Plant Physiol. (1987) 83, 265-271
0032-0889/87/83/0265/07/$01.00/0

Electrophoretic Characterization of a Detergent-Treated Plasma Membrane Fraction from Corn Roots[1]

Received for publication June 20, 1986 and in revised form September 4, 1986

SEAN R. GALLAGHER[2] AND ROBERT T. LEONARD*
Department of Botany and Plant Sciences, University of California, Riverside, California 92521

ABSTRACT

Experiments were conducted to determine conditions essential for electrophoretic characterization of a detergent-extracted plasma membrane fraction from corn (*Zea mays* L.) roots. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) initially gave poor resolution of polypeptides in the plasma membrane fraction and, upon detergent treatment for purification of the proton-pumping adenosine triphosphatase (ATPase), showed no enrichment for a 100 kilodalton catalytic subunit characteristic of the ATPase. In contrast to SDS-PAGE, phenol urea acetic acid (PAU)-PAGE clearly resolved two polypeptides in the 100 kilodalton region that were enriched during detergent treatment and indicated at least one polypeptide forms a phosphorylated intermediate characteristic of the ATPase. Problems with SDS-PAGE were found to be caused, in part, by a combination of endogenous proteases and heat-induced aggregation of high molecular weight proteins. The usually standard procedure of boiling the sample prior to SDS-PAGE caused the aggregation of the 100 kilodalton polypeptides. By controlling for proteases using chymostatin and/or phenylmethane sulfonyl floride, and not boiling the sample prior to electrophoresis, two polypeptides were clearly resolved by SDS-PAGE in the 100 kilodalton region of Triton X-114-extracted membranes from corn, oat, barley, and tomato.

The transport of most of the essential mineral nutrients into root cells involves ion carriers in the plasma membrane which are most likely integral proteins or protein complexes which span the membrane (23). Purification and characterization of carrier proteins requires the ability to extract plasma membrane vesicles with detergents without significant denaturation of integral proteins and the ability to follow purification of plasma membrane polypeptides by electrophoresis (17, 24, 35).

The selection of detergents and the determination of extraction conditions for purification of integral proteins cannot be based strictly on theoretical grounds, but must be determined empirically (16, 17). There are many detergents available for use in removing proteins from the membrane, and there are several types of strategies which can be used to purify integral membrane proteins (17, 35, 36). The two approaches most often used involve removal of peripheral proteins by treatment with low concentrations of detergent followed by recovery of the integral protein still associated with the extracted membrane (negative purification) and/or solubilization of the membrane at a detergent concentration near its critical micelle concentration and separation of integral proteins by, for example, rate zonal centrifugation or affinity chromatography (positive purification). The success of these approaches is greatly influenced by the initial proportion of the protein of interest in the membrane and the extent of purification is usually less than 6-fold (11).

The routine application of SDS-PAGE to analysis of polypeptide components of the plant plasma membrane fraction has proved difficult and often produced gels with low resolution, particularly in the high mol wt range. Hence, there are relatively few reports on the analysis of the polypeptide composition of plasma membrane fractions (4, 5, 9, 10, 29, 32, 38). The basis for the difficulties is not clearly understood, although aggregation of polypeptides and protease degradation are likely to be involved.

The plasma membrane of all plant cells contains an ATP-driven H^+ pump which plays a central role in energy transfer to nutrient ion transport (23, 32, 34). This ATPase is the only carrier protein in the plasma membrane which has been extensively characterized and yet its precise role in ion transport is not clearly understood. Most attempts to purify the enzyme so that its function can be more precisely determined by reconstitution into lipid vesicles have resulted in only partial purification (3, 10, 21, 27, 31, 37). However, Anthon and Spanswick (2) have recently succeeded in obtaining a highly purified ATPase preparation from tomato roots.

In this paper, we describe electrophoretic conditions essential for following purification of membrane-associated polypeptides by SDS-PAGE.

MATERIALS AND METHODS

Plant Material. Corn seeds (*Zea mays* L. WF9 × M017) were germinated at 27°C for 3 d in low light and then transferred to aeroponic tanks and grown in a greenhouse for an additional 18 to 21 d (5). Approximately 200 g of roots were excised, rinsed with cold distilled H_2O, and excess water removed with the aid of a lettuce drier.

Preparation of Plasma Membrane Fraction. Plasma membrane vesicles were isolated as previously described (13). Briefly, roots were homogenized (mortar and pestle) in 250 mM sucrose, 3 mM EDTA, 2.5 mM DTT, and 25 mM Tris-Mes, pH 7.7 (2.4 ml medium/g tissue). The filtered homogenate was centrifuged at 13,000*g* for 20 min, followed by 80,000*g* for 30 min. The 13,000 to 80,000*g* pellet was suspended in 10 ml of 250 mM sucrose, 1 mM DTT, and 1 mM Tris-Mes, pH 7.2 (suspension buffer), and applied to four, 36 ml-34/45% (w/w) sucrose step gradients in 1 mM Tris-Mes, pH 7.2 and 1 mM DTT. Following centrifugation of the gradients at 82,500*g* for 2 h, the plasma membrane fraction was collected at the 34/45% sucrose interface, diluted with suspension buffer, and centrifuged at 80,000*g* for 1 h. The final pellet was made to 15 to 20 mg protein/ml with suspension buffer and stored in 0.25 ml aliquots under liquid N_2.

