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Solubilization of Plant Membrane Proteins for Analysis by Two-Dimensional Gel Electrophoresis

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ABSTRACT

A plasma membrane-enriched fraction prepared from barley roots was analyzed by two-dimensional gel electrophoresis. Four methods of sample solubilization were assessed on silver stained gels. When membranes were solubilized with 2% sodium dodecyl sulfate followed by addition of Nonidet P-40, gels had high background staining and few proteins because of incomplete solubilization. Gels of membranes solubilized in urea and Nonidet P-40 had a geater number of proteins but proteins with molecular weights greater than 85,000 were absent and proteins with low molecular weights were diffuse. High molecular weight proteins were present in gels of membranes solubilized in 4% sodium dodecyl sulfate followed by acetone precipitation but background staining and streaking remained a problem. Gels of the best quality were obtained when membrane proteins were extracted with phenol and precipitated with ammonium acetate in methanol; background staining and streaking were diminished and proteins were clearly resolved. This method makes possible the resolution required for meaningful qualitative and quantitative comparisons of protein patterns on two-dimensional gels of plant membrane proteins.

The analysis of proteins by high resolution 2D[1] PAGE has become increasingly popular following the pioneering work of O'Farrell (16). The most important application of 2D PAGE is the resolution of large numbers of polypeptides in complex protein mixtures. This technique may be used to analyze and compare synthesis, turnover, and modification of many proteins during development or in response to environmental changes. For example, 2D gels of plant proteins have revealed changes in patterns of protein synthesis that are induced by hormones (12, 19, 22, 27, 28), wounding (18, 21), aging (17), and water stress (2). To compare 2D gels, it is essential that proteins are well resolved, that gels are substantially free of streaking, smearing, and background staining, that gels lack artifacts due to proteolysis, and that protein patterns are reproducible from gel to gel. Although 2D separations of proteins solubilized from whole plant tissues (2, 6, 17–19, 27) or from *in vitro* translation reactions (22, 28) meet these criteria, plant membrane proteins seem to be resistant to 2D gel analysis. When proteins from plasma membrane-enriched fractions of soybean roots (3–5) and rye seedlings (23, 24) have been analyzed on 2D gels, the gels were characterized by streaking and smearing of protein patterns and by high background staining. This apparent resistance of plasma membrane proteins to 2D analysis is probably the result of incomplete disruption of all protein complexes and aggregates during sample solubilization (8, 20). SDS gives good solubilization of membrane proteins (1), but because of its anionic nature, proteins solubilized in SDS cannot be applied directly to isoelectric focusing gels. In this paper, we examine four methods of membrane solubilization, two of which include SDS, for preparation of samples for isoelectric focusing. Solubilization of a plasma membrane-enriched fraction from barley roots was used to assess the electrophoretic separations of these protein preparations on silver stained 2D gels.

MATERIALS AND METHODS

Plant Material. Seeds of barley (*Hordeum vulgare* L. cv California Mariout 72) were sown on moist cheesecloth above aerated Johnson's medium (10) and grown at 22°C in the dark for 7 d.

Membrane Preparation. Microsomal suspensions were obtained from barley roots as previously described and the plasma membrane-enriched fraction was isolated based on the distribution of microsomal membranes on linear sucrose gradients (9). Microsomal suspensions were applied to discontinuous sucrose gradients consisting of 10 ml of 22% (w/w), 13 ml of 30% (w/w), and 13 ml of 40% (w/w) sucrose in 1 mM DTT and 1 mM EDTA adjusted to pH 7.2 with Tris. The gradients were centrifuged for 2 h at 80,000*g* and the plasma membrane-enriched fraction was collected with a syringe by puncturing the centrifuge tube at the 30/40% sucrose interface. The fraction was washed with 150 mM KCl and pelleted as previously described (9).

Solubilization of Membrane Proteins. The centrifuge tubes containing membrane pellets were inverted on ice for 10 min and excess supernatant removed before addition of solubilization buffer. Four methods were used to solubilize the plasma membrane-enriched fraction; all solutions containing ampholytes had 1.6% pH range 5 to 7 and 0.4% pH range 3.5 to 10.

