

# Plasma Membrane from Muskmelon Leaves: Purification and Lipid Composition during Growth at 15 or 30C

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**Abstract.** Using an aqueous polymer two-phase [polyethylene glycol (PEG) 3400/dextran T500, 6.2% : 6.2%, w/w] partitioning procedure combined with isopycnic fractionation, plasma membranes derived from muskmelon (*Cucumis melo* L. var. *reticulatus* Naud.) leaf blades have been isolated and examined for marker enzyme activity, density, and molecular composition. After aqueous polymer partitioning, plasma membranes were centrifuged on a linear sucrose density gradient, and a single band was found at the 31% (w/w) sucrose (1.13 g-cm<sup>-3</sup>). Identification of plasma membranes was performed by the combination of K<sup>+</sup>-stimulated ATPase, pH 6.5, vanadate inhibition of ATPase and KNO<sub>3</sub>-insensitive ATPase activity. Plasma membranes from seedling leaves grown for 5 days at 15C had the highest concentration of total phospholipids, the lowest concentration of proteins, and a total sterol concentration not significantly different from leaves grown at 30C. The total sterol to total phospholipid ratio of the plasma membrane from leaves grown for 5 days at 15C was ≈1:1; from leaves grown for 10 days at 15C or 5 days at 30C the ratio was ≈2:1; and from leaves grown for 10 days at 30C the ratio was ≈3:1. The plasma membrane phospholipid saturated to unsaturated fatty acid ratio from leaves grown for 5 days at 15C was ≈0.8:1.0; from leaves grown for 10 days at 15C or 5 days at 30C the ratio was ≈1.0:1.0; and from leaves grown for 10 days at 30C it was 1.4:1.0.

Gombos and Vigh (1986) have shown that the plasma membrane of blue-green algae has the primary role in thermal adaptation of this organism. There is evidence to indicate that the plasma membrane of *Cucumis melo* L. is directly involved in heat acclimation of muskmelon leaves (Lester, 1985, 1986). However, it appears that age-related changes are more important in enhancing muskmelon leaf cellular thermostability than is growing temperature (Lester, 1986). This fact indicates that the dynamics of membrane reorganization during development are the probable mechanism regulating muskmelon leaf thermostability.

Isolation and purification of plasma membrane are essential for measurement of heat- and age-related effects on membrane molecular composition. In this study, an aqueous polymer two-phase partitioning system in tandem with a linear sucrose density gradient was used to obtain relatively pure, chlorophyll-free plasma membrane isolates from green muskmelon leaves. The purpose of this study was to isolate, purify, and characterize the plasma membrane from muskmelon leaves grown at 15 or 30C for 5 or 10 days in order to directly measure the influence of growing temperature and aging on plasma membrane molecular composition.

## Materials and Methods

*Plant material.* 'Perlita' muskmelon seeds were germinated and grown in 1 vermiculite : 1 sphagnum peat (v/v) and were fertilized as described by Lester and Dunlap (1985). Seedlings were grown in a glasshouse at 20C minimum nights and 35C maximum days under a 12-hr photoperiod supplemented with (300 μmol·s<sup>-1</sup>·m<sup>-2</sup>) high-pressure sodium lights. After first true leaf emergence, the seedlings were moved to growth chambers

held at either 15 or 30 ± 2C with 85% ± 3% RH and a 12-hr photoperiod (440 μmol·s<sup>-1</sup>·m<sup>-2</sup>) for 5 or 10 days.

