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Isolation and Characterization of Tonoplast from Chilling-Sensitive Etiolated Seedlings of *Vigna radiata* L.[1, 2]

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ABSTRACT

Tonoplasts were isolated in a high purity from etiolated young seedlings of *Vigna radiata* L. (mung bean) utilizing a sucrose density gradient system. The excised hypocotyls were homogenized in a sorbitol-buffer system and the 3,600 to 156,000*g* pellets obtained after the differential centrifugations were suspended in a sorbitol medium and loaded on a linear sucrose density gradient. After centrifugation at 89,000*g* for 2 hours, tonoplasts were banded at the sample load/sucrose interface. Assessed by electron microscopy and marker enzymes, the purity and the quantity were found to be sufficient for biochemical and biophysical analyses. The tonoplasts were associated with NO_3^--sensitive and vanadate-insensitive ATPase. The tonoplast ATPase was stimulated by proton ionophores such as carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and gramicidin D, suggesting a proton-pumping enzyme. In the presence of ATP and Mg^{2+}, a proton gradient was formed in the isolated tonoplast vesicles as assessed by fluorescence quenching of quinacrine. The tonoplasts contained several kinds of mannosylated or glycosylated glycoproteins and a major protein (65 kilodaltons) which was unique to the membranes.

A great deal of information has been accumulated on the proton ATPase located on the tonoplast from several plants (1, 7, 19, 27). The majority of the publications involve measurements carried out on intact vacuoles isolated by the osmotic lysis of enzymically isolated protoplasts under conditions that preserve the integrity of the tonoplasts (11, 26).

To get more insight into the biochemical and molecular properties of the tonoplast ATPase and the membrane *per se*, we need membranes both of high purity and in a large quantity sufficient for analytical uses. For this purpose, tonoplast-enriched fractions are separated from crude microsomal membranes by centrifugation on a linear or discontinuous sucrose density gradient or a dextran density gradient (2, 7, 8, 10, 16, 18, 20).

ATPases are known to be associated with wide variety of membrane systems in plant cells. Recently, ATPases have reported to be associated with ER (4, 5), Golgi membranes (6), and plastid envelopes (15), and they have been partially characterized. The tonoplast-enriched fractions separated after a density gradient are usually contaminated with those endomembranes associated with ATPases. Tonoplasts could also be separated in a high purity from the intact vacuoles isolated from enzymically prepared protoplasts following an osmotic lysis. However, there is difficulty in getting a large quantity of the tonoplasts.

This paper reports the isolation of tonoplast from etiolated seedlings of *Vigna radiata* by a method in which sorbitol was used as an osmotic stabilizer throughout crude membrane preparation prior to a sucrose density gradient centrifugation. This method has permitted us to isolate tonoplast in a high purity and in a large quantity on subsequent sucrose density gradient.

MATERIALS AND METHODS

Plant Materials. Seeds of *Vigna radiata* L. (mung bean) were imbibed and germinated at 26°C in darkness in a plastic box installed with a stainless steel mesh as reported elsewhere (23). After 4-d germination, the hypocotyls were excised from the etiolated young seedlings and used as the experimental materials.

Membrane Preparation. The excised hypocotyls were washed once in distilled H_2O and prechilled at 10°C before homogenization. The chilled tissues (140 g fresh weight) were sliced into 180 ml of homogenizing medium prechilled at 0°C and immediately homogenized with Polytron PT 30 at the medium speed setting for 30 s. The medium used for homogenization contained 0.25 M sorbitol, 75 mM MOPS[3]-KOH, pH 7.6, 5 mM EGTA, 2 mM SHAM, 2 mM PMSF, 2.5 mM potassium metabisulfite, 1.5% soluble PVP (mol wt 24,000), 10 μg/ml BHT, and 0.5% defatted BSA. The homogenate was passed through four layers of gauze and subjected to differential centrifugations at 3,600*g* for 10 min and then 156,000*g* for 20 min. The obtained 3,600 to 156,000*g* pellets were suspended in 0.25 M sorbitol, 5 mM MOPS-KOH (pH 7.3), 1 mM EGTA, 0.2 mM PMSF, 10 μg/ml BHT, 10 mM KCl, and 1 mM DTT (sorbitol-MOPS-buffer system) and centrifuged at 156,000*g* for 20 min. The washed pellets were suspended in 7 ml of the sorbitol-MOPS-buffer system as used above and loaded onto 30 ml of a linear sucrose density gradient (15–45% w/w) made up in the MOPS-buffer system (sorbitol-sucrose density gradient system). After centrifugation at 89,000*g* for 2 h on a Hitachi SW-27, 1.2 ml aliquots were fractionated from the top of the gradient and used for analysis of enzyme activities and protein content. For the reference experiment, the sorbitol as the osmotic-stabilizer was replaced by sucrose throughout the procedure (sucrose-sucrose density gradient system).