1) 2% SDS Followed by Addition of NP-40 (3). The membrane pellet was suspended in a SDS buffer: 2% (w/v) SDS, 1 mM EDTA, 1 mM DTT, 10 μg/ml BHT, and 375 mM Tris-Cl (pH 8.9). The solution was heated for 3 min at 80°C and insoluble material removed by centrifugation (178,000*g* for 15 min in a Beckman Airfuge[2]). Two volumes of a sample dilution buffer consisting of 9.5 M urea, 8% (v/v) NP-40, 5% (v/v) 2-mercaptoethanol, and 2% (v/v) ampholytes were added to the sample.

2) Urea and NP-40 (16). Membranes were solubilized with 9.5 M urea, 2% (v/v) NP-40, 2% (v/v) ampholytes, and 5% (v/v) 2-mercaptoethanol for 1 h at room temperature. Insoluble material was removed by centrifugation (178,000*g* for 15 min).

3) 4% SDS Followed by Acetone Precipitation. The membrane pellet was suspended in a SDS buffer: 4% (w/v) SDS, 2% (v/v) 2-mercaptoethanol, 20% (w/v) glycerol, and 2 mM PMSF in 100

[1] Abbreviations: 2D, two-dimensional; BHT, butylated hydroxytoluene; NP-40, Nonidet P-40; PMSF, phenylmethylsulfonyl fluoride; TEMED, *N,N,N'*,*N'*-tetramethylethylenediamine.

[2] Mention of a specific product name by the United States Department of Agriculture does not constitute an endorsement and does not imply a recommendation over other suitable products.

mM Tris-Cl (pH 8.5). The solution was heated for 3 min at 80°C and insoluble material removed by centrifugation (15,000 rpm for 10 min in an Eppendorf microfuge). Four volumes of cold (−20°C) acetone were added and the solution incubated for 20 min at −20°C. The precipitate was washed with cold (−20°C) 80% (v/v) acetone, and the pellet dried under a stream of N_2 gas. The pellet was solubilized in a urea buffer consisting of 9 M urea, 4% (v/v) NP-40, 2% (v/v) 2-mercaptoethanol, and 2% (v/v) ampholytes (7). The sample was incubated for 1 h at room temperature and insoluble material removed by centrifugation (178,000*g* for 15 min).

4) Phenol Extraction (17). The membrane pellet was suspended in 0.5 ml of extraction buffer (0.7 M sucrose, 0.5 M Tris, 30 mM HCl, 50 mM EDTA, 0.1 M KCl, 2% [v/v] 2-mercaptoethanol, and 2 mM PMSF) and homogenized with a glass homogenizer fitted with a Teflon® plunger. The sample was adjusted to 1.5 ml with extraction buffer and incubated for 10 min at 4°C. An equal volume of water saturated phenol was then added. After 10 min with shaking at room temperature, the phases were separated by centrifugation. The phenol phase was recovered and reextracted with an equal volume of extraction buffer. Proteins were precipitated from the phenol phase by addition of 5 vol of 0.1 M ammonium acetate in methanol and incubated at −20°C overnight. The precipitate was washed three times with the ammonium acetate in methanol and once with acetone. The pellet was dried and solubilized as in method 3.

All samples were loaded immediately after preparation, although it was found that similar 2D gel patterns were obtained when samples were stored at −70°C.

Two-Dimensional Gel Electrophoresis. Although analysis of proteins by 2D gel electrophoresis is done by standard methods, gels of high quality can be obtained only when techniques are optimized. Dunbar (7) has recommended that this can be accomplished by analyzing samples of proteins with patterns that have been well characterized. We have found that if the techniques used for 2D gels are not optimized, none of the sample solubilization methods used in this study will result in quality gels.