Assays. ATPase activity and TCA-precipitable protein were

[1] Supported in part by National Science Foundation grant PCM 8025004.

[2] Present address: Biological Sciences Department, Stanford University, Stanford, CA 94305.

measured as previously described (13).

Detergent Treatment of the Plasma Membrane Fraction. Peripheral proteins were extracted from the plasma membrane fraction by treatment with SDS or with Triton X-100 or 114 (TX-100 or 114).[3] For SDS extraction, the 4-fold concentrated solubilization buffer consisting of 0.2 M imidazole, 8 mM EDTA, and 24 mM Na_2ATP (all adjusted to pH 7.5 at room temperature, about 22°C) was mixed with SDS and water. This was added to the undiluted plasma membrane fraction at room temperature over a 10-min period with constant mixing to give the detergent concentrations indicated in the "Results" and final concentrations of buffer components equal to 50 mM imidazole, 2 mM EDTA, and 6 mM Na_2ATP. The final protein concentration was 1 to 2 mg/ml. A protein-to-detergent ratio (w/w) of 5 to 6 was critical to avoid significant inactivation of ATPase activity. The detergent-plasma membrane mixture was applied to a 34/42% (w/w) sucrose in 25 mM imidazole and 1 mM EDTA, pH 7.5 (imidazole buffer A) step gradient and centrifuged at 82,500*g* for 4 h. The SDS-extracted membranes were collected from the interface, diluted in imidazole buffer A to 38 ml, and centrifuged at 160,000*g* for 4 h. The final pellet was suspended in suspension buffer plus 30% (w/v) glycerol and stored under liquid N_2.

For TX-100 or TX-114 extraction, the plasma membrane fraction was diluted to about 1 mg/ml in 10 mM imidazole, 2 mM EDTA, 10% (w/v) glycerol, and 1 mM DDT pH 7.5 (imidazole buffer B). TX-100 or TX-114 was added to the diluted plasma membrane fraction all at one time with constant stirring at ice temperature to give a final detergent concentration of 1% (w/v). The TX-100 or TX-114 usually dissolved in 15 to 20 min. The detergent-plasma membrane mixture was centrifuged at 100,000*g* for 30 min. The pellet was suspended in a minimal amount of imidazole buffer B and stored under liquid N_2. Prior to electrophoresis, the TX-100 or TX-114-extracted membrane fraction was washed by dilution to 38 ml with imidazole buffer B and centrifuged at 100,000*g* for 1 h to remove excess TX-100 or TX-114, which can interfere with electrophoresis.

Electrophoresis. Two methods of electrophoretic fraction of polypeptides were used. SDS-PAGE was performed using 1.5 × 140 × 160 mm discontinuous polyacrylamide gels backed with Gel-Bond by the procedure of Laemmli (22) as modified by Hames (15). PAU-PAGE was performed using 0.75 × 140 × 160 mm (Fig. 2) or 1.5 × 140 × 160 mm (Fig. 1B) continuous polyacrylamide gels backed with Gel-Bond by a procedure described in detail elsewhere (14).

For SDS-PAGE, 50 μg of protein in 10 μl was mixed with 10 μl of 2× sample buffer (0.125 M Tris-HCl, pH 6.8, 10% SDS, 0.2 M DTT, 10% glycerol, and 0.002% bromophenol blue). When added, the protease inhibitors chymostatin (500 μg/ml) and PMSF (5 mM) were included in the sample buffer without heating. If indicated, the sample was heated at 100°C for 3 min in a boiling water bath to insure reduction. The samples were centrifuged 5 min in a Beckman Microfuge, and a 20 μl aliquot of the supernatant was applied per sample well. Gels were electrophoresed at 20 mamp constant current for about 5 h.

For PAU-PAGE, 50 μg of protein in 10 μl was mixed with 50 μl of exchange solution (50% phenol, 2 M urea, and 25% acetic acid). A 1:5 ratio of aqueous sample to exchange solution was not exceeded since the exchange solution can only mix with a limited amount of water. If the sample was to be reduced (required for mol wt standards), an equal volume of 0.3 M DTT was added and the sample was heated at 100°C for 3 min prior to addition of the exchange solution. An aliquot of 50 μl of sample was electrophoresed per well. Gels were electrophoresed at 100 V (constant voltage) for 30 min followed by 500 V for 5 h at room temperature or 700 V for 16 h at 4°C.

Following electrophoresis, SDS-PAGE gels were immersed in 500 ml staining solution (40% methanol, 7% acetic acid, 0.025% Coomassie blue R 250) and left overnight with gentle shaking. The gels were destained for 1 h in 500 ml of the above solution without dye and finally placed in a storage solution (5% methanol, 7% acetic acid). The storage solution was changed daily until the background dye was completely removed. PAU gels were initially placed in 500 ml fixative (50% methanol, 12% TCA) for 30 min with shaking. The PAU gel was then stained for protein as described for SDS-PAGE.

