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# Preparation of membrane proteins for analysis by two-dimensional gel electrophoresis☆

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**Abstract**

In order to separate hydrophobic membrane proteins, we have developed a novel two-dimensional electrophoresis system. For the iso-electric focusing, agarose was used as a supporting matrix and *n*-dodecyl-β-D-maltopyranoside was used as a surfactant. In combination with a previously developed Tris/MES electrophoresis system in the second dimension, distinct spots were reproducibly detected from hydrophobic membrane proteins whose grand average hydropathicity (GRAVY) exceed 0.3. In contrast to the immobilized pH gradient system, *c*-type heme was also visualized in this system.


*Keywords:* Membrane protein; Proteomics; IEF; Iso-electric focusing; Agarose; *n*-dodecyl-β-D-maltopiranoside

## 1. Introduction

Many kinds of membrane protein complexes perform their unique and important function embedded in or at the surface of biological membranes [1]. In their active form, those complexes are comprised of several different subunit hydrophobic membrane proteins and extrinsic proteins. For example, complex I in mitochondrial respiratory chain from bovine heart mitochondrion contains 46 different subunit proteins [2], photosystem II (PS II) complexes in cyanobacteria, algae and plants are comprised of over 20 different subunit proteins [3–6]. To investigate the structure and function of those large membrane protein complexes, precise determination of subunit proteins is one of the most important criteria. For this purpose, two-dimensional electrophoresis using iso-electric focusing (IEF) as a first dimension can be a powerful tool because of its high resolution [7]. However, it is well known that IEF two-dimensional electrophoresis is not suitable for the analysis of membrane proteins [2,7–9]. Carroll et al. failed to detect hydrophobic ND1 to ND6 and ND4L subunits of complex I from bovine heart on their IEF two-dimensional gels although they detected those proteins by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [2]. Kashino et al. successfully applied one-dimensional SDS-PAGE for the proteomic analysis of multi-subunit PS II complexes without using IEF [6].

Other than these specific membrane protein complexes, total membrane proteins on a specific membrane such as brain plasma membrane from mouse or thylakoid membrane from plant chloroplast are now intriguing proteome targets (e.g., [10,11]). In spite of the importance of the function of membrane proteins and the accumulating interest in membrane proteins, the analytical tool for them is not so powerful. Hydrophobic proteins whose grand average hydropathicity (GRAVY) value is larger than 0.1 (in *Saccharomyces cerevisiae*), 0.15 (in *Bacillus subtilis*) or 0.3 (in *Escherichia coli*) are rarely detected on IEF two-dimensional electrophoresis although these organisms have proteins with GRAVY values as high as ∼1.7 [9].

*Abbreviations:* Chl, Chlorophyll; CMC, Critical micelle concentration; DAB, Diaminobenzidine tetra-hydrochloride; DDM, *n*-dodecyl-β-D-maltopyranoside; DTT, Dithiothreitol; PS II, Photosystem II; GRAVY, Grand average hydropathicity; IEF, Iso-electric focusing; IPG, Immobilized pH gradient (gel strip); LDS, Lithium dodecyl sulfate; MALDI, Matrix-assisted laser desorption ionization mass spectroscopy; MES, 2-(*N*-Morpholino)ethanesulfonic acid; PAGE, Polyacrylamide gel electrophoresis; SDS, Sodium dodecyl sulfate

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Besides the intense efforts to improve the methods to effectively solubilize membrane proteins for IEF [7,12], other analytical methods have also developed and operated in many cases. For example, blue native PAGE, alone or in combination with SDS-PAGE in the second dimension, is a powerful tool to separate membrane protein complexes [13]. Doubled SDS-PAGE was effectively applied for complex I [8]. Partitioning [11] or solvent extraction [14,15] of membrane proteins also works in combination with one-dimensional SDS-PAGE and MS.

