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Note

Technical Improvement to 2D-PAGE of Rice Organelle Membrane Proteins

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Cytosolic and membrane-associated proteins prepared from rice cells were separated and compared by two different 2D-PAGE methods, isoelectric focusing (IEF)/SDS-PAGE and nonequilibrium pH gradient electrophoresis (NEPHGE)/SDS-PAGE. Although IEF/SDS-PAGE of the cytosolic proteins showed sufficient resolution, some mitochondrial and basic microsomal membrane-associated proteins were weakly or hardly detectable on the 2D gel. High-quality and -quantity separation of the organelle membrane-associated proteins was accomplished by NEPHGE/SDS-PAGE, the advantage of this method being more critical in tightly membrane-bound proteins that were unwashable with NaCl. These results indicate that NEPHGE/SDS-PAGE is a useful tool for the proteomic analysis of rice membrane-associated proteins.

Key words: *Oryza sativa* L.; membrane protein; 2D-PAGE; NEPHGE

Since O'Farrell[1] introduced the useful technique of high-resolution two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), the procedure of 2D-PAGE has been improved by several researchers[2–6] and it has become one of the most powerful tools for the separation and quantification of proteins from a complex mixture.[7–12] However, further technical improvement for separating organelle membrane proteins by 2D-PAGE was still necessary. In the present communication, we report a suitable procedure by 2D-PAGE for the proteomic analysis of rice organelle membranes.

Suspension-cultured cells derived from the embryo of rice seed (*Oryza sativa* L. cv. Nipponkai) were fractionated by differential centrifugation into the mitochondria (10,000×*g* pellet), microsomes (100,000×*g* pellet) and cytosol (100,000×*g* supernatant).[13] These cell-fractionated preparations were extracted with phenol/chloroform/methanol according to the procedure of Hurkman and Tanaka[14] to remove the nonprotein components that would otherwise interfere with isoelectric focusing. Each preparation was dissolved and adjusted to 1.0 ml with an extraction buffer consisting of 100 mM Tris-HCl, 10 mM EDTA, 100 mM KCl, and 2% (v/v) 2-mercaptoethanol (pH 7.5). The suspension was mixed with an equal volume of water-saturated phenol and shaken vigorously for 10 min at room temperature. After centrifugation at 10,000×*g* for 10 min, the lower phenol phase was shaken again with an equal volume of the extraction buffer. The final phenol phase was mixed with 6 volumes of 0.1 M ammonium acetate in methanol and incubated overnight at −20°C. The precipitate obtained by centrifugation at 10,000×*g* for 10 min was washed three times with 0.1 M ammonium acetate in methanol and once with acetone, before being dried. The extraction procedure was critical to obtain the high-quality and -quantity separation of rice membrane-associated proteins by 2D-PAGE. Extraction with acetone or

Table 1. Comparison of the Conditions for First-dimensional Gel Electrophoresis by IEF and NEPHGE

	IEF	NEPHGE
Gel solution	**Final conc.**	
Urea	8 M	8 M
30% Acrylamide/1.6% Bis	4%/0.21%	4%/0.21%
20% NP-40	1.0%	1.0%
40% Ampholine (pH 3.5–10)	1.0%	0.33%
40% Ampholine (pH 7–9)	—	0.33%
40% Ampholine (pH 5–8)	1.0%	0.33%
10% APS	0.2%	0.2%
TEMED	3.0%	3.0%
Electrode buffer	**Position**	
20 mM NaOH (cathode)	Upper	Lower
15 mM Phosphoric acid (anode)	Lower	Upper
Running conditions		
Prerunning	200 V, 15 min	—
	300 V, 15 min	—
	400 V, 30 min	—
Running	400 V, 19 h	300 V, 4 h
		600 V, 30 min

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Abbreviations: 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; APS, ammonium persulfate; CBB, Coomassie brilliant blue; IEF, isoelectric focusing; NEPHGE, nonequilibrium pH gradient electrophoresis; NP-40, nonidet P-40; TEMED, N,N,N′,N′-tetramethyl ethylenediamine