*Isolation of membranes.* All procedures were a modification of Kjellbom and Larsson (1984) and were carried out at 4C. Leaf blades without petioles (75 g) were washed in ice-cold water for 5 rein, then torn and placed in a mortar with 250 ml homogenizing buffer: 0.5 mM sucrose, 50 mM Hepes-KOH, pH 7.5, 5 mM ascorbic acid, 5 mM EDTA, 5 mM dithiothreitol (DTT), and 0.6% (w/v) polyvinylpyrrolidone (PVPP). Ascorbic acid and PVPP were added just before homogenization. Leaf blades were macerated in the homogenizing medium for 5 min with a pestle, placed under vacuum for 10 rein, then macerated an additional 5 min. The material was transferred to a tall beaker and homogenized for 30 sec at medium speed, using a Polytron homogenizer (Brinkman Instr., Westbury, N. Y.) equipped with a PTA-50 generating probe. The homogenate was filtered through two layers of Miracloth (Calbiochem, La Jolla, Calif.) and centrifuged at 14,000× g for 20 min. A microsomal pellet was obtained from the 14,000× g supernatant by centrifugation at 100,000× g for 30 min. This pellet was homogenized at the slowest speed, using a Polytron homogenizer, in 10 ml of suspension buffer: 0.33 M sucrose, 3 mM KCl, and 5 mM potassium phosphate, pH 7.8. The homogenized suspension (9 g) was added to 27 g aqueous polymer buffer containing 6.2% (w/w) dextran T500, 6.2% (w/w) PEG 3400 at final weight. The phase system was mixed well and phase separation was accelerated by centrifugation in a swinging bucket centrifuge at 1500× g for 3 min. Upper and lower phases were separated and the lower phase was washed with equal volumes of 6.2% (w/w) fresh upper phase. The upper phases were washed with equal volumes of fresh 6.2% lower phase until a chlorophyll-free preparation was achieved (about three washes). The upper phases were pooled and a plasma membrane-enriched pellet was recovered by centrifugation at 100,000× g for 30 min. The lower phases plus interface material were pooled, and the intercellular material was pelleted at 100,000× g for 30 min. Individual pellets were suspended in 1 ml of assay buffer: 250 mM sucrose,

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25 mM Tris-Mes, 1 mM EDTA, and 5 mM DTT and kept on ice for immediate use or pelleted, flushed with  $N_2$ , and stored at  $-80^\circ C$ . Protein content of membrane fractions was determined by the method of Bradford (1976). Chlorophyll was determined on both upper- and lower-phase pellets according to the modified aqueous acetone procedure of Arnon (1949). Pellets were extracted in 1 ml 80% (v/v) acetone at room temperature, centrifuged at  $25,000\times g$  for 10 min and absorbance was measured according to the modified formula:  $[(A_{645} - A_{710})0.0202 + (A_{663} - A_{710})0.00802]/\text{mg protein} \times \text{dilution}$ .

**Enzyme analysis.** ATPase (EC 3.6.1.8) was assayed at pH 6.5 or 9.0 and 38C by the method described in Hodges et al. (1972), 1-ml reaction mixtures contained 3 mM Tris-ATP, 3 mM  $MgSO_4$ , 30 mM Tris-Mes  $\pm$  50 mM KCl. The pH-activity profile required 100 mM Tris-Mes. UDPase (EC 3.6.1.6) was assayed at pH 6.5 and 38C as described by Nagahashi and Seibles (1986); 1-ml reaction mixtures contained 3 mM UDPase, 3 mM  $MnSO_4$ , 30 mM Tris-Mes  $\pm$  0.03% (v/v) Triton X-100. ATPase and UDPase enzyme assays were stopped by adding 2 ml of 2 N HCl containing 1.25% (w/v) ammonium molybdate. Inorganic phosphate was determined by the method described in Peterson (1978). Monovalent ion stimulation of ATPase activity was assayed in the same manner as ATPase (pH 6.5) activity by adding either 50 mM solutions of KCl, NaCl, RbCl, or Tris-Cl. Latency of K-stimulated, Mg-dependent ATPase activity was assayed in the same manner as ATPase (pH 6.5) activity  $\pm$  50 mM KCl, plus 0%, 0.01%, 0.02%, 0.05%, or 0.10% Triton X-100. Nucleoside diphosphatase or triphosphatase was assayed in the same manner as ATPase (pH 6.5) activity using 3 mM solutions of ADP, CTP, IDP, GTP, or UTP as substrate. Vanadate and nitrate effect on ATPase was assayed by adding to the assay 0.1 mM vanadate in the presence of 50 mM KCl or 50 mM  $KNO_3$  without KCl as described by Bennett et al. (1982).