Enzyme Assays. Activities of ATPase, antimycin A-insensitive NADH Cyt *c* reductase, Cyt *c* oxidase, acid phosphatase were analyzed according to the methods reported elsewhere (22, 25). Protein was analyzed by the method of Bradford (3).

Proton-Transport Assay. The transmembrane H^+-transport activity was measured by quenching of quinacrine fluorescence

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[3] Abbreviations: MOPS, 3-(*N*-morpholino)propanesulfonic acid; SHAM, salicylhydroxamic acid; PMSF phenylmethylsulfonyl fluoride; BHT, *t*-butylated hydroxytoluene; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; DES, diethylstilbestrol; DIDS, 4,4'-diisothiocyano-2,2'-stilbene disulfonic acid.

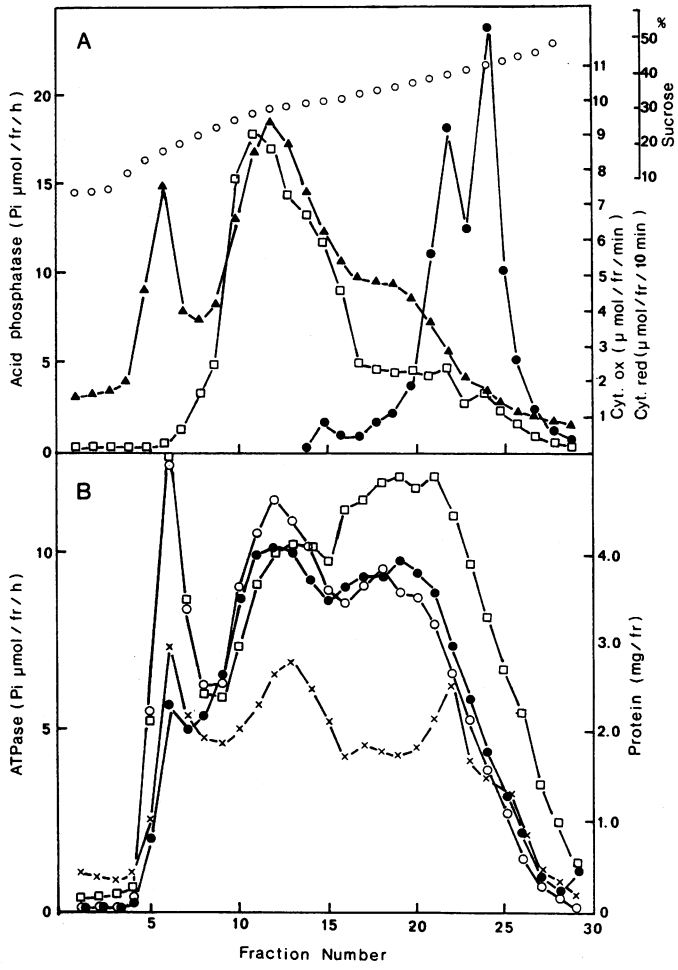

FIG. 1. Sucrose density gradient of crude membranes isolated in sorbitol-containing buffer. Tissues were homogenized in a medium contained 0.25 M sorbitol as an osmoticum and the 3,600 to 156,000*g* pellets were obtained. The pellets were suspended in 0.25 M sorbitol, 5 mM MOPS/KOH (pH 7.3), 1 mM EGTA, 10 mM KCl, 0.2 mM PMSF, 10 µg/ml of BHT, 1 mM DTT, and loaded on a linear sucrose density gradient (15–45%) made up in the same buffer system as used above. After the centrifugation at 89,000*g* for 2 h, 1.2 ml aliquots were collected from the top of the gradient. (A), (▲), acid phosphatase; (□), NADH Cyt *c* reductase; (●), Cyt *c* oxidase. (B), (○), control ATPase: (●), ATPase with presence of 50 mM KNO_3; (□), ATPase with presence of 0.016% Triton X-100; and (×), protein. The ATPase assay mixture basically contained 3 mM ATP, 3 mM $MgSO_4$, and 25 mM Tris-Mes (pH 6.5). The assay was performed at 30°C for 30 min.