Considering the fact that the membrane proteins are the intriguing targets of proteomics, a precise and simple analytical system is necessary. The precise separation by two-dimensional electrophoresis using IEF as a first dimension [7], with its lower cost, seems still to be superior to other methods. We have developed two-dimensional electrophoresis using IEF as a first dimension to separate very hydrophobic membrane proteins using agarose as a supporting matrix and *n*-dodecyl-β-D-maltopyranoside (DDM) as a surfactant. To this challenge, highly purified PS II complexes [6] were applied to this system and the separation capacity was tested. All known hydrophobic proteins including the most hydrophobic protein in PS II complexes, PsbD protein (GRAVY = 0.361), as well as hydrophilic subunit proteins in PS II complexes larger than 10 kDa made clear spots on this two-dimensional electrophoresis.

## 2. Experimental

### 2.1. Chemicals

IEF grade agarose, Pharmalyte, Ampholine and immobilized pH gradient gel strip (IPG; Immobiline DryStrip pH3–10, 11 cm) were purchased from GE Healthcare Bio-Science (Piscatawary, NJ, USA), thiourea, isopropanol, lithium dodecyl sulfate (LDS), *N*-*p*-Tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin and Sigmacote were from Sigma (St. Louis, MO, USA), DDM and CHAPS were from Anatrace (Maumee, OH, USA), urea, dithiothreitol (DTT) and Coomassie blue R-250 were from Nacalai tesque (Kyoto, Japan), D-sorbitol was from Wako (Osaka, Japan). Molecular weight marker (broad range) and p*I* marker (2-D SDS-PAGE Standards) were purchased from Bio-Rad (Hercules, CA, USA). All other analytical grade chemicals were purchased from Sigma, GE Healthcare Bio-Science, Wako, Nacalai tesque or Takara Bio (Ohtsu, Shiga, Japan).

### 2.2. Equipment

The IEF for our novel system was operated using AE-6310 with a heat exchanger, AE-6370 (ATTO, Tokyo, Japan) connected to a low temperature circulator, CCA-1100 (Eyela, Tokyo, Japan). Voltage was supplied by AE8130 (ATTO) for IEF. The IPG IEF was performed on temperature controlled Multiphor II with EC 703 programmable power supply (GE Healthcare Bio-Science). The electrophoresis in the second dimension was operated using a regular slab size electrophoresis box with AE8130 or other equivalent power supplies.

### 2.3. Sample preparation

PS II complexes were purified from a cyanobacterium, *Synechocystis* sp. PCC 6803, as described in [6]. The final protein sample was suspended in a solution containing 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)-NaOH (pH 6.0), 10 mM $MgCl_2$, 5 mM $CaCl_2$, 25% glycerol and 0.04% DDM making ∼2 mg chlorophyll (Chl)/mL (∼16 mg protein/mL).

### 2.4. Two-dimensional electrophoresis with IPG IEF

Immobiline DryStrip was re-hydrated overnight by placing it on top of a sample solution (236 μL) so that the gel side was down in a re-swelling tray (GE Healthcare Bio-Science) and covered by DryStrip Cover Fluid (GE Healthcare Bio-Science). The re-swelling sample solution consisted of 5 M urea, 2 M thiourea, 1% CHAPS, 15% isopropanol, 0.35% DTT, 1.3% Ampholine, which contained 70 or 15 μg Chl of PS II complexes (equivalent to ∼560 or ∼120 μg proteins) per gel strip. Ampholine was added to ensure a steady pH gradient. After re-swelling, green Chl color spread evenly on the gel. The IPG strip was then transferred to the focusing tray and covered by DryStrip Cover Fluid as detailed in the manufacturer's instruction manual. Electrophoresis in the first dimension was stopped after 41,000–42,000 V h was reached using a 7-step program (30 V h at 50 V, 100 V h at 150 V, 150 V h at 250 V, 300 V h at 500 V, 400 V h at 750 V, 2000 V h at 1000 V and 38,000–39,000 V h at 2000 V). The IPG gel strip was removed from the tray and the excess mineral oil was drained off. The strip was then placed onto the slab gel containing 18–24% gradient acrylamide and 6 M urea with Tris/MES buffer system [16] for the second dimension. It was fixed on the top of the stacking gel with 0.5% agarose solution containing 12.5 mM Tris (pH 6.8), 0.46% SDS and 1% mercaptoethanol for the sake of denaturation. This agarose solution was made by adding ×25 stock solution containing Tris, SDS and mercaptoethanol after agarose was melted by microwaving. After electrophoresis at 11 mA to reach ∼180 mA h, the polypeptides were visualized by Coomassie blue. Otherwise, the gel was subjected to heme-staining [6].