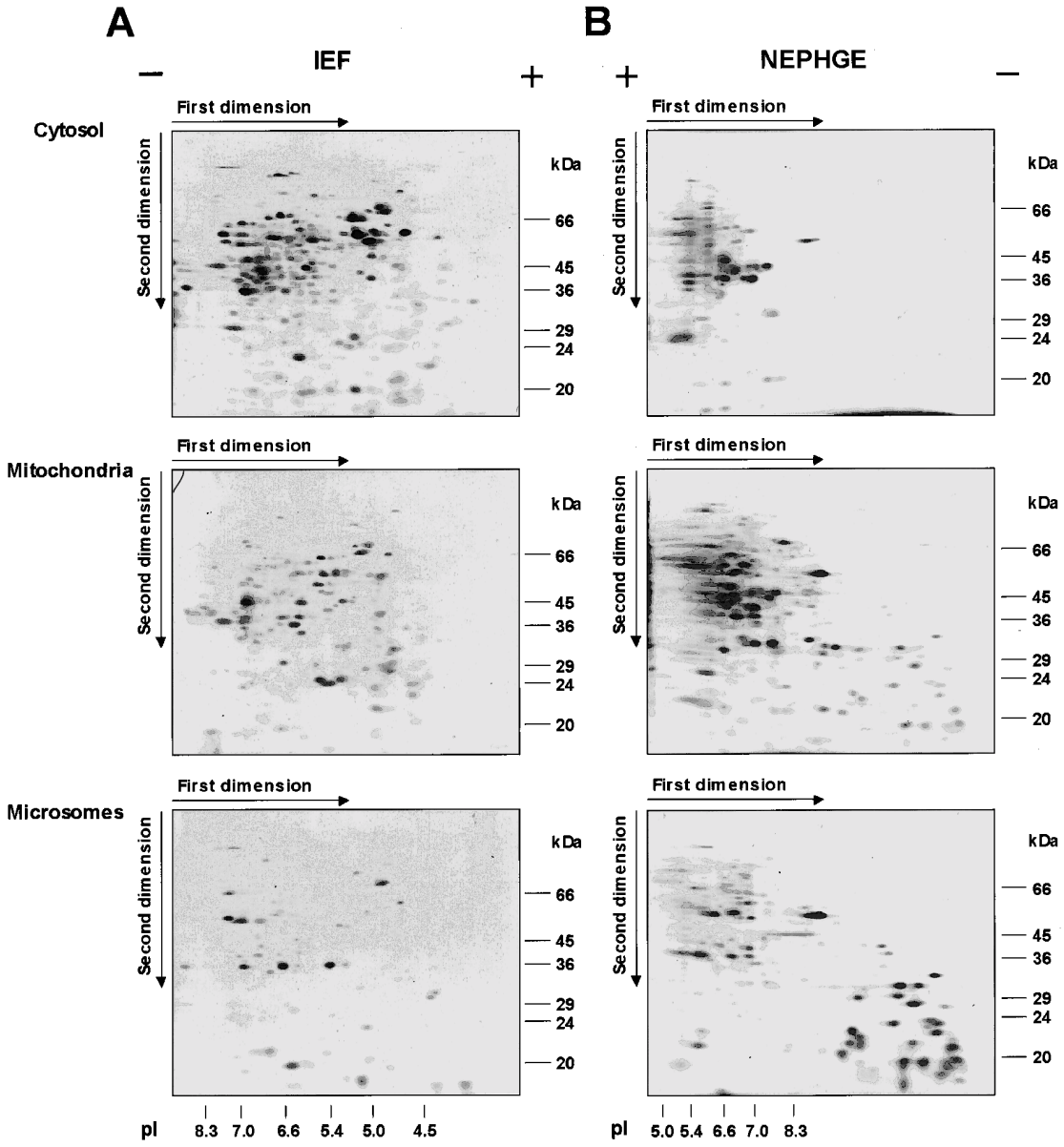

Fig. 1. Two Different 2D-PAGE Patterns of the Proteins Extracted and Prepared Distinct Compartments of Rice Cells.
Each protein sample (200 μg) extracted from the cytosol- (top panel), mitochondria- (middle panel), or microsome-enriched fraction (bottom panel) was separated by IEF/SDS-PAGE (A) and NEPHGE/SDS-PAGE (B). The separated proteins in the 2D gels were stained with CBB.

ether alone was not enough to achieve good separation (data not shown). The dried precipitate was dissolved in a lysis buffer consisting of 9 M urea, 3% (v/v) nonidet P-40 (NP-40), 2% (v/v) 2-mercaptoethanol, and 2% (v/v) ampholine (pH 3.5–10). Finally, the sample was centrifuged at 100,000 $\times g$ for 10 min to remove any insoluble materials.