as reported before (11). An aliquot of separated tonoplast vesicles (equivalent to 180 µg protein) was preincubated in a reaction mixture containing 0.25 M sorbitol, 25 mM Tris-Mes (pH 7.0), 50 mM KCl, and 10 µM quinacrine-HCl. After the temperature equilibration at 23°C, ATP (Tris-salt) was added to a final concentration of 5 mM. The fluorescence quenching was initiated upon the subsequent addition of $MgSO_4$ in a final concentration of 5 mM. The relative fluorescence intensity was followed at excitation and emission wavelengths of 425 and 500 nm, respectively. Gramicidin D (2 µM) was introduced into the reaction mixture for the reversal of the quenching as a result of the collapse of the proton gradient formed between inside and outside of the tonoplast vesicles.

Electron Microscopy. The isolated tonoplast fraction was tested under the electron microscope. The membrane sample was fixed, dehydrated, and embedded as reported elsewhere (25).

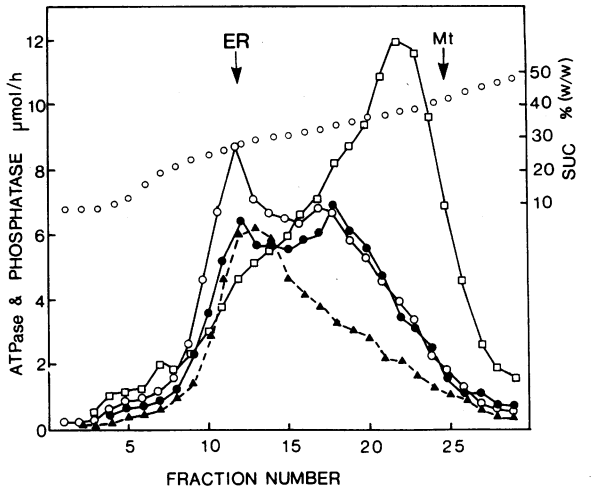

FIG. 2. Sucrose density gradient of crude membranes isolated in sucrose-containing buffer. Tissues were homogenized in a medium contained 0.25 M sucrose as an osmoticum and the 3,600 to 156,000*g* pellets were obtained. The pellets were suspended in 0.25 M sucrose, 5 mM MOPS-KOH (pH 7.3), 1 mM EGTA, 10 mM KCl, 0.2 mM PMSF, 10 µg/ml of BHT, 1 mM DTT, and loaded on a linear sucrose density gradient made up in the same medium as used above. After centrifugation at 89,000*g* for 2 h, 1.2 ml aliquots were collected from the top of the gradient. (○), control ATPase; (●), ATPase with presence of 50 mM KNO_3; (□), ATPase with presence of 0.016% Triton X-100; (▲), acid phosphatase. ER and mitochondria (Mt) were banded at indicated positions (arrows).

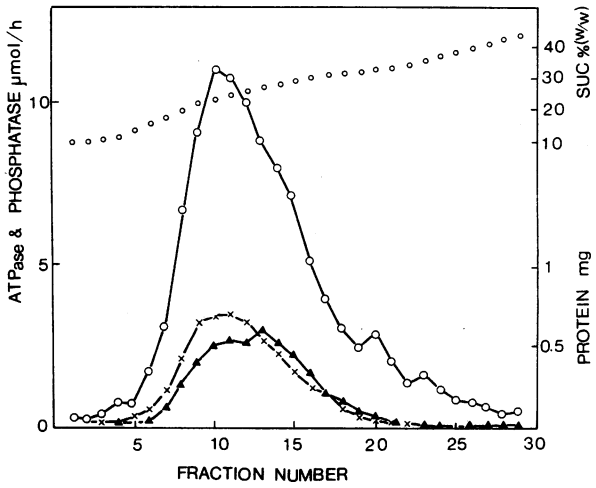

FIG. 3. Isopycnic sucrose density gradient of the interfacial membrane fraction. The interfacial membrane fraction obtained as in Figure 1 was pelleted and briefly sonicated in 10% sucrose made up in the buffer system contained the same ingredients as described in Figure 2 and subjected to a linear sucrose density gradient (15–45%, w/w). After centrifugation at 89,000*g* for 15 h, 1.2 ml aliquots were collected from the top of the gradient. (○), ATPase; (▲), in acid phosphatase; (×), protein.