### 2.5. Two-dimensional electrophoresis with agarose-based IEF

The first dimensional gel was made manually, fresh each time. The IEF grade agarose was used as a supporting matrix in the IEF. The gel solution contained 1% agarose, 0.66 M sorbitol [17], 1% DDM, 5 M urea, 2 M thiourea, 2 mM DTT, 10% Pharmalyte and PS II complexes of 10 μg Chl/mL (equivalent to approximately 80 μg protein/mL). The gel solution was made as follows. To make 4 mL of gel solution (for 4 to 5 capillary gels), 0.04 g agarose and 0.48 g sorbitol was added to 2.0 mL of $H_2O$ in a 20 mL Erlenmeyer flask. Loosely covered by plastic wrap, to prevent excess evaporation, agarose was melted using the microwave. Immediately, 1.2 g urea and 0.6 g thiourea were added to the resulting agarose solution, and dissolved completely by gentle but quick shaking of the flask. Upon the dissolution

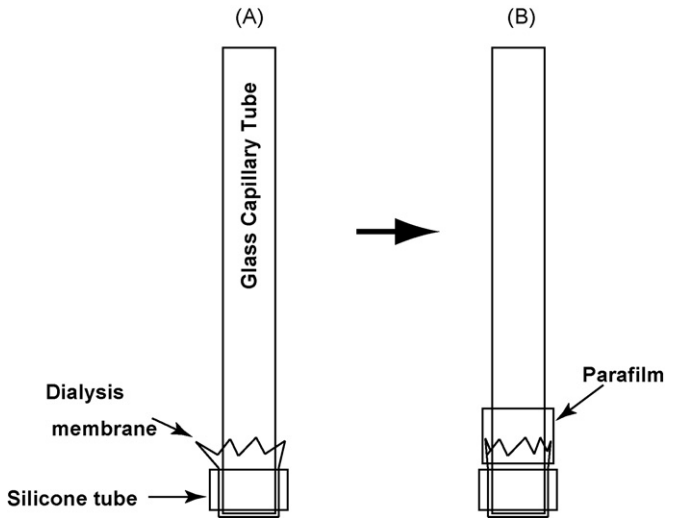


Fig. 1. Scheme to prepare capillary tube for the sample loading.

of urea and thiourea, the temperature of the solution drastically decreases, but, the solution will not become a gel at room temperature for several tens of minutes leaving an enough time for the following process to occur at room temperature. The microwave in the presence of urea and thiourea was not used [18]. DDM (200 μL) was added from 20% stock solution yielding 1% DDM. Then, Pharmalyte (pH 3–10, 400 μL) and DTT (8 μL from 1 M stock solution) were added and mixed. PS II complexes equivalent to 40 μg Chl (∼320 μg proteins) were added and the resulting sample gel solution was sonicated for 1 min using Bransonic B2200 (Branson Ultrasonics, Danbury, CT, USA) to ensure the full dissociation of subunit polypeptides. The gel solution was applied to vertically placed glass capillary tubes up to the top assuring to remove air bubbles using a narrow, flexible capillary tube. The size of the glass capillary tube was 2.5 mm × 7 mm × 130 mm (I.D. × O.D. × length) making ∼650 μL inner volume. The inner surface was coated by silicone in advance by Sigmacote (Sigma). This silicone coating is very efficient for taking the gel from the glass capillary tube after the electrophoresis. The bottom end of capillary tube was sealed using dialysis membrane, size 27 (cut off size is ∼14,000; Wako), which was previously boiled and washed to remove preservatives. A short silicone band (7 mm × 10 mm × 6 mm, I.D. × O.D. × length) was used to hold the dialysis membrane to the bottom end of capillary tube (Fig. 1A) and Parafilm was additionally used to prevent the leakage of gel solution from the small gap interface between the membrane and glass surface (Fig. 1B). The capillary tube containing gel solution was placed at 4 °C for 15 ∼ 24 h to make the solution gelatinize.