Two-dimensional gel electrophoresis was done according to O'Farrell (16) with modifications. Samples containing approximately 80 μg of protein were loaded at the acidic end of the focusing gels and overlaid with 10 μl of 5 M urea. The upper (anode) buffer was 0.2% (v/v) H_2SO_4 and the lower (cathode) buffer was 0.5% (v/v) ethanolamine; isoelectric focusing was conducted for 17 h at 250 V plus 1 h at 800 V (28). Following focusing, the pH gradient of one gel was measured by the method of Booz and Travis (3). The other gels were equilibrated for 1.5 h in two changes of 2.3% (w/v) SDS, 10% (w/v) glycerol, and 0.05% (w/v) DTT in 62.5 mM Tris-Cl (pH 6.8). Focusing gels were either immediately run on a second dimension gel or stored frozen. Gels to be stored at −70°C were equilibrated for 30 min and frozen on dry ice in capped tubes with equilibration buffer. Frozen gels were brought to room temperature and equilibrated for 1 h in fresh buffer; buffer was changed after 30 min.

The second dimension SDS gels were 0.75 mm thick and consisted of a 14.8 cm separation gel of 10% acrylamide overlaid with a 1.2 cm stacking gel of 4% acrylamide. The focusing gel was sealed to the SDS gel with 0.5% agarose in equilibration buffer without DTT. Additional pieces of 1% agarose containing 0.1% bromophenol blue (tracking dye) were sealed at each end of the focusing gel. The upper (cathode) buffer was 25 mM Tris, 195 mM glycine, and 0.1% (w/v) SDS; the same buffer was used for the lower (anode) reservoir except that it was diluted with an equal volume of water. The buffer in the lower reservoir was cooled with a cooling coil connected to a refrigerated water bath set at 12°C. Electrophoresis was done at 25 mamp/gel constant current until the tracking dye had traversed the stacking gel and then at 30 mamp constant current until the dye had reached the bottom of the gel.

Two-dimensional gels were fixed and silver stained by the method of Morrissey (13) and destained by the method of Oakley *et al.* (14). Stained gels were stored at 4°C to slow diffusion of reaction product from the protein spots. Since the increased sensitivity of the silver stain technique results in artifactual bands (50,000–68,000 mol wt) attributable to skin proteins (15), disposable gloves were worn whenever handling materials, equipment, and gels. Marker proteins used were: β-galactosidase (mol wt 116,000), BSA (68,000), ovalbumin (45,000), carbonic anhydrase (29,000), and trypsin inhibitor (21,000).

Protein Determination. Protein determinations were made by the method of Lowry *et al.* (11). Total membrane protein was determined following precipitation in 10% (w/v) TCA. Samples for protein determination of soluble and insoluble fractions were obtained as follows:

1) 2% SDS Followed by Addition of NP-40. Samples were solubilized in the SDS buffer, centrifuged, and the pellet and supernatant fractions obtained. Protein was precipitated from the supernatant with acetone as outlined in method 3 of "Solubilization of Membrane Proteins."

2) Urea and NP-40. Solubilization was done in the absence of 2-mercaptoethanol and ampholytes because they interfered with the assay. Following solubilization, samples were centrifuged at 178,000*g* for 15 min to obtain soluble and insoluble fractions.

3) 4% SDS Followed by Acetone Precipitation. As in 1.

4) Phenol Extraction. Both insoluble protein from the aqueous phase and soluble protein from the phenol phase were recovered by precipitation with ammonium acetate in methanol. For all methods, protein in solution was assayed directly; protein precipitates were solubilized in 1 N NaOH and aliquots assayed.

Chemicals. Chemicals for electrophoresis were obtained from Bio-Rad (Electrophoresis Purity Reagents: acrylamide, bisacrylamide, SDS, TEMED, ammonium persulfate), BRL (Ultra Pure: urea, phenol), Sigma (2-mercaptoethanol, NP-40, glycerol, ethanolamine, mol wt markers), LKB (ampholytes), and Baker (agarose: Standard Low Electroendosmosis for Electrophoresis). All other chemicals were standard reagent grade laboratory chemicals. Water from a Millipore Milli-R04 reverse osmosis system was used for all solutions.