Phosphorylation of ATPase. The phosphorylated intermediate of the ATPase was identified after PAU-PAGE followed by autoradiography. The phosphorylation reaction (5, 6) contained 40 μM $MgSO_4$, 30 mM Tris-Mes, pH 6.5, 40 μM Tris-ATP, and 300 μg protein from the plasma membrane fraction in a volume of 0.7 ml. To start the reaction, 0.3 ml of 40 μm Tris-[γ^{32}P]ATP (15 μCi at 1250 mCi/mmol), 40 μm $MgSO_4$, and 30 mM Tris-Mes, pH 6.5 were added. After 20 s at ice temperature and constant stirring, the reaction was stopped by the addition of 1.0 ml of 10% TCA, 40 mM NaH_2PO_4, 5 mM $Na_2P_2O_7$, 1 mM ATP (sodium salt). Protein was collected by centrifugation at 27,000*g* for 10 min at 2°C. The pellet was suspended in 1 ml of 30 mM HCl and centrifuged again as above. The pellet was suspended in 100 μl of PAU-PAGE exchange solution at ice temperature, and 25 μl/well was applied to a 0.75 mm PAU gel which was electrophoresed at 100 V (constant voltage) for 30 min followed by 700 V for about 16 h. The gel was fixed by gentle agitation in 50% (v/v) methanol and 10% TCA (w/v) for 30 min, then for two changes of 40% methanol in 7% acetic acid for 3 h each. All steps including electrophoresis and fixing were at 4°C. For drying, the gel was soaked for 10 min in 7% acetic acid and 5% glycerol and then laid flat in an oven (50°C) and dried onto the Gel-Bond overnight. The dried gel was placed against Kodak X-Omat XAR-5 x-ray film for 16 h at −20°C with Cronex Lightening Plus intensifying screens. After autoradiography, the PAU-PAGE gel was rehydrated (Gel-Bond permitted this with minimum damage) and stained for protein as described above.

RESULTS

Detergent Treatment of the Plasma Membrane Fraction. The utility of SDS for extracting peripheral proteins from corn root plasma membrane vesicles was investigated. ATPase activity was used as a marker for determining the state of integral membrane components following detergent treatment. Maximal recovery of ATPase activity in the pellet after SDS treatment occurred at a protein-to-detergent ratio of 5 to 7 (mg protein/mg detergent). Protein-to-SDS ratios lower than 5 resulted in a progressive loss of ATPase activity without extraction of additional protein from the membrane. When the SDS-extracted membranes were centrifuged to equilibrium in a continuous sucrose density gradient, there was a peak of protein at about 30% (w/v) sucrose and a peak of K^+-stimulated ATPase activity at about 37% sucrose (not shown). SDS-extracted membranes recovered at the 34/42% (w/w) interface of a preparative sucrose gradient showed 3- to 4-fold higher ATPase activity than untreated plasma membrane vesicles (ATPase activities in μmol Pi/mg protein·h for untreated plasma membranes were 22 and 33, and for SDS-extracted membranes, 95 and 131 in the absence and presence of 50 mM KCl, respectively). Most of the increase in ATPase activity with SDS treatment represented purification rather than detergent stimulation because stimulation of ATPase activity by SDS was 30 to 40% under optimal conditions (not shown). However, considerable variability in ATPase activity was observed following SDS treatment. For this reason, the utility of TX-114 for

[3] Abbreviations: TX-100, Triton X-100 and TX-114, Triton X-114, octylphenoxypolyethoxyethanol; PMSF, phenylmethane sulfonyl floride; PAU, phenol-acetic acid-urea; % T, percent w/v of total monomer (acrylamide + *N,N'*-methylenebis acrylamide).

extracting membrane proteins was examined.

TX-100 and TX-114 differ only slightly by the average oxyethylene units in the structures and are considered equivalent for use in protein purification. TX-100 has been used to preferentially remove peripheral proteins from corn (30), oat (37), and *Acer pseudoplantanus* (3) membrane preparations. Addition of 1% TX-114 to the plasma membrane fraction from corn roots increased ATPase specific activity by about 30% and increased sensitivity to 50 μM vanadate (Table I). The TX-114-extracted membranes showed about a 2-fold increase in ATPase activity and greater inhibition by vanadate as compared to the untreated plasma membrane fraction. The sensitivity to vanadate of ATPase in the TX-114-extracted plasma membrane fraction approached the 80 to 90% inhibition reported by O'Neill and Spanswick (27) for untreated plasma membrane vesicles from corn roots.

Extraction with 1% TX-114 removed about 75% of the protein in the plasma membrane fraction and should have resulted in a 4-fold increase in ATPase specific activity (Table I). Failure to routinely obtain such an increase in activity indicates that some ATPase was inactivated or extracted by the procedure.