To run IEF, the vertically fixed capillary tube (and apparatus previously described) was embedded in the anode reservoir buffer (1 L). The anode reservoir buffer was prepared by adding 670 μL of phosphoric acid (85% solution) to 1 L of distilled water. During the IEF, the anode reservoir buffer was kept at 4 °C by a heat exchanger AE-6370 connected to a low temperature circulator. The cathode reservoir buffer (∼150 mL) contained 0.2 M NaOH. The IEF started at 50 V, and after 30 min, the voltage was increased to 100 V for 30 min and then, to 300 V, which was maintained for ∼24 h. Before stopping, the voltage was decreased to 50 V for ∼30 min to avoid any local higher temperature in the gel.

Using 1 mL size pipetting valve, the gel was carefully taken out directly onto the stacking gel of the second dimension. The slab gel in the second dimension contained 16% acrylamide and 6 M urea with Tris/MES buffer system [16]. The agarose rod gel in the first dimension was fixed onto the stacking gel by 1 mL of 0.8% agarose (Agarose L03, Takara). The agarose was melted by microwaving. After cooling to ∼40 °C, one tenth volume of the denaturing solution containing 5.2% LDS, 40 mM DTT, 172 mM Tris (pH 6.8), 0.5 M sucrose and 0.01% pyronin Y [16] was added and the resulting agarose solution was applied on top of the gel. The electrophoresis in the second-dimension was performed at room temperature at 11 mA to reach ∼180 mA h.

### 2.6. Detection and identification of polypeptides on the gel

After electrophoresis in the second dimension, polypeptides were detected by Coomssie blue R-250, silver staining, heme staining or Western blotting. After identifying PsbA (anti-PsbA antibody, AgriSera, Vännäs, Sweden), PsbB, PsbC and PsbD [19] by Western blotting [16], other polypeptides were determined by comparing the relative mobility with polypeptides in PS II complexes [6]. Heme staining was performed as described in [6] using 3,3-diaminobenzidine tetra-hydrochloride (DAB) after two-dimensional electrophoresis with IPG. For two-dimensional electrophoresis using agarose-based IEF as a first dimension, heme was detected by chemiluminescence using WestFemto (Pierce, Rockford, IL, USA) and Fuji LAS1000 plus (Fuji Film, Tokyo, Japan) after electroblotting onto polyvinylidene fluoride (PVDF) membrane [1]. Low content polypeptides were detected by silver staining. After Coomassie staining, the gel was thoroughly washed in a solution containing 15% methanol and 10% acetic acid, and then, $H_2O$ followed by an incubation in a 320 μM DTT solution for over 30 min. After soaking with 0.2% $AgNO_3$ solution for at least 30 min, development was performed by a solution containing 3% $Na_2CO_3$ and 1.9% formaldehyde. The development was stopped by 10% acetic acid. Excess development with high back ground was reduced by a solution containing 0.75% potassium ferricyanide and 9.6% sodium thiosulfate.

### 2.7. MALDI mass spectrometry

Coomassie stained polypeptides on a two-dimensional gel were excised and subjected to in-gel digestion with trypsin. The extracted peptides were purified using a reversed-phase micro-column (ZipTipC18, Millipore, Bedford, MA, USA) and analyzed by matrix-assisted laser desorption ionization mass spectroscopy (MALDI). MALDI was performed on a Voyager DEPRO instrument (PerSeptive Biosystems Inc., Framingham, MA) using α-cyano-4-hydroxycinnamic acid as the matrix.