RESULTS

Silver stained gels of proteins solubilized from the plasma membrane-enriched fraction of barley roots with 2% SDS followed by addition of NP-40 had relatively few proteins and were characterized by horizontal and vertical streaking and high background staining (Fig. 1A). Since the sample was loaded at the acidic end of the focusing gel, the high background staining in this region of the SDS gel indicates incomplete solubilization of the membrane sample. Incomplete solubilization would result in protein complexes and aggregates that remain at the top of the focusing gel or move slowly into the gel during focusing.

When the urea/NP-40 buffer of O'Farrell (16) was used to solubilize membrane proteins, a greater number of protein spots was present on the silver stained 2D gels (Fig. 1B). This increase in number of proteins coupled with decreased staining in the acidic region of the gel indicates a more complete disaggregation of protein complexes during membrane solubilization. A comparison of the amount of protein solubilized from membrane preparations with 2% SDS/NP-40 and with the urea/NP-40 buffer supports this hypothesis. Approximately 14 to 16% of the membrane protein was solubilized by the 2% SDS/NP-40 method whereas approximately 70 to 80% was solubilized with the urea/NP-40 buffer (Table I). Except for the decreased staining in the acidic region of the gel, horizontal and vertical streaking were not diminished appreciably when membrane fractions were solubilized in the urea/NP-40 buffer. Although there was an