Electrophoretic Analysis of Polypeptides in the Plasma Membrane Fraction. An attempt was made to use SDS-PAGE to analyze the polypeptide composition of untreated and detergent-extracted plasma membrane fractions. In particular, preparations enriched in ATPase should show a prominent protein band at about 100,000 D corresponding to the catalytic subunit of the ATPase (6, 7, 30, 38, 41). With procedures for SDS-PAGE described by Laemmli (22), polypeptides in the plasma membrane fraction were poorly resolved and a significant amount of Coomassie blue positive material was retained at the top of the resolving gel (Fig. 1A). This was in marked contrast to the well-resolved polypeptides of mitochondrial and supernatant fractions from corn roots and red cell membrane from rabbit (14). Extraction of the plasma membrane fraction with deoxycholate (5), SDS, or TX-100 (or TX-114) should enrich for 100 kD polypeptides, but SDS-PAGE of such preparations (Fig. 1A) showed poor resolution, much Coomassie blue staining material at the top of the resolving gel, and no major polypeptides at the 100 kD region of the gel.

An alternative electrophoretic procedure which utilizes the denaturing effects of phenol and urea and the intrinsic charge characteristic of proteins to separate polypeptides according to mol wt (43) was modified for a slab gel format (14).

PAU-PAGE of the plasma membrane fraction revealed the presence of polypeptides with a molecular mass of about 100 kD (Fig. 1B). The various detergent treatments resulted in the expected enrichment for two closely spaced polypeptides at the 100 kD region of the gel and for other polypeptides in other regions of the gel (Fig. 1B). Coomassie blue staining material did not accumulate at the top of the gel using the PAU-PAGE procedure.

The untreated and detergent-extracted plasma membrane fractions shown in Figure 1 were incubated with [γ^{32}P]ATP to label the catalytic subunit of the ATPase (6, 7, 30, 38, 41) and then the polypeptides were separated by PAU-PAGE. The autoradiogram of the gel showed radioactivity which could be chased by unlabeled ATP (*i.e.* rapidly turning-over phosphorylated intermediate) in the 100 kD region for each of the detergent-extracted preparations and for the untreated plasma membrane fraction (not shown). Figure 2 shows the autoradiogram, and the rehydrated and stained gel which gave the autoradiogram, for a TX-114-extracted plasma membrane fraction which was incubated with [γ^{32}]ATP to label the catalytic subunit of the ATPase. Some resolution was lost because of the dehydration/rehydration process prior to staining (note weak staining of protein standards in lane 1 of Fig. 2A). Following phosphorylation for 20 s or 5 min (lanes 2 and 5 of Fig. 2B), the only radioactive polypeptides which entered the gel were in the 100 kD region, and radioactivity in this region was markedly reduced by a 40-s chase with unlabeled ATP (lanes 4 and 6 of Fig. 2B). On this gel, inhibition of phosphorylation by 100 μM vanadate was not apparent because of unequal application of protein (compare lanes 2 and 3 of Fig. 2A) and because the phosphorylation reaction showed less sensitivity to vanadate than ATPase activity (compare data presented below and Table I). These data confirmed results of Briskin and Leonard (6) indicating that the catalytic subunit of the corn root ATPase has a molecular mass of about 100 kD.

PAU-PAGE of detergent-extracted plasma membranes revealed the presence of two closely spaced bands in the 100 kD region (Fig. 1B), but it was not possible to determine from the autoradiogram which of the polypeptides was phosphorylated and, therefore, which one corresponds to the catalytic subunit of the ATPase. A TX-114-extracted plasma membrane preparation was phosphorylated, and the polypeptides were separated by PAU-PAGE. The gel was surface stained for protein at 0°C, the bands in the 100 kD region were excised and radioactivity determined by liquid scintillation counting. A similar number of cpm was found in both bands, and the number of counts was reduced 37% (upper, band 1) and 47% (lower, band 2), respectively, by 100 μM vanadate. An unlabeled ATP chase reduced the radioactivity in bands 1 and 2 by 78 and 83%, respectively. These results indicate that both bands in the 100 kD region of the gel (Fig. 1B) may contain polypeptides which form a phosphorylated intermediate characteristic of the catalytic subunit of the ATPase. However, excising such closely spaced bands without cross contamination is difficult, and these results should be viewed with caution.

Problems with SDS-PAGE of Plasma Membrane Preparations. The high background staining and diffuse banding of the proteins after SDS-PAGE (Fig. 1A) could be a result of proteo-

Table I. *ATPase Activity of the Plasma Membrane Fraction from Corn Roots following Treatment with TX-114*

Corn plasma membrane protein (2.68 mg) was diluted to 1 mg/ml and treated with 1% TX-114 as described in "Materials and Methods." Plasma membrane plus TX-114 denotes the ATPase activity of a subsample of the solubilization mix containing TX-114, prior to centrifugation. Percentage of inhibition by vanadate represents percentage of control activity inhibited by 50 μM vanadate in presence of 50 mM K^+.