## 3. Results and discussion

For the assessment of the resolution in two-dimensional electrophoresis with IEF as a first dimension, we used highly purified PS II complexes from a cyanobacterium, *Synechocystis* 6803. The polypeptide composition of them were already analyzed precisely and reported in [6].

Fig. 2 shows the separation pattern of PS II complexes on two-dimensional electrophoresis using IPG. The major spots are extrinsic hydrophilic polypeptides in PS II complexes (PsbO, PsbQ, PsbU and PsbV proteins). PS II complexes contain one copy of each of these polypeptides as well as hydorophobic polypeptides such as PsbA, PsbB, PsbC, PsbD and PsbE [3–5]. Because of the stoichiometric presence of these proteins, it is expected that these hydrophobic membrane proteins will form spots in similar size as hydrophilic ones. However, no distinct spots of membrane proteins were observed. Since the gel system adopted here in the second dimension is proved to be suitable for the analytical separation of the hydrophobic membrane proteins [6,16], the main problem on the poor separation of membrane proteins should be in the IPG electrophoresis in the first dimension as well as their poor solubility at the isoelectric point. Excess loading of sample should not be the reason for poor resolution in this case since decreasing the loading sample to around one fifth (560 versus 120 μg proteins) did not improve the separation pattern of membrane proteins (see Fig. 7A for the result using 120 μg proteins). It is noteworthy that significant smear appeared at the interface between stacking and resolving gels (see below).

The difficulty of the effective separation of hydrophobic membrane proteins may come from the structure of matrix of IPG in the first dimension. Conventional O'Farrell's two dimensional gel electrophoresis is also not suitable to separate hydrophobic proteins [20]. The hydrophobic membrane proteins might form large aggregates even in the presence of surfactants and urea/thiourea especially at around their isoelectric point. This is also one of the reasons for the significant smear at the interface between stacking and resolving gels; the smooth elution of heavily aggregated hydrophobic protein from the IPG gel might be disturbed by the sieve of IPG matrix. We used CHAPS as a surfactant, as is usually used for membrane proteins [21]. To improve the appearance of the membrane proteins in the second dimension, the survey of detergents may be one of the options. However, we thought that the separation could be improved by making the sieve mesh in the matrix much larger. Dextran matrix was used to purify photosystem I complexes from cyanobacterium whose molecular weight is ~1000 kDa [22]. Agarose gel whose sieve mesh is not made by covalent bonding different from acrylamide has been successfully applied for large polypeptides up to 500 kDa [17]. We adopted agarose as a supporting matrix for the IEF in the first dimension. Additionally, our preliminary investigation showed a better resolution by using 1% DDM as a surfactant compared to 4% *n*-heptyl-β-D-thioglucopyranoside or 1% CHAPS (data not shown). DDM is superior for the solubilization of many kinds of membrane protein complexes [1]. The small critical micelle concentration (CMC) of DDM is also desirable since lower amounts of detergent is enough to keep solubilized