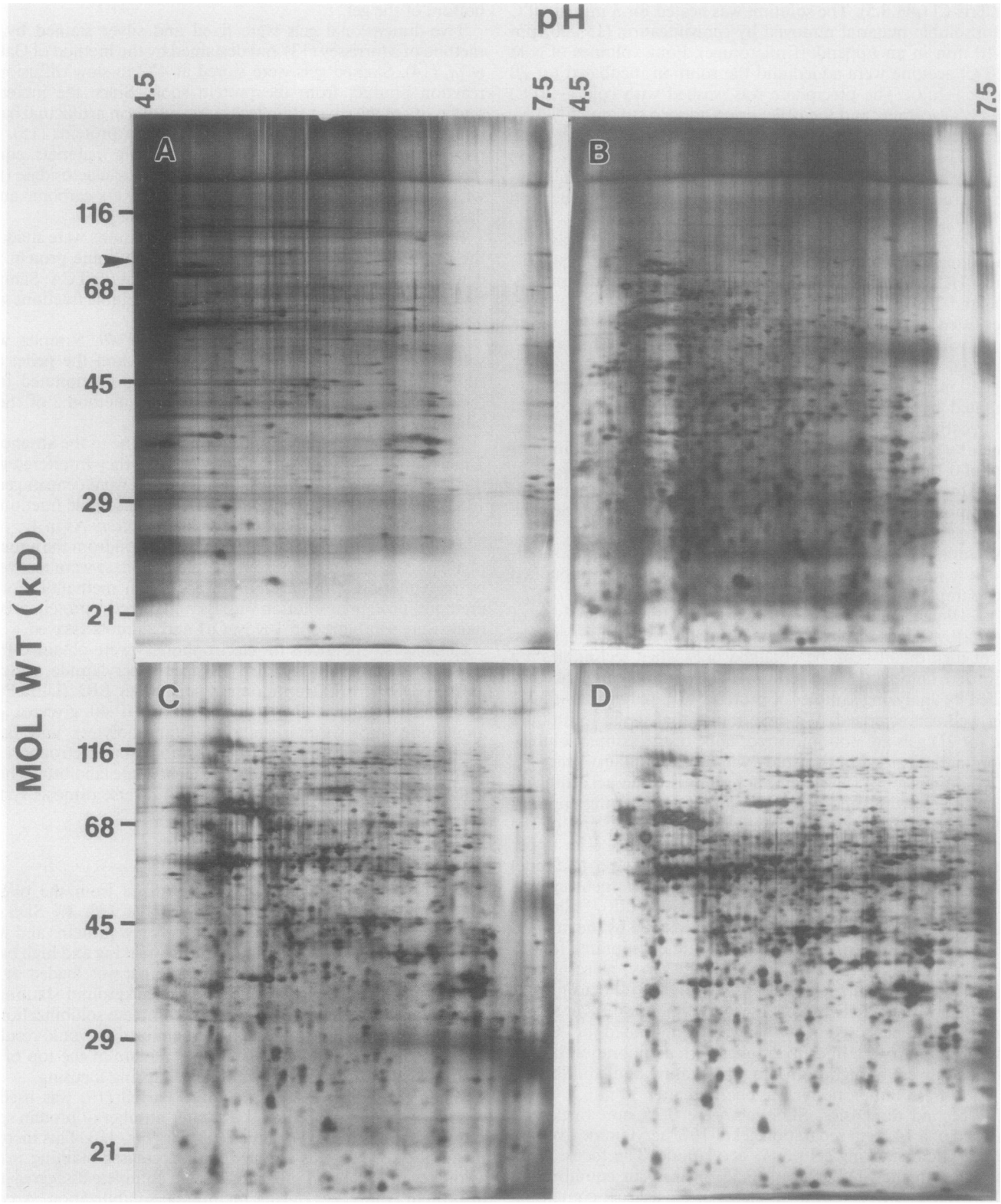

FIG. 1. Two-dimensional electrophoretograms of proteins solubilized from a plasma membrane-enriched fraction from barley roots. A, Membranes were initially solubilized in a buffer containing 2% SDS to which was added two volumes of buffer containing urea and NP-40; B, membranes were solubilized with the urea lysis buffer of O'Farrell (16); C, membranes were solubilized with a buffer containing 4% SDS, precipitated and rinsed with acetone, and resolubilized in a urea buffer; D, membrane proteins were extracted with phenol, precipitated with ammonium acetate in methanol, rinsed with acetone, and solubilized in a urea buffer. Arrow indicates calculated position for mol wt of 85 kD.

Table I. *Solubilization of Protein from Plasma Membrane-Enriched Fractions of Barley Roots by Four Methods*

Method	Protein[a]		Solubilized	
	Soluble	Insoluble	Of original	Of soluble + insoluble
	μg		%	
2% SDS/NP-40	63	335	14.0	15.8
Urea/NP-40	316	78	70.2	80.2
4% SDS/acetone precipitation	320	63	71.1	83.6
Phenol extraction	335	60	74.4	84.8

[a] Pellets containing 450 μg of protein were solubilized and assayed as described in "Materials and Methods." Data in this table are from a representative experiment.

increased number of protein spots, there were noticeably few proteins that had mol wt greater than 85,000. In addition, protein spots in the low mol wt region of the gel were diffuse and poorly resolved. The addition of 2 mM PMSF to the urea/NP-40 buffer did not increase the number of high mol wt proteins (data not shown).

Two-dimensional gels of proteins recovered by acetone precipitation of fractions solubilized with 4% SDS (Fig. 1C) had less horizontal and vertical streaking and reduced background staining. Compared to 2D gels of membrane samples solubilized with the urea/NP-40 buffer, gels of membrane samples solubilized with 4% SDS had a greater number of high mol wt proteins. Protein spots in the low mol wt region of the gel were less diffuse and better resolved. Approximately 71 to 84% of the membrane protein was solubilized by this method (Table I).

The 2D gels of proteins extracted by the phenol method had low background staining and diminished horizontal and vertical streaking (Fig. 1D). The 2D gel pattern obtained when proteins were extracted with the phenol method was very similar to that obtained when membrane samples were solubilized with 4% SDS. Approximately 74 to 85% of the membrane protein was solubilized by the phenol extraction method (Table I). The phenol extraction method also worked well for mitochondrial and microsomal fractions and fractions enriched in tonoplast and ER from barley roots, for a plasma membrane-enriched fraction prepared from rye epicotyls, and for a microsomal fraction prepared from abscission zone tissue of red kidney bean (data not shown).