	Volume	Protein	Specific Activity		Total Activity		Vanadate Inhibition
			+K	−K	+K	−K	
	ml	*mg*	*μmol/mg·h*		*μmol/h*		%
Untreated plasma membrane	2.00	2.69	43.3	31.4	116.5	84.3	37.4
Plasma membrane + TX-114	1.70	2.29	56.9	44.4	130.2	101.6	59.4
TX-114-extracted plasma membrane	0.80	0.69	92.7	51.1	63.7	35.1	76.2

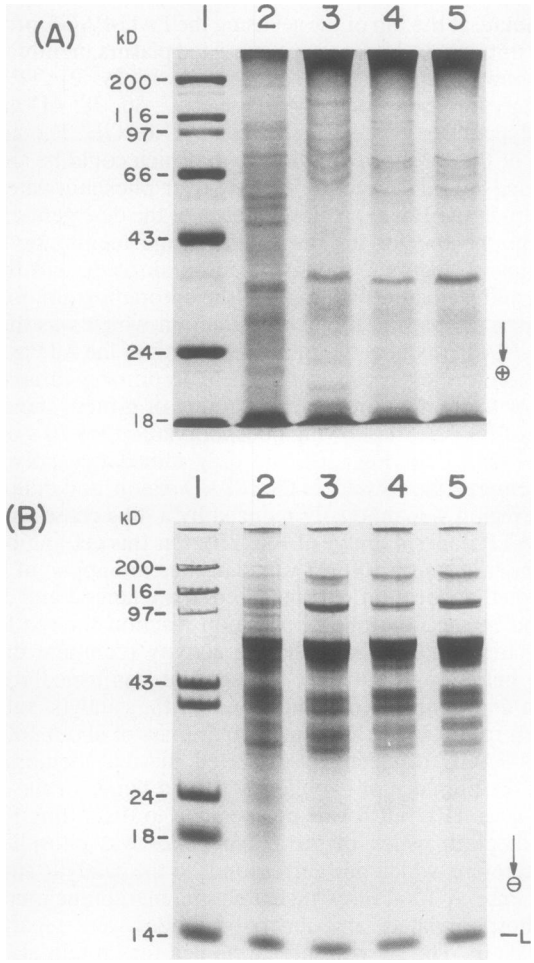

FIG. 1. Comparison of SDS-PAGE and PAU-PAGE of untreated and detergent-extracted plasma membrane preparations from corn roots. A, SDS-PAGE with a 10.3% T resolving gel. Lanes 2 to 5 each contained 50 μg total protein from untreated plasma membrane (lane 2), SDS-extracted plasma membrane (lane 3), deoxycholate (5, 6) extracted plasma membrane (lane 4), and TX-100-extracted plasma membrane (lane 5). Lane 1 contained the mol wt standards which were: myosin (200 kD), β-galactosidase (116 kD), phosphorylase a (97 kD), BSA (66 kD), ovalbumin (43 kD), trypsinogen (24 kD), and β-lactoglobulin (18 kD). All samples were chemically reduced by heating to 100°C for 3 min in SDS-PAGE solubilization buffer containing 0.2 M DTT. B, PAU-PAGE with a 7.7% resolving gel and 50 μg of the same membrane preparations in the lanes described in (A). All samples contained lysozyme (L), but were not chemically reduced. Mol wt standards (same as A, except for the addition of lysozyme, 14 kD) were not chemically reduced, which resulted in an anomalous mobility for BSA (14).

lytic degradation of the plasma membrane proteins during homogenization and isolation, as well as during electrophoresis (15, 28, 42). Protease activity was detected in both the homogenate and plasma membrane fraction of corn roots (in the absence or presence of 5% SDS), and this activity could be inhibited by 5 μg/ml chymostatin or 1 mM PMSF (12). Inclusion of protease inhibitors during homogenization, cell fractionation, and during electrophoresis significantly improved resolution with SDS-PAGE (Figs. 3 and 4). For the plasma membrane fraction, isolation in the presence of protease inhibitors followed by incubation in 5% SDS for 1 h at room temperature (20°C), still resulted in a significant decrease in high mol wt polypeptides and a corresponding increase in low mol wt polypeptides on SDS-PAGE, indicating that proteases are active during solubilization in SDS sample buffer prior to boiling (Fig. 4B). Boiling the sample inactivates proteases (lanes 6 and 7 in Fig. 4B), but created the problem discussed below. Inclusion of protease inhibitors in the SDS solubilization buffer reduced, but did not completely eliminate, protease activity (Fig. 3).

Boiling plasma membrane fractions in solubilization buffer prior to SDS-PAGE resulted in aggregation of high mol wt polypeptides and accumulation at the top of the gel (Figs. 1A, 3, and 4). This problem was particularly apparent for detergent-extracted plasma membrane preparations (Figs. 1A and 4). The inability to resolve the 100 kD catalytic subunit by SDS-PAGE was because heating plasma membrane preparations in SDS solubilization buffer at 100°C for 3 min caused selective aggregation of the 100 kD polypeptides (Fig. 4).