The ultra-thin sections were routinely stained or with the phosphotungstic acid-CrO_3 method. The stained sections were viewed in a Hitachi JEM 100 electron microscope.

Slab SDS-PAGE. The SDS-solubilized membrane proteins were separated by slab SDS-PAGE as reported (22) and the polypeptide bands were viewed either with Coomassie blue stain or concanavalin A-peroxidase stain (28).

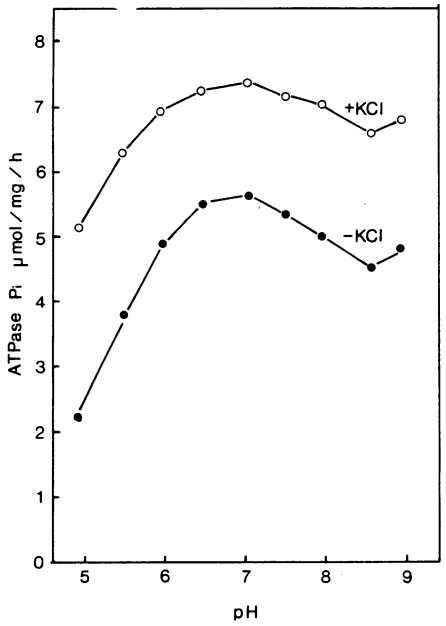

FIG. 4. pH-activity profile of tonoplast ATPase. ATPase assay mixture contained 3 mM ATP-Tris, 3 mM $MgSO_4$, 25 mM Tris-Mes in various pH values and an aliquot of membrane sample in a final volume of 0.25 ml with absence (●) or presence (○) of 50 mM KCl. Reaction was performed at 30°C for 30 min.

Table I. *Effects of Inhibitors on Tonoplast ATPase*

Reaction mixture contained 3 mM ATP, 3 mM $MgSO_4$, 25 mM Tris-Mes (pH 7.0), 50 mM KCl, various concentration of inhibitors and 50 μl aliquots of membrane sample in a final volume of 250 μl. Reaction was performed at 30°C for 30 min. DES, DCCD, and DIDS were made up in 30% ethanol. The final concentration of ethanol was 0.1%.

Inhibitor	Activity	
	μmol/h·mg protein	*% control*
None	5.19	100
Sodium vanadate (50 μM)	4.71	91
NaN_3 (5 mM)	5.29	102
KNO_3 (50 mM)	3.17	61
Sodium molybdate (1 mM)	4.39	85
DES (50 μM)	5.11	99
DCCD (50 μM)	3.53	68
DIDS (30 μM)	4.31	83
Ethanol (0.1%)	5.21	100

RESULTS

Figure 1 indicates activity profiles of various marker enzymes after a sucrose density gradient centrifugation of crude membrane fraction prepared and loaded in sorbitol-buffer system (sorbitol-sucrose system). A massive white band was formed at the interface of sample load and sucrose layer (sorbitol-sucrose interface). The interfacial band was enriched in KNO_3-sensitive ATPase and cofractionated with acid phosphatase. This fraction was clearly separated from ER (antimycin A-insensitive NADH Cyt *c* reductase), mitochondria (Cyt *c* oxidase), and plasma membranes (Triton X-100 stimulative ATPase). The ATPase cofractionating with the interfacial band was less sensitive to vanadate, whereas the Triton X-100 stimulative ATPase associated with heavier fractions (fractions 18–24) was severely inhibited by vanadate (data not shown), suggesting location of plasma membranes in these fractions. In the sorbitol-sucrose density gradient system used in the present study, plasma membranes

Table II. *Substrate Specificity of Tonoplast ATPase*

Reaction mixture contained 3 mM $MgSO_4$, 25 mM Tris-Mes (pH 7.0), 50 mM KCl, 3 mM of substrate, 1 mM Na-molybdate, and 50 μl aliquots of membrane sample in a final volume of 250 μl. Reaction was performed at 30°C for 30 min.