2D-PAGE was performed by two different procedures, IEF and NEPHGE/SDS-PAGE. To make 12 first-dimensional IEF or NEPHGE disc gels (11.5 cm $\times \phi$3 mm), 7.2 g of urea, 2 ml of acrylamide stock (30% acrylamide and 1.6% bis-acrylamide), 6.24 ml of H_2O, 0.75 ml of 20% (w/v) NP-40, 0.75 ml of 40% (w/v) ampholine, 30 μl of 10% (w/v) ammonium persulfate (APS), and 45 μl of N,N,N′,N′-tetramethyl ethylenediamine (TEMED) were mixed and poured into glass tubes. The components of ampholine for IEF and NEPHGE were in the pH range of 3.5–10/5–8 (1:1) and 3.5–10/5–8/7–9 (1:1:2), respectively. The electrode buffers and running conditions for IEF and NEPHGE, as well as those of the gel components are summarized in Table 1. Eighty μl of each sample (approximately 200 μg of proteins) was loaded on to a disc gel. After first-dimensional PAGE, the gel was placed in an equilibration solution consisting of 0.06 M Tris-HCl (pH 6.8), 2.5% (w/v) SDS, 5% (w/v) 2-mercaptoetanol, and 10% (v/v) glycerol for 10 min while gently shaking twice.

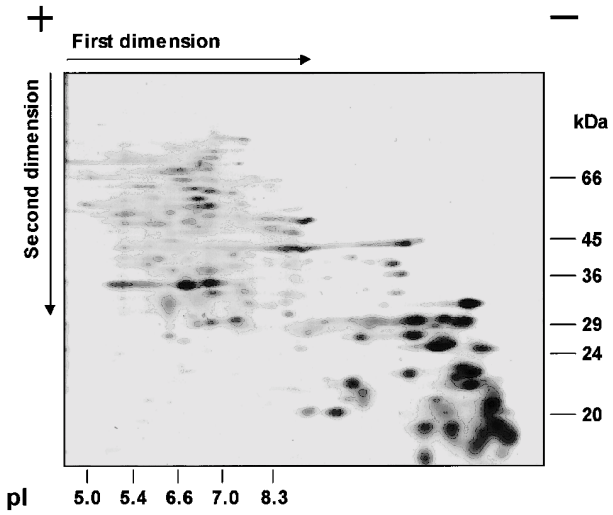

Fig. 2. Separation Profile of the Proteins Extracted from the NaCl-Unwashable Fraction in Rice Microsomes by NEPHGE/SDS-PAGE.

Microsomal membranes were treated with 0.3 M NaCl and centrifuged at 100,000 × *g* for 30 min. The proteins (200 μg) in the NaCl-unwashable fraction were separated by NEPHGE/SDS-PAGE.