Major Polypeptides of the Detergent-Extracted Plasma Membrane Fraction. Electrophoresis of the TX-100- or TX-114-extracted plasma membrane preparation from corn roots under conditions which reduced the adverse effects of proteases and heat-induced aggregation revealed the presence of several major molecular mass classes of polypeptides which appear to be components of various integral membrane proteins or protein complexes (Figs. 1B and 4). Molecular mass estimates for polypeptides in the region of the gel which contained the catalytic subunit of the ATPase ranged from about 95 (SDS-PAGE) to 110 (PAU-PAGE) kD. The apparent molecular mass of the catalytic subunit of the ATPase was not significantly altered by chemical reduction of the sample prior to electrophoresis, but reduction did have a marked effect on resolution of polypeptides in this region of the gel (Fig. 4). However, in SDS-PAGE, polypeptides with a molecular mass of about 45 kD shifted with reduction to form two bands at about one-half the molecular mass (Fig. 4). For PAU-PAGE, the corresponding shift in molecular mass was from 65 kD to three bands at about one-half the molecular mass (14). In both gel systems, reduction resulted in a decrease in the Coomassie Blue staining polypeptides at these molecular masses and a proportional increase at the lower molecular masses.

Inclusion of protease inhibitors during isolation of the plasma membrane fraction, subsequent extraction with detergent, and sample preparation for electrophoresis had a significant effect on the position and relative proportion of polypeptides in the region of the SDS gel corresponding to the catalytic subunit of the ATPase (Fig. 4). Without inhibition of protease activity at any stage of sample preparation, both bands in this region of the gel showed a decrease in molecular mass of about 1.5 kD. Inhibition of protease activity during isolation of the plasma membrane fraction and during detergent extraction reduced the amount of the 90 kD polypeptides relative to those at 95 kD (Fig. 4B). Estimates (from densitometry scans) of the relative amount of polypeptides in the region of the gel which corresponds to the catalytic subunit of the ATPase indicated that about 10 to 15% of the Coomassie blue staining polypeptides in the TX-114-extracted fraction could be the catalytic subunit of the ATPase (not shown). The prominent band at 45 kD (Fig. 4) in unreduced samples accounted for over 50% of the Coomassie blue staining polypeptides in the TX-114-extracted plasma membrane fraction. It was difficult to make such estimates for gels of the untreated plasma membrane fraction because of the relatively large number of bands on the gels and the low resolution as compared to gels of the TX-114-extracted plasma membrane fraction. It appeared that the catalytic subunit of the ATPase accounts for 3 to 6% of the Coomassie blue staining polypeptides in the plasma membrane fraction.

The major polypeptides found in the detergent-extracted plasma membrane fraction from corn roots were also observed in similar proportions in plasma membrane-rich preparations from oat, barley, and tomato roots (not shown). However, protease inhibitors found to be effective with corn were not neces-

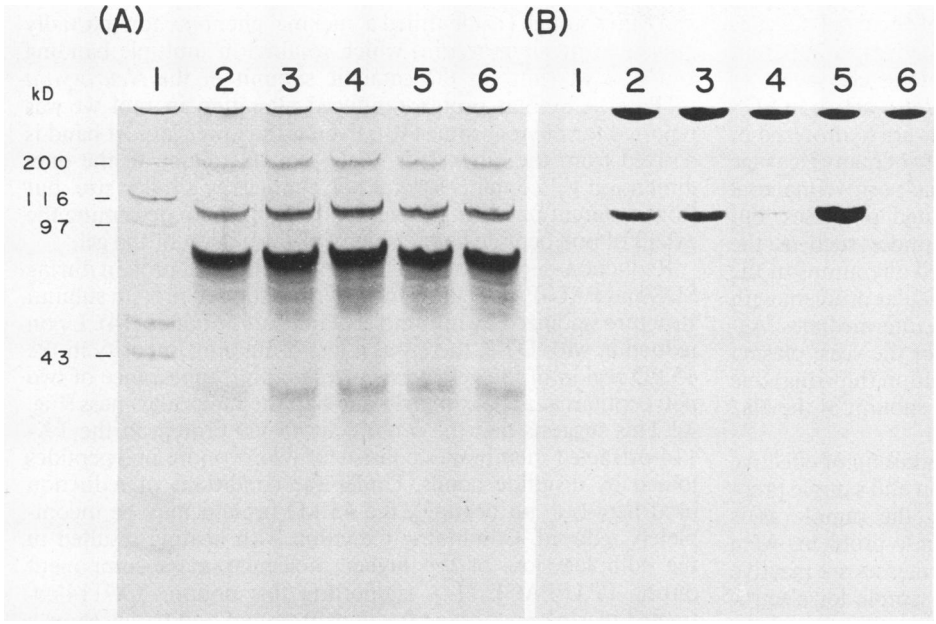

FIG. 2. PAU-PAGE and corresponding autoradiogram of TX-114-extracted, phosphorylated plasma membrane fraction from corn roots. A, Rehydrated and stained gel showing mol wt standards (see Fig. 1, lane 1, for details), 20 s phosphorylation reaction (lane 2), 20 s phosphorylation in presence of 100 μM vanadate (lane 3), 5 min phosphorylation followed by 40 s unlabeled ATP chase (lane 4), 5 min phosphorylation (lane 5), and 20 s phosphorylation followed by 40 s unlabeled ATP chase (lane 6). Approximately 75 μg protein was applied to each of lanes 2 to 6. The gel was rehydrated and stained after autoradiography. B, Autoradiogram of the dried gel shown in A.