Substrate	Activity	
	μmol/h·mg protein	*% control*
ATP	6.88	100
ADP	1.21	17.6
AMP	0.58	8.4
CTP	1.45	21.1
GTP	2.93	42.6
ITP	2.69	39.1
IDP	1.71	24.9
UTP	3.32	48.3
UDP	1.63	23.7
p-Nitrophenylphosphate	0.55	8.0

Table III. *Effects of Divalent Cations on Tonoplast ATPase*

Reaction mixture contained 3 mM ATP, 3 mM divalent cation as Cl salt, 25 mM Tris-Mes (pH 7.0), 50 mM KCl, 1 mM Na-molybdate and 50 μl of membrane suspension in a final volume of 250 μl. Reaction was performed at 30°C for 30 min.

Divalent Cation	Activity	
	μmol/h·mg protein	*% control*
None	2.60	100
Mg^{2+}	5.12	197
Mn^{2+}	4.40	169
Co^{2+}	2.56	99
Ca^{2+}	1.76	68
Zn^{2+}	0.68	26
Cu^{2+}	0.37	14
Cd^{2+}	0	0
Sr^{2+}	1.90	73
Hg^{2+}	0.14	5

Table IV. *Effects of Monovalent Ions on Tonoplast ATPase*

Reaction mixture contained 3 mM ATP, 3 mM $MgSO_4$, 25 mM Tris-Mes (pH 7.0), 1 mM Na-molybdate, and 50 mM monovalent cation or anion and 50 μl of membrane suspension in a final volume of 250 μl. Reaction was performed at 30°C for 30 min.

Ions	Activity	
	μmol/h·mg protein	*% control*
Cations		
None	5.40	100
KCl	7.24	134
NaCl	6.90	128
LiCl	6.46	120
NH_4Cl	8.78	163
Choline-Cl	6.48	120
Anions		
None	5.40	100
KCl	7.33	136
KNO_3	3.90	72
KNO_2	7.85	145
KF	4.61	85
KI	5.67	105
K_2SO_4	5.38	99

Table V. *Effect of Proton Ionophores on Tonoplast ATPase*

Reaction mixture contained 3 mM ATP, 3 mM $MgSO_4$, 25 mM Tris-Mes (pH 7.0), 50 mM KCl, and 50 μl aliquots of membrane sample in a final volume of 250 μl. Reaction was performed at 30°C for 30 min in the presence of 0.25 M glucose.

Ionophore	Activity	
	μmol/h·mg protein	*% control*
None	3.96	100
FCCP (10 μM)	5.78	146
Gramicidin D (2 μM)	5.38	136

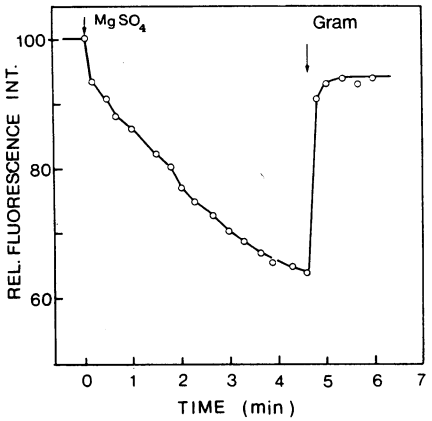

FIG. 5. H^+-transport activity measured by quenching of quinacrine fluorescence. An aliquot of tonoplast vesicles (180 μg protein) was preincubated in a reaction mixture containing 0.25 M sorbitol, 25 mM Tris-Mes (pH 7.0), 50 mM KCl, and 10 μM quinacrine. Following temperature equilibration at 23°C, ATP (Tris-salt) was added to a final concentration of 5 mM. Fluorescence quenching was initiated upon the subsequent addition of $MgSO_4$ to a final concentration of 5 mM. Gramicidin D (2 μM) was added where indicated.

were banded at the slightly lower density of the gradient than in ordinary sucrose-sucrose density gradient system (Fig. 2).

When the whole procedure was carried out by using sucrose-buffer system instead of sorbitol as the osmotic stabilizer and subjected to a sucrose density gradient centrifugation (sucrose-sucrose density gradient system), no interfacial band was formed at the boundary of the sample load and the sucrose gradient (Fig. 2). The KNO_3-sensitive ATPase was cofractionated with ER at the 25 to 26% sucrose layers. In other plant materials, tonoplasts are reported to be equilibrated around those densities. Thus, a difference is quite evident in the banding behaviors of tonoplasts between the sorbitol-sucrose and the sucrose-sucrose density gradient systems.