**Isopycnic sucrose density gradient.** Upper-phase plasma membrane-enriched pellets were suspended in 2 ml of storage buffer modified to 20% sucrose (w/w) then layered on a 36-ml linear sucrose gradient of 20% to 50% (w/w) sucrose in 40 mM Tris-Mes, 1 mM EDTA (pH 8.0), and 1 mM DTT, then centrifuged at  $75,000\times g$  for 15 hr at 2C. After centrifugation, 1.5-ml fractions were collected from the bottom of the gradient with a proportioning pump at a flow rate of  $0.5 \text{ ml}\cdot\text{min}^{-1}$  at 4C.

**Total sterol and phospholipid determination.** Lipids were extracted from isolated gradient fraction using the procedure of Uemura and Yoshida (1984). Total sterols were eluted through a Sep-Pak silica cartridge (Waters Assoc. Inc., Milford, Mass.) by adding 5 ml of chloroform, then 3 ml of 10% acetone in chloroform. Acylated steryl glycosides and diglycerides, if present, were eluted by addition of 10 ml of acetone. Phospholipids were eluted by addition of 10 ml 1 chloroform : 1 methanol (v/v), followed by 10 ml of methanol. Quantitative analyses of sterols were performed according to Zlatkis and Zak (1969) by dissolving sterols in 5 ml of chloroform, 3 ml of acetic anhydride, and 0.1 ml of concentrated sulfuric acid for 10 min in darkness. The absorbance was measured at 415 nm and campesterol was used as the standard. Quantitative analyses of phospholipids was performed according to Peterson (1978).

**Fatty acid determination.** Further purification of phospholipids involved TLC separation along with phospholipid standards on nonactivated silica Gel G plates (Analabs, Norwalk, Conn.) developed at 21C in filter-lined chambers with a 130:60:8 solvent system of chloroform, methanol, and 7 N sodium hydroxide, (by volume), respectively. Phospholipids were visu-

alized with Rhodamine 6G and then scraped from the plate and methylesters were prepared by the transesterification  $BCl_3$ -MeOH method of Klopfenstein (1971). Fatty acid methylesters were analyzed by GC using a supelcowax 10 (Supelco, Inc. Bellefonte, Pa.) capillary column  $30 \text{ m} \times 0.25 \text{ }\mu\text{m}$  and quantified using methyl heptadecanoate as the internal standard.

## Results

The upper- (PEG 3400) and lower- (dextran T500) phase separation in the 6.2% :6.270 (w/w) phase system showed that 89% of the plasma membrane marker activity was recovered in the upper phase (Table 1). Only 10% of the Golgi body marker (Triton X-100 stimulated UDPase, pH 6.5), no endoplasmic reticulum or mitochondrial marker (NADH-cytochrome-C reductase and ATPase, pH 9.0, respectively), and no chlorophyll was recovered in this upper fraction. Tonoplast membrane marker ( $KNO_3$  inhibition of ATPase) was determined just on the upper-phase fraction (data not shown) and demonstrated no activity. The lower-phase fraction, which should contain all microsomal membranes, except the plasma membrane, showed 100%, 81%, and 12% of the total activity for markers of ER, mitochondria, Golgi body, and plasma membrane, respectively, and 100% for chlorophyll content (Table 1). Table 2 shows the nucleotide and monovalent ion specificity of the plasma membrane ATPase. The greatest nucleotide activity occurred with ATPase, and with GTPase showing 48% of the ATPase activity. The greatest monovalent ion stimulation of ATPase occurred with KCl, with the activity measured in the presence of  $Na^+$  being 84% of the activity measured in the presence of  $K^+$ .

The pH curves for ATPase activity assayed on upper-phase fractions were highest at 6.5 with KCl and at 7.0 without KCl (data not shown). Depending on pH, the specific activity of Mg-ATPase marker was increased 8% to 16% in the presence of KCl. The specific activity of KCl-stimulated Mg-ATPase (pH 6.5) activity was stimulated by 0.01% and inhibited by 0.1% Triton X-100 (data not shown).

In the presence of DTT, plasma membrane, and Golgi body marker, phosphohydrolase activity was preserved. Otherwise,  $>30\%$  reduction in phosphohydrolase activity would occur following isolation and isopycnic centrifugation (data not shown).