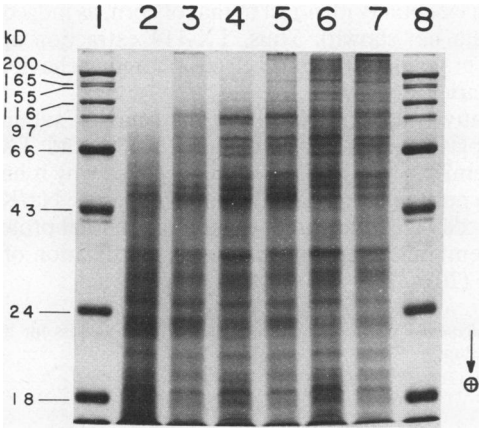

FIG. 3. Effect of proteases and protease inhibitors on SDS-PAGE of the plasma membrane fraction from corn roots. Lanes 2, 4, and 6 contained plasma membrane isolated without added protease inhibitors, and lanes 3, 5, and 7 contained plasma membrane isolated in the presence of 1 mM PMSF and 25 μg/ml chymostatin. For lanes 2 to 5, the plasma membrane fractions were suspended in 5% SDS-PAGE solubilization buffer (see "Materials and Methods") and incubated for 1 h at room temperature (20–22°C) prior to electrophoresis. For lanes 6 and 7, the plasma membrane fractions were added directly to 100°C solubilization buffer for 3 min prior to electrophoresis. For lanes 4 and 5, 5 mM PMSF and 500 μg/ml chymostatin was added to the solubilization buffer. Lanes 2 to 7 each contained a total of 50 μg of protein. Lanes 1 and 8 contained the same mol wt standard as in Figure 1, except for the addition of RNA polymerase (155 and 165 kD). All samples were reduced by addition of 0.2 M DTT to the solubilization buffer.

sarily effective at inhibiting proteases from other plant species (12).

DISCUSSION

The problem of low resolution of plasma membrane polypeptides in SDS-PAGE (Fig. 1A) was the result of two interacting factors, protease degradation and heat-induced aggregation, particularly of high molecular polypeptides (Fig. 3). Protease activity created two artifacts during isolation and electrophoresis of the membrane proteins; during homogenization, proteolytic activity caused subtle, discrete alterations in the mobility of the major

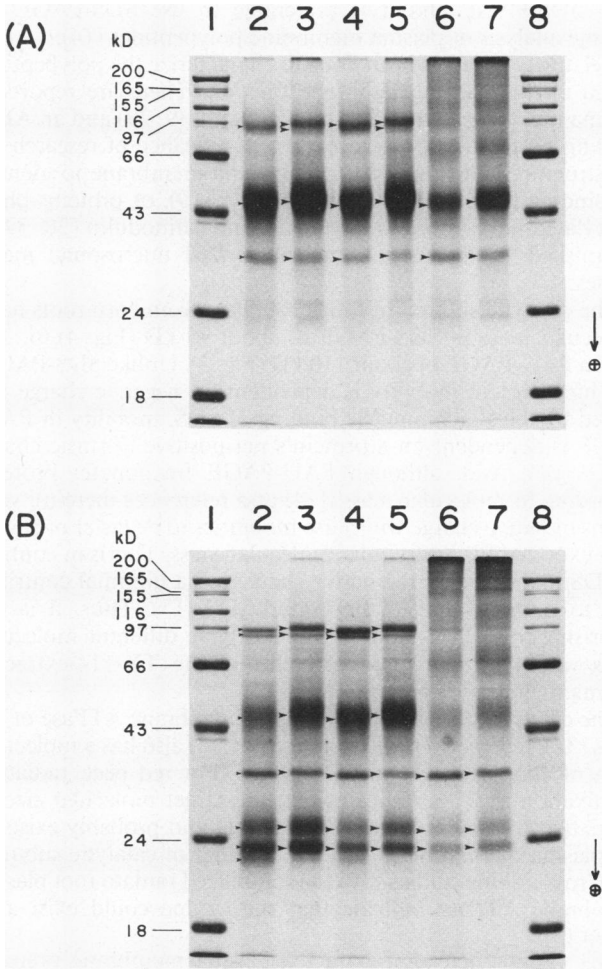

FIG. 4. Effect of protease inhibitors and chemical reduction on SDS-PAGE of the TX-114-extracted plasma membrane fractions from corn roots. (A) and (B) are unreduced or reduced (0.2 M DTT), respectively, 50 μg/lane samples of TX-114-extracted plasma membrane prepartions. The samples applied to lanes 1 to 8 were prepared and treated exactly as described for these lanes in Figure 3, except for extraction with TX-114.

polypeptides, including those in the region of the catalytic subunit of the ATPase (Fig. 4). The discrete changes in apparent mol wt are because of low availability of protease cleavage sites in integral membrane proteins. Proteases are also active in SDS (Fig. 3) (28, 42), and SDS-solubilized proteins are hydrolyzed in a nondiscrete fashion because of the availability of many cleavage sites. This leads to smearing of Coomassie blue-positive material in SDS-PAGE. Boiling the sample inactivated proteases, but caused aggregation of high mol wt polypeptides such as the catalytic subunit of the ATPase. Heat-induced alterations in the electrophoretic pattern of a protein are a particular problem with transport ATPases that form phosphorylated intermediates. Aggregation caused by heat has been reported for the yeast plasma membrane ATPase (25), while a loss of resolution (blurring) due to heating has been reported for the catalytic subunit of the Na/K ATPase (33) and *Neurospora* ATPase (1).