When the interfacial band obtained by the sorbitol-sucrose density gradient system was pelleted and resuspended in 10% sucrose-buffer system with brief sonication, and then subjected to a linear sucrose density gradient centrifugation, a single band with KNO_3-sensitive ATPase was equilibrated at the sucrose layers of 25 to 26% (Fig. 3).

Figure 4 shows a pH-activity profile of the ATPase associated with the interfacial membrane fraction. The pH curve was observed to be broad toward alkaline pH and the optimum was around pH 7.0, slightly higher than that of the plasma membrane ATPase as has been reported (23). The moderate KCl stimulation was observed in the wide range of pH.

Table I shows the effects of various inhibitors upon the ATPase. The enzyme was inhibited by KNO_3, DCCD, and DIDS, while no inhibition or only a slight inhibition was observed with azide, vanadate, and DES. A slight inhibition by molybdate was due to the included phosphatase activity.

As shown in Tables II and III, the ATPase had high specificities both for ATP and Mg^{2+}. Unlike the plasma membrane ATPase, Co^{2+} had no stimulatory effect (23).

Table IV shows the effects of monovalent ions upon the ATPase. The ATPase was stimulated more or less by all the chloride salts used. On the other hand, no stimulation was detected by K_2SO_4. This means that the stimulation by the chloride salts may be due to the accompanying Cl^- ion.

Table V shows the effect of proton ionophores upon the ATPase activity. The ATP was stimulated to some degree by the addition of 10 μM FCCP and 2 μM gramicidin D. This is indicative of the presence of a proton-driving ATPase in the interfacial membranes. This was also confirmed by the experiments using fluorescence quenching of quinacrine introduced into the suspension of the interfacial membranes (Fig. 5). The fluorescence was markedly quenched by the addition of Mg^{2+} and ATP into the membrane suspension and the quenching was almost completely reversed by the addition of gramicidin D.

Figure 6 shows electron micrographs of the isolated interfacial membranes. The fraction consisted of vesicles with relatively uniform size (a) and most of them were not stained by phosphotungstic acid-CrO_3 which is a specific stain for the plasma membrane.

As can be assessed by the KNO_3-sensitive, vanadate-insensitive

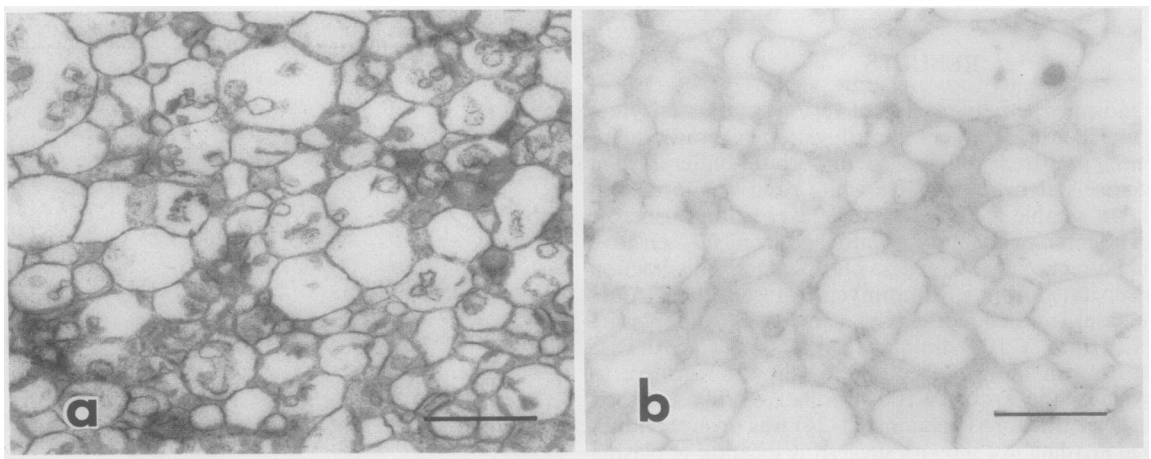

FIG. 6. Electron micrographs of the tonoplast-enriched interfacial fraction. Before viewing with electron microscope, the thin sections were stained either with lead citrate-uranyl acetate (a) or with phosphotungstic acid-CrO_3 stain (b). Bars represent 1 μm.