Upper-phase, plasma membrane-enriched pellets fractionated over a linear sucrose gradient of 20% to 50% (Fig. 1A), showed that protein was concentrated in the linear fractions corresponding to densities between  $1.11$  to  $1.14 \text{ g}\cdot\text{cm}^{-3}$  (Fig. 1B). The peak concentration of protein occurred at a density of  $1.13 \text{ g}\cdot\text{cm}^{-3}$ . The greatest phosphohydrolase activity of the plasma membrane marker was coincident with the protein peak (Fig. 1C). The Mg-ATPase, pH 6.5 minus KCl, also co-fractionated at  $1.13 \text{ g}\cdot\text{cm}^{-3}$  and had  $\approx 13\%$  less specific activity when compared to the KCl-stimulated Mg-ATPase activity. Inhibitors of ATPase were tested on all protein-enriched sucrose gradient fractions (Fig. 1D). Vanadate, as  $Na_3VO_4$  inhibited the plasma membrane-associated KCl-Mg-ATPase activity by  $> 80\%$ , whereas  $NO_3^-$ , an inhibitor of tonoplast marker, had no effect. The Golgi membrane marker, Triton-stimulated UDPase at pH 6.5, showed the greatest activity within fractions of  $1.11$  to  $1.12 \text{ g}\cdot\text{cm}^{-3}$  and almost no activity at the density of  $1.13 \text{ g}\cdot\text{cm}^{-3}$  (Fig. 1E).

Plasma membrane fractions from leaves grown for 5 days at 15C had the highest concentration of total phospholipids, the lowest concentration of proteins, and total sterols nearly equal in concentration to the plasma membrane from leaves grown for 5 or 10 days at 30C (Table 3). The plasma membrane sterol to phospholipid ratio ( $\mu\text{mol} : \mu\text{mol}$ ) increased as growing tem-

Table 1. Specific phosphohydrolase activity ( $\mu\text{mol Pi}/\text{mg per hr}$ ) and total phosphohydrolase activity (%) and chlorophyll concentration associated with muskmelon leaf microsomal membranes partitioned into aqueous two-phase polymer, PEG 3400 (upper) or dextran-T500 (lower) 6.2% : 6.2% (w/w). Plasma membrane marker (Mg-ATPase pH 6.5 plus KCl), endoplasmic reticulum marker (NADH cytochrome-C reductase), and Golgi body marker (UDPase pH 6.5 plus Triton X).

Phase	Mg-ATPase pH 6.5 plus KCl		NADH cytochrome-C reductase		UDPase pH 6.5 plus Triton X		Chlorophyll	
	$\mu\text{mol Pi}/$ $\text{mg per hr}$	%	$\mu\text{mol NADH}/$ $\text{mg per hr}$	%	$\mu\text{mol Pi}/$ $\text{mg per hr}$	%	$\text{mg}\cdot\text{mg}^{-1}\cdot\text{ml}^{-1}$	%
Upper	63.0	89	0.0	0	3.0	10	0.00	0
Lower	7.7	11	1.2	100	26.0	90	0.23	100

Table 2. Nucleotide and monovalent ion specificity of the membrane-bound ATPase activity from muskmelon leaf plasma membrane isolated by two-phase partitioning and isopycnic sucrose gradients. The liberation of Pi from the various phosphate substrates at 3 mM was determined at 37C and pH 6.5 in 33 mM Tris·Mes and 3 mM  $\text{MgSO}_4$ . Monovalent ion specificity was the same as ATPase plus 50 mM of the various ions. Specific activity is presented in  $\mu\text{moles Pi}$  released/mg protein per hr.

Substrate	ATPase activity ( $\mu\text{mol Pi}/\text{mg per hr}$ )	Ion	ATPase activity ( $\mu\text{mol Pi}/\text{mg per hr}$ )
ATP	51.0	KCl	12.0
GTP	24.4	NaCl	2.0
UTP	21.4	RbCl	0.0
IDP	18.4	Tris·Cl	0.0
CTP	17.9		
ADP	14.8		

perature increased and leaves aged (Table 3). The total sterol to total phospholipid ratio of the plasma membrane from leaves grown for 5 days at 15C was 50% that of plasma membrane from leaves grown for 10 days at 15C or 5 days at 30C and  $\approx 40\%$  that of plasma membrane from leaves grown for 10 days at 30C. The plasma-membrane phospholipid, saturated to unsaturated fatty acid ratio also increased as growing temperature increased and leaves aged (Table 3). From leaves grown for 5 days at 15C, the plasma-membrane fatty acids were more unsaturated; while from leaves grown for 10 days at 15C or 5 days at 30C the fatty acids were nearly equal in saturation to unsaturation, and from leaves grown for 10 days at 30C the plasma-membrane fatty acids were more saturated.