Best results with SDS-PAGE required the addition of effective inhibitors of proteases during cell fractionation and sample preparation for electrophoresis, and not boiling the sample as is commonly done prior to electrophoresis. These problems were not encountered with PAU-PAGE because proteases are inactive in the phenol and urea used to prepare the sample for electrophoresis and the sample is not usually boiled in this procedure. However, the PAU-PAGE procedure is technically more difficult than SDS-PAGE and it is preferable to use SDS-PAGE for routine analysis of plasma membrane polypeptides (14).

Relatively little has been done to characterize the polypeptides found in the plasma membrane fraction. There are reports of plasma membrane-associated glycoproteins (9, 29) and an ABA-binding protein (18). However, the significance of research for the structure and function of the plasma membrane to identify Ca-binding proteins (9), auxin receptors (19), or proteins phosphorylated in response to auxin, Ca, and calmodulin (26, 39) is diminished by the use of uncharacterized microsomal membranes.

The catalytic subunit of the H^+-ATPase from corn roots has a molecular mass in SDS-PAGE of about 95 kD (Fig. 4) (6, 41), and in PAU-PAGE of about 110 kD (Fig. 2). Unlike SDS-PAGE, in which protein mobility is dependent on negative charge imparted by the stoichiometric binding of SDS, mobility in PAU-PAGE is dependent on a protein's net positive intrinsic charge at low pH. And, although PAU-PAGE fractionates proteins according to molecular mass ([14] and references therein), variations in native charge will cause mobilities to be faster or slower than expected for a particular molecular mass. This is in contrast to SDS-PAGE where the native charge has a minimal contribution to mobility due to the bound SDS (15). Thus, it is not surprising that PAU- and SDS-PAGE give different molecular mass values for the major proteins in the TX-114-extracted plasma membrane preparation.

The catalytic subunit of the plasma membrane ATPase of oat roots (32), red beet (7), and tomato roots (2) also has a molecular mass of about 100 kD in SDS-PAGE. For red beet, radiation inactivation analysis indicates that the target molecular size of the native ATPase is larger than 228 kD and probably exists in the plasma membrane as, at least, a dimer of catalytic subunits (8). Cross-linking studies with the solubilized tomato root plasma membrane ATPase indicate that the enyme could exist as a trimer (2).

The various detergent-extracted plasma membrane preparations contained two polypeptide bands in the region of the gel which contained the catalytic subunit of the ATPase (Figs. 1B and 4). The presence of two bands may be due to the action of proteases because addition of protease inhibitors reduced the amount of the lower mol wt band (Fig. 4B) and polypeptides in both bands appeared to form a rapidly turning over phosphorylated intermediate which is characteristic of the ATPase. Addison and Scarborough (1) identified a 'nicking' phenomenon (partially prevented by chymostatin) which resulted in multiple banding and mol wt shifts in the catalytic subunit of the *Neurospora* ATPase. A similar protease-induced alteration in mol wt was reported for phytochrome (40). Even if the lower mol wt band is derived from the other, it is likely that this region of the one-dimension gel contains several polypeptides of similar size, but from different proteins. Studies are in progress to determine the extent of polypeptide heterogeneity in this region of the gel.

Reduction-dependent shifts in the mobility of a protein during SDS and PAU-PAGE allow the determination of protein subunit structure stabilized by intramolecular disulfide bonds (14). Upon reduction with DTT, there was a loss of staining intensity in the 45 kD region of the gel and a corresponding appearance of two polypeptides at approximately one-half the molecular mass (Fig. 4). This suggests that the prominent 45 kD protein in the TX-114-extracted membranes consists of two or more polypeptides joined by disulfide bonds. Under the conditions of reduction used here (*i.e.* no heating), the 45 kD protein may be incompletely reduced. Combining reduction with heating resulted in the complete loss of the higher molecular mass component during PAU-PAGE (14), supporting this notion. TX-114-extracted plasma membrane fractions from oat and barley show a major 45 kD protein and reduction-dependent changes in banding pattern essentially identical to that of corn, as judged by SDS-PAGE (data not shown). Thus, TX-114 extraction appears to enrich for proteins with similar electrophoretic behavior in corn, oat, and barley.

The relatively low proportion of the catalytic subunit in the enriched plasma membrane fraction (3–6%) and TX-114-extracted membranes (10–15%) further explains why it has proven difficult to purify the ATPase. By comparison, the Na/K ATPase of animal cells accounts for 30 to 40% of the total protein in the plasma membrane preparation used for purification of the Na/K ATPase (20).

Acknowledgment—We are grateful to Charles W. Hotchkiss for his excellent technical assistance.

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