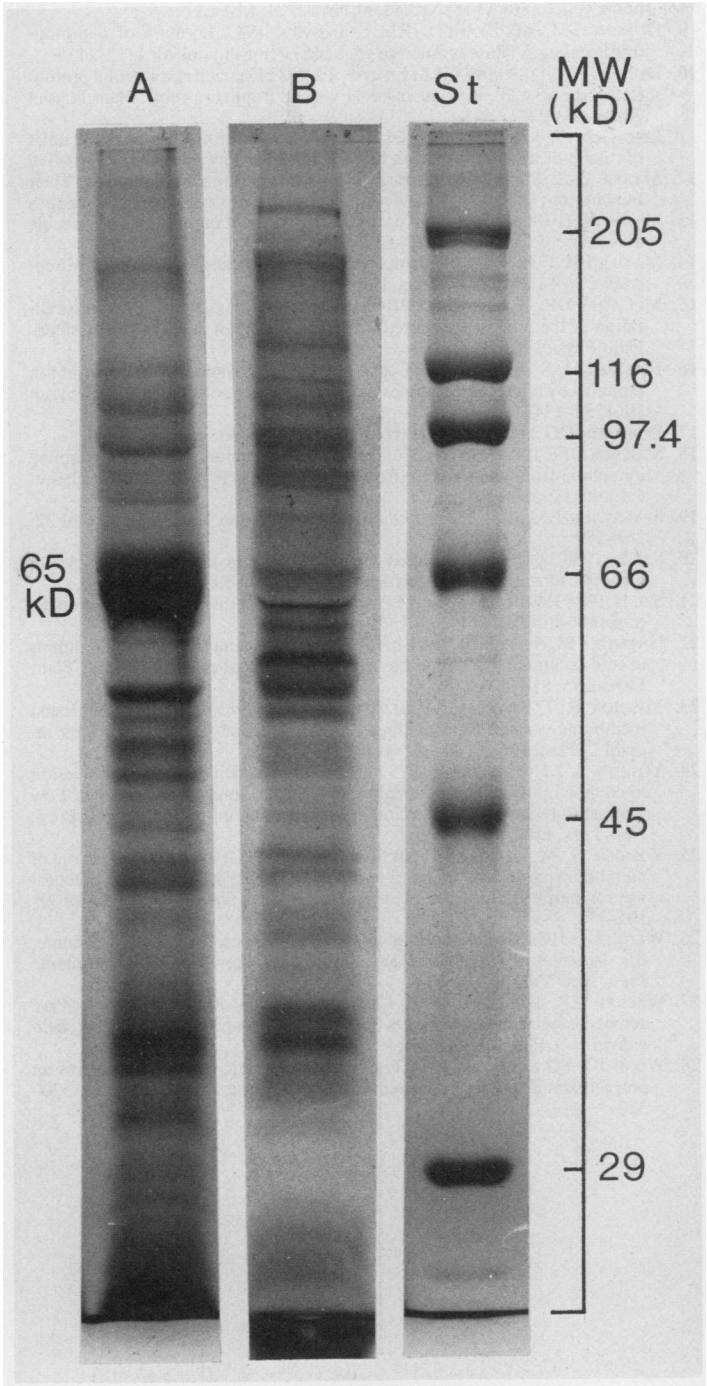

FIG. 7. SDS-PAGE of tonoplast polypeptides. Tonoplast samples were solubilized in 2% SDS-65 mM Tris-HCl (pH 6.8) and 50 mM DTT with boiling for 3 min. Aliquots of the solubilized membrane samples (30 μg) were electrophoresed on 1 mm thick slab gel (10% polyacrylamide in separating gel) as reported previously (22). After the electrophoresis, the gels were stained either with Coomassie blue (A) or Con A-peroxidase stain (B) for glycopeptides (28). St, molecular marker proteins.

ATPase and by the absence of other marker enzymes, and together with the morphological observation, the interfacial membrane fraction isolated on the sorbitol-sucrose density gradient system was considered to be highly pure tonoplasts.

Figure 7 shows SDS-PAGE of the polypeptides (A) and glycopeptides (B) of the tonoplasts. The major band (65 kD) was characteristics of the tonoplasts proteins and it could hardly be found in other membranes such as plasma membranes, ER, Golgi membranes, and mitochondria (T. Kawata and S. Yoshida, unpubished data). It is of interest to note that the tonoplasts contained several glycoproteins (Fig. 7B).