### Discussion

This investigation has shown that relatively pure plasma membrane from green muskmelon leaves can be "quickly" obtained by aqueous polymer separation in tandem with isopycnic sucrose gradient centrifugation and is suitable for further membrane investigations. Following isopycnic centrifugation, plasma membranes from muskmelon leaves equilibrate at a density of  $1.13 \text{ g}\cdot\text{cm}^{-3}$  ( $\approx 31\%$  w/w sucrose). This density fraction was shown to be free of markers for mitochondrial membrane, Golgi membrane, and tonoplast. The relatively light density for muskmelon leaf plasma membrane was consistent with previous work on green tissues. Pea (*Pisum sativum* L.) epicotyl (Pierce and Hendrix, 1979) plasma membrane had a density of  $1.13 \text{ g}\cdot\text{cm}^{-3}$  and orchard grass (*Dactylis glomerata* L.) culms (Yoshida and Umera, 1984) had plasma membrane density of 1.14 to 1.15  $\text{g}\cdot\text{cm}^{-3}$  ( $\approx 34\%$  w/w sucrose). Non-green tissues such as oat (*Avena sativa* L.) roots (Hodges et al., 1972) and etiolated mung

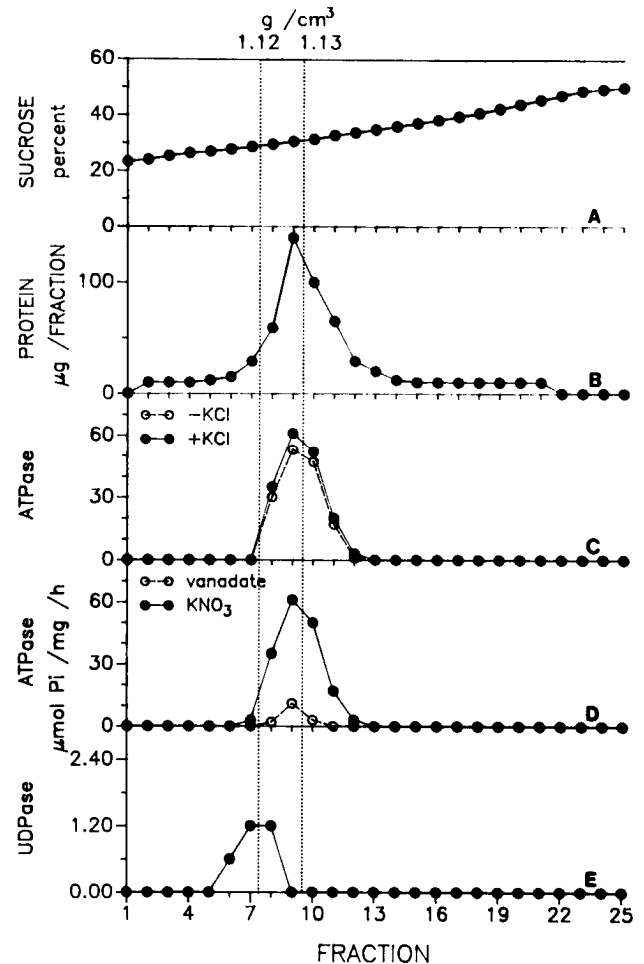


Fig. 1. Sucrose density (20% to 50% w/w) gradients of upper-phase (polyethylene glycol 6.2%, w/w) fraction membrane pellet from 300 g (fresh weight) of muskmelon leaf homogenates. A pellet was suspended in 20% sucrose (w/w), 40 mM Tris·Mes, pH 8.0, 1 mM EDTA, 1 mM DTT and centrifuged at  $75,000\times g$  for 15 hr at 2C, then twenty-five 1.5-ml fractions were collected. (A) Sucrose %. (B) Protein. (C) ATPase activity assayed at pH 6.5  $\pm$  KCl. (D) Vanadate or  $\text{KNO}_3$  inhibition/stimulation of ATPase activity at pH 6.5. (E) Triton X-100-stimulated UDPase assayed at pH 6.5. Each value represents six replications.