DISCUSSION

Solubilization of membrane proteins for 2D gels has proven to be difficult. Ames and Nikaido (1) found that the combination of urea, NP-40, and mercaptoethanol originally reommended for sample solubilization (16) did not fully solubilize all the membrane proteins of *Salmonella tryphimurium*. They developed a SDS solubilization procedure based on that described by O'Farrell (16) that could be combined with isoelectric focusing. Membrane preparations were initially solubilized with 2% SDS and then a sample dilution buffer containing urea, NP-40, and mercaptoethanol was added.

The strategy of using both ionic and nonionic detergents has been used for 2D gel analyses of proteins in plasma membrane-enriched fractions from soybean roots (3–5) and rye seedlings (23, 24). Coomassie stained 2D gels of soybean plasma membrane proteins were characterized by horizontal streaking and heavy background staining in the neutral, high mol wt regions of the gels. The silver stained 2D gels of plasma membrane proteins of rye seedlings, as would be expected, revealed more protein spots, but the high level of background staining limited resolution and photographic reproduction of the gels. When plasma membrane-enriched samples of barley roots were solubilized by this method, the silver stained gels similarly had high background staining and relatively few protein spots, a finding we can attribute to inadequate solubilization of the membrane samples.

When the O'Farrell lysis buffer (16) was used to solubilize the plasma membrane fraction from barley roots, the 2D gels revealed many more proteins. Although a larger number of proteins was solubilized by this method, there were few proteins with mol wt greater than 85,000. Addition of PMSF to the lysis buffer prior to solubilization of the membrane sample did not result in the appearance of additional high mol wt proteins. The observation that PMSF is not effective and that proteases are active in the urea lysis buffer has previously been reported (6, 21).

A solubilization buffer containing 4% SDS and 2 mM PMSF resulted in good separation of membrane proteins on one-dimensional SDS gels (WJ Hurkman, unpublished observation). Therefore, we modified this procedure for analysis of membrane proteins by 2D gel electrophoresis. The membrane samples were solubilized in the SDS buffer and the proteins were then precipitated and rinsed with acetone to remove the SDS (25) prior to solubilizing the proteins in the urea buffer. Not only were protein spots better resolved and background staining reduced, but a greater number of high mol wt proteins were present in the gels. This increase in high mol wt proteins may be attributable to a decrease in proteolysis. Solubilization of leaf proteins in SDS buffer significantly decreased proteolysis of the large subunit of ribulose bisP carboxylase/oxygenase (6).

One disadvantage of 2D gels of membrane proteins solubilized in SDS was high background staining. Nucleic acids interact with proteins and ampholytes and can adversely affect resolution obtained on focusing gels (7, 8, 16, 20). They are also stained by silver stains resulting in background streaking of 2D gels (7). Nucleic acids can be removed by enzymic digestion using specific nucleases (16, 23); however, this procedure could add additional proteins to the sample which can make interpretation of protein patterns more complex and could introduce contaminating proteolytic activity (7). Nucleic acids are conventionally extracted by a two-phase phenol/buffer isolation method where proteins are partitioned in the phenol phase and nucleic acids in the aqueous phase. Recovery of proteins from the phenol phase has been used to prepare protein extracts of plant tissues for analysis by 2D gel electrophoresis (17, 26). The 2D gels of proteins solubilized from the plasma membrane-enriched fraction of barley consistently had low background staining and improved resolution. Interestingly, the protein pattern was similar to that of the gels of membranes solubilized with 4% SDS, suggesting that the phenol extraction method prevents proteolysis to the same extent as the 4% SDS method.

In this paper, we have shown that plant membrane proteins can be separated with good resolution by 2D gel electrophoresis. Of the four methods we assessed, one method (2% SDS/NP-40) failed to solubilize membrane samples adequately while the other three methods solubilized approximately the same percentage of protein (Table I). The best quality gels were obtained when proteins were first solubilized and then precipitated. Both the 4% SDS method and the phenol extraction method seemed to minimize proteolysis and optimize extraction of membrane proteins. Phenol extraction resulted in the best resolution of proteins on 2D gels, probably because this method removes nonprotein components that interfere with isoelectric focusing.

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