DISCUSSION

A great deal of attention has been focused on the physiological roles of vacuoles (12, 14, 17). After establishment of the reliable marker for tonoplast, *i.e.* KNO_3-sensitive proton-pump ATPase (21), we can easily identify the membrane by following the marker all through the isolation procedure. However, no convenient technique to separate the tonoplasts in high purity and also in large quantity has been established as yet.

In the present study, a method to purify the tonoplasts from homogenates of etiolated young seedlings of *V. radiata* was established by using a sorbitol-sucrose gradient system. The whole procedure from preparation of homogenate to the subsequent density gradient may be completed within 3 h. This seems to be an advantageous factor for the separation of the tonoplasts, since vacuoles, in general, are known to be a lytic compartment in the plant cells and containing a large amount of lytic enzymes (14, 17).

Assessed by the degree of contamination by the other marker enzymes specific for the other membranes, together with the ultrastructural observations, the isolated tonoplasts seem to be of high purity. Cl^--stimulated, NO_3^--sensitive, and proton-motive ATPase located in the isolated tonoplasts obtained in the present study is well established in other plant materials (2, 8–10, 16, 19, 20, 27), although the purity with other plant materials is doubtful. According to our recent studies, Mg^{2+}-ATPases are found to be associated with a wide variety of membrane systems including ER, Golgi-derived membranes isolated from the same plant materials as used here (T Kawata, S Yoshida, unpublished data). Upon an ordinary sucrose gradient (sucrose-sucrose density gradient system), tonoplasts co-sediment with ER and partially with Golgi membrane-vesicles at 24 to 25% sucrose. The mutual separation is not feasible. In the present study, after sucrose density gradient of crude membranes which were prepared in a sorbitol medium (sorbitol-sucrose density gradient system), the tonoplast vesicles were collected at the sample load/sucrose interface (about 1.05 g/cm^3) in a relatively uniform size (0.3–1.4 μm) without contamination of other membrane materials. Since the equilibrated density of the tonoplast on an ordinary sucrose-sucrose density gradient system was around 1.06 to 1.07 g/cm^3, the banding of tonoplast vesicles at sample load/sucrose interface is unlikely to be due to the effective density of the tonoplast *per se* but presumably due to an inclusion of sorbitol (0.25 M) in the tonoplast vesicles during membrane preparation in a sorbitol-buffer system, making them apparently lighter in the sucrose gradient. The possibility seems to be also supported by the fact that plasma membrane showed apparently lighter density (32–36%, Fig. 1) in the sorbitol-sucrose density gradient system than in the sucrose-sucrose density gradient system (36–37%, Fig. 2). It is important to note that effectiveness of the sorbitol-sucrose density gradient system for the isolation of tonoplast is guaranteed only when plant tissues are disrupted with minimum mechanical forces so that tonoplasts are transformed into sealed vesicles with intact semipermeability to solutes. As illustrated in Figure 6, most of the tonoplasts seem to be isolated in sealed vesicles with relatively uniform size.

The SDS-PAGE of tonoplast proteins showed a unique pattern with relatively large quantity of a major band (65 kD) and thus easily discriminated from those of the other membranes, such as ER, Golgi membranes, mitochondria (T Kawata, S Yoshida, unpublished data), and plasma membranes (23). Although the detailed information on the chemical composition of any purified tonoplast is quite limited, according to Marty and Branton

(13), it has been reported that polypeptides both from purified beet root tonoplasts and the vacuolar sap contain much glycoprotein. In the present study, it was confirmed that the tonoplasts of *V. radiata* also contain several kinds of mannosylated or glycosylated glycoproteins. The physiological functions of these glycoproteins in the tonoplast remain future problems to be investigated.

In our earlier studies on ultrastructural changes in chilling-sensitive cultured cells of *Cornus stolonifera* L. in response to cold treatment, an aggregation of the inner-membrane particles of tonoplast *p*-face (cytoplasmic fracture face) and subsequent disruption of the membranes were observed during the early process of cold treatment (24). Considering the fact that the plant vacuoles are a lytic compartment containing several types of hydrolytic enzymes (14, 17), the observed changes in the tonoplast structure during chilling of the cells may have a prominent role in determining cell survival. To elucidate the mechanism of chilling injury of plant cells, further detailed studies on the cause of the tonoplast lesion during chilling are clearly needed. For this purpose, experiments are going on in our laboratory using tonoplast samples prepared from *V. radiata* which is highly chilling-sensitive plants.

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