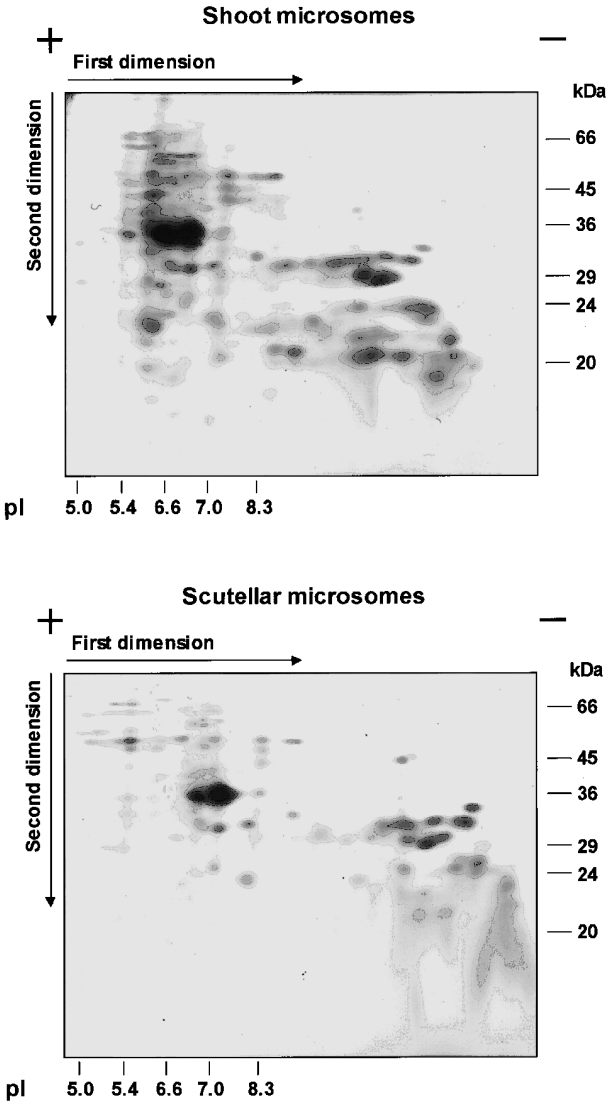

Fig. 3. Separation Profiles of the Proteins Extracted from Microsomes in the Shoot and Scutellar Tissues of Rice Seedlings by NEPHGE/SDS-PAGE.

Rice seeds were germinated for 4 days in the dark at 30°C. The microsomal membrane proteins prepared from the shoot and scutellar tissues of the seedlings (300 μg and 200 μg, respectively) were separated by NEPHGE/SDS-PAGE.

The gel was then placed on a second-dimensional SDS-slab gel and sealed with 0.5% (w/v) agarose dissolved in an equilibration solution without 2-mercaptoethanol. The separation gel (10 × 14 cm, 1 mm in thickness) contained 0.1% (w/w) SDS and 19% (w/w) acrylamide (acrylamide/bisacrylamide, 30:0.135) in a 0.375 M Tris-HCl buffer (pH 8.8), while the stacking gel contained 0.1% SDS and 5% (w/w) acrylamide (acrylamide/bisacrylamide, 30:0.8) in a 0.125 M Tris-HCl buffer (pH 6.8). Electrophoresis was carried out according to the procedure of Laemmli[15] at a constant current of 6–7 mA per plate. The separated proteins on the 2D gels were visualized by staining with Coomassie brilliant blue (CBB) R-250. The isoelectric point and molecular size of each protein were evaluated by using the Bio-Rad 2D-SDS-PAGE standards. The protein spots in the 2D gels were detected and characterized by an imaging analyzer with PDQuest 2D gel analysis software (Bio-Rad, Japan).

A comparison of the separation profiles of the proteins extracted and prepared from different compartments of rice cells between IEF and NEPHGE/SDS-PAGE are shown in Fig. 1. In the case of IEF/SDS-PAGE, the cytosolic proteins showed good separation with many protein spots spread widely on the gel (Fig. 1A, top panel). However, the total CBB-stained intensity of the mitochondrial and microsomal membrane-associated proteins on the 2D gel (Fig. 1A, middle and bottom panels) was weak compared with that of the cytosolic proteins. The stain intensity was not improved by any changes in the gel and running conditions (data not shown). Some proteins perhaps aggregated on the top of gel during IEF. As shown in Fig. 1B (middle and bottom panels), many more proteins, particularly the basic proteins, were separated and detected by NEPHGE/SDS-PAGE than by IEF/SDS-PAGE. The imaging analysis of 2D gels indicated that the total number and volume of protein spots detected on the NEPHGE/SDS gels were approximately twofold those on the IEF/SDS gels (data not shown). It must be stressed that the intensity and number of spots appearing in the acidic and neutral areas was also higher, although it is known that NEPHGE/SDS-PAGE is useful for the separation of basic proteins.[16] The separation of rice membrane-associated proteins by NEPHGE/SDS-PAGE was highly

reproducible even when the amount of proteins loaded on to the disk gel was increased to 350 μg (data not shown). The advantage of the NEPHGE/SDS-PAGE analysis was also critical in tightly membrane-bound proteins that were unwashable with NaCl (Fig. 2). The reason why NEPHGE/SDS-PAGE gave such good separation of the membrane-resident proteins might be that the basic proteins rapidly enter the gel and separate from the acidic proteins in the early stage of electrophoresis; therefore, little aggregation of the acidic and basic proteins occurs in the loaded sample. In contrast, NEPHGE/SDS-PAGE was inappropriate for rice cytosolic proteins (Fig. 1B, top panel). Microsomal membrane proteins prepared from the shoot and scutellar tissues of rice seedlings germinated for 4 days in the dark at 30°C were separated by NEPHGE/SDS-PAGE as well as those of suspension-cultured cells (Fig. 3). It was easy to compare and analyze the resident proteins in microsomal membranes prepared from different tissues by NEPHGE/SDS-PAGE. Overall these results indicate that NEPHGE/SDS-PAGE is a useful tool for the proteomic analysis of rice membrane-associated proteins.

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