are in agreement with those of Bennett and Spanswick, 1983; Lew and Spanswick, 1985.

ATPase activity(μmol Pi/protein .min) was measured in the presence of 3mM $MgSO_4$, 3 mM Tris-ATP, 50mM KCl, 30mM Mes-Tris (pH 6.5), 0.03% asolectin, and various concentrations of inhibitor as indicated.

ATPase activity (μmol Pi/protein .min) was assayed in the presence of 3 mM Tris-ATP, 3 mM divalent cations, 50 mM monovalent cations, and 30 mM Mes-Tris (pH 6.5).

The Effect of Herbecides on the Purified ATPase Activity

The results illustrated in Table 5. represent the inhibitory effect of acifluorfen-sodium, fomesafen, and butachlor at various concentrations (0, 1.0, 10, 50, 100, 150 µM) of each on the purified ATPase activity. The herbicides studied here induce important inhibitions of the enzyme activity. The percentage of inhibition ranged from (15%-65%) with acifluorfen-sodium concentratios (1.0-100µM), from (10%-54%) with fomesafen concentrations (1.0-100µM), and from (5%-46%) with butachlor concentrations (1.0-100µM), which is in agreement with the results of Ratterman and Balke, 1987; El-Kassabany, 2004.

It could be concluded that the interaction of these herbicides with plasma membrane ATPase activity constitutes is an essential mechanism of their mode of action in plants.

ATPase assays were carried out as described in ˙Materials and Methods. Aliquots were added to the enzyme and the mixture incubated at 38˚C for 5 min. prior to starting ATPase assay by addition of the reaction mixture.

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Table 1. ATPase Purification and Yield

Fraction	ATPase	Activity			Protein	Yield
	Asolectin		Yield		Amount	
	-	+	Unit	%		
	μmol Pi/mg protein.min		μmol Pi/min		(mg)	(%)
PM	0.41±0.03	0.55±0.02	3.53±0.19	100	6.50±0.51	100
0.1% DP	0.76±0.02	1.36±0.11	3.58±0.22	101.4	2.60±0.40	40
1% DS	1.32±0.14	1.80±0.20	0.62±0.03	17.6	0.36±0.02	5.5
GGPF	1.56±0.12	2.34±0.21	0.17±0.02	4.8	0.08±0.02	1.2
PM	0.30±0.03	0.38±0.02	1.82±0.03	100	4.67±0.12	100
0.1% DP	0.65±0.02	0.91±0.04	1.90±0.02	104.4	2.07±0.11	44.3
0.1% AS	1.04±0.06	1.10±0.10	0.39±0.03	21.4	0.36±0.03	7.7
GGPF	1.13±0.08	1.26±0.12	0.14±0.02	7.7	0.15±0.02	3.2
PM	0.52±0.02	0.58±0.02	2.77±0.12	100	4.14±0.08	100
0.1% DP	1.02±0.07	1.43±0.06	2.93±0.11	105.8	1.70±0.05	41.1
0.1% A+1%DS	2.61±0.21	3.61±0.13	0.87±0.04	31.4	0.60±0.03	14.5
GGPF	2.73±0.14	6.23±0.22	0.27±0.02	9.8	0.12±0.02	2.9

Table 2. The Effect of Inhibitors on Purified ATPase

Inhibitor	ATPase Activity
None	4.22 ± 0.11
Oligomycin (5µg/ml)	3.99 ± 0.12
NaN₃ (1mM)	3.85 ± 0.14
Ammonium molybdate (100µM)	3.98 ± 0.10
Ouabain (50µM)	3.87 ± 0.12
KNO₃ (100mM)	3.38 ± 0.13
DCCD (100µM)	1.41 ± 0.05
DES (100µM)	1.52 ± 0.06
Vanadate (50µM)	0.37 ± 0.02
Gramicidin S (100µM)	1.60 ± 0.07

Table 3. The Effect of Divalent and Monovalent Cations on Purified ATPase

Cation	ATPase Activity
KCl	
+ MgSO₄	4.52 ± 0.11
+ MnSO₄	2.58 ± 0.07
+ CoCl₂	2.26 ± 0.06
+ ZnSO₄	0.0
+ CaCl₂	0.0
+ CuSO₄	1.98 ± 0.04
MgCl₂	
+ KCl	5.55 ± 0.12
+ NaCl	5.12 ± 0.14
+ LiCl	3.63 ± 0.11
+ NH₄Cl	5.32 ± 0.12

Table 4. Substrate Specificity of Purified ATPase

Substrate	ATPase Activity
AMP	0.05 ± 0.002
ADP	0.32 ± 0.02
ATP	3.91 ± 0.11
GTP	1.19 ± 0.03
UTP	1.17 ± 0.02
CTP	0.68 ± 0.04

Table 5. The Effect of Various Herbicides on Purified ATPase

Herbicide	Concentration (μM)	ATPase Activity (% of control)
Acifluorfen-sodium		
	0.0	100 ± 2
	1.0	85 ± 4
	10	72 ± 5
	50	60 ± 6
	100	35 ± 5
	150	35 ± 4
Fomesafen		
		100 ± 3
	0.0	90 ± 4
	1.0	78 ± 6
	10	67 ± 7
	50	46 ± 8
	100	46 ± 6
	150	
Butachlor		
	0.0	100 ± 6
	1.0	95 ± 8
	10	87 ± 7
	50	72 ± 8
	100	54 ± 5
	150	54 ± 6

الملخص العربي

فصل وتنقية إنزيم الأدينوزين ثالث الفوسفاتيز من الغشاء السيتوبلازمى لخلايا أوراق السبانخ. دراسة خصائصة وتأثير مبيدات الحشائش علية.

شفيقة أحمد الكسبانى

المعمل المركزي للمبيدات- مركز البحوث الزراعية- الصبحية- الإسكندرية.

تــم فصـــل إنزيم الأدينوزين ثالث الفوسفاتيز من الغشاء السيتوبلازمى لخلايا أوراق السبانخ باستخدام المنظفات DOC الأيونى و Aminoxid WS35 الغير أيونى. وتم تنقيتة باستخدام الفصل الطبقى وطريقة الطرد المركزى لنظام تدرج الجليسرول. وقد ثبت أن هذا الانزيم نقى بقياس نشاطة النوعي الذي زاد حوالي 11 مــرة. وبدراسة خواصة وجد أنة متخصص لمادة التفاعل الادينوزين ثالث الفوسفات وحساس للمثبطات المعروفة مثل الفانادات وأرتفاع نشاطة بالايونات. تم تقييم فعالية ثلاثة من مبيدات الحشائش، أسيفلوروفين الصود يوم، فوميسافين وبــيوتاكلور علــى نشـــاط الأنزيم النقى. وقد وجد أن هذة المركبات لها تأثير تثبيطـــي على نشاط الانزيم أسيفلوروفين الصود يوم، فوميسافين، و بيوتاكلور على التوالي.

ومن هذا يتضح أن الأغشية السيتوبلازمية للخلايا النباتية تعتبر هدف هام لهذة المبيدات في النبات.