bean (*Vigna radiata* L.) hypocotyls (Yoshida et al., 1986) have a plasma membrane density of 1.17 to 1.18  $\text{g}\cdot\text{cm}^{-3}$  ( $\approx 38\%$  to 40% w/w sucrose).

Compositional analysis of purified plasma membrane indi-

Table 3. Comparison of the plasma membrane isolated from 'Perlita' muskmelon leaves grown for 5 or 10 days at 15 or 30C for protein, total sterols ( $\mu\text{mol}/\text{mg}$  membrane), total phospholipids ( $\mu\text{mol}/\text{mg}$  membrane), sterol to phospholipid ratio (sterol : phos) and phospholipid saturated to unsaturated fatty acid ratio (sat : unsat).

Growing conditions [Days/temp ( $^{\circ}\text{C}$ )]	Protein ( $\text{mg}\cdot\text{g}^{-1}$ dry wt)	Sterols ( $\mu\text{mol}\cdot\text{mg}^{-1}$ )	Phospholipids ( $\mu\text{mol}\cdot\text{mg}^{-1}$ )	Sterol : Phos ( $\mu\text{mol}\cdot\text{mg}^{-1}$ : $\mu\text{mol}\cdot\text{mg}^{-1}$ )	Fatty acid (sat : unsat)
5/15	0.05	1.69	1.27	1.3:1.0	0.77:1.0
10/15	0.11	1.24	0.52	2.6:1.0	0.98:1.0
5/30	0.15	1.51	0.60	2.5:1.0	1.01:1.0
10/30	0.16	1.59	0.49	3.3:1.0	1.40:1.0
LSD = 0.05	0.03	0.18	0.09		

icates that a definite and characteristic relationship exists between growing temperature and leaf age. When seedling muskmelon leaves are grown for 5 days at 15C vs. 5 days at 30C, the plasma membrane from leaves grown at 15C initially has significantly less protein, more unsaturated than saturated fatty acids, more total phospholipids, but no significant difference in total sterols. As muskmelon leaves age from 5 to 10 days, regardless of growing temperature, the fatty-acid composition becomes more saturated and there is a further decline in plasma membrane total phospholipids. This results in an ever-increasing sterol to phospholipid ratio. Although growing temperature has an impact on cell metabolism, it is secondary to aging in adapting the plasma membrane for thermostability (Lester, 1985), which indicates that cells from leaves grown under low vs. high temperatures will require different lengths of time to achieve equal physiological development and subsequent equal membrane thermostabilities. Others have reported similar membrane protein (Smaraka and Szarak, 1986), phospholipid (Kinney et al., 1987), sterol (Uemura and Yoshida, 1984), and fatty-acid ratio (Christianson, 1978) changes after growing plants at low vs. high temperatures. Also, lipid saturation has been directly linked to tomato leaf cell thermostability (Tal and Shannon, 1989).

In a heat injury study, Lester (1986) showed that, in 5-day-old 'Perlita' muskmelon leaves grown at 15 vs. 35C, then subjected to 50C for 15 min, heat injury was 70% and 48%, respectively. After 9 days at 15 or 35C, heat injury of leaves was 35% and 20%, respectively, indicating that thermostability of muskmelon leaves increases with age and that in 10-day-old or older leaves, heat tolerance is not significantly affected by growing temperature (Lester, 1985). It would appear that inherent properties (probably enzymes) expressed with age are responsible for regulating the compositional molecular state of the plasma membrane and subsequent leaf thermostability.

The observed thermostability of *Cucumis melo* leaves (Lester, 1985, 1986) appears to be a predetermined molecular event that, with age, develops the fluid state of the plasma membrane to function at relatively high growing temperatures.

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