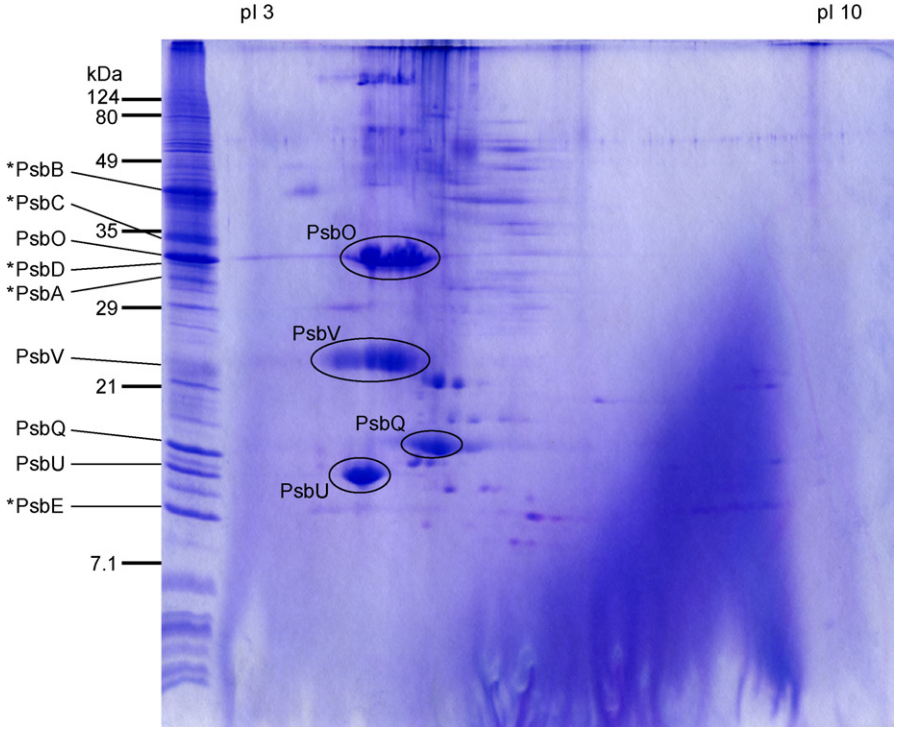


Fig. 2. Two-dimensional separation of PS II complexes using IPG. PS II complexes equivalent to 70 μg Chl (~560 μg protein) were applied to IPG strips (pH 3–10) in the first dimensional separation. The second-dimensional gel contained 18–24% gradient acrylamide and 6 M urea in Tris/MES system. In the second-dimension electrophoresis, PS II complexes equivalent to 7 μg Chl were applied to the left end of the gel. PS II polypeptides larger than ~10 kDa are indicated. The large iso-electrically focused spots are marked on the gel. Asterisks on the polypeptide names indicate hydrophobic membrane proteins.

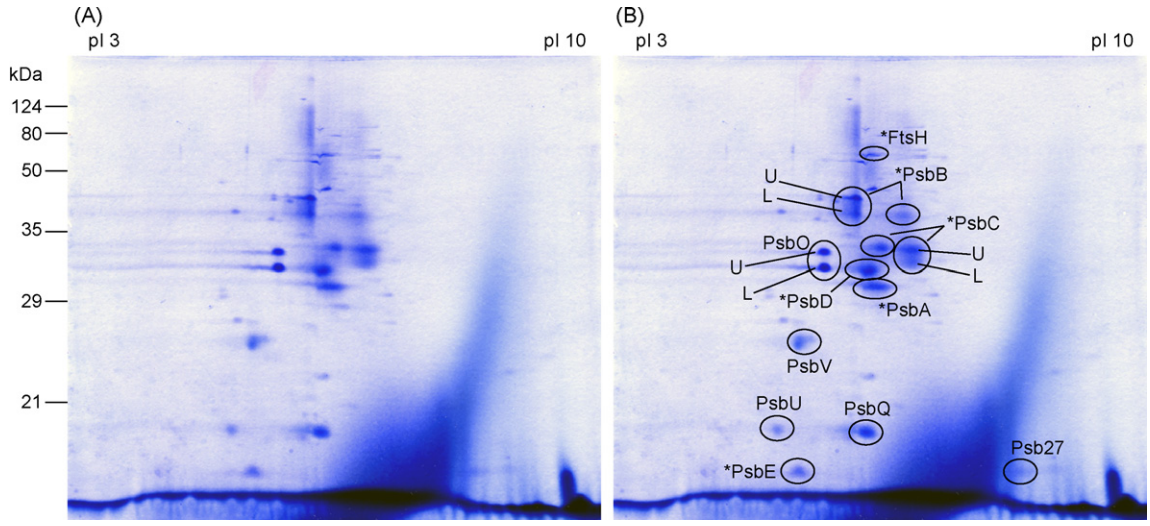


Fig. 3. Two-dimensional separation of PS II complexes using agarose-based IEF. PS II complexes equivalent to ~7.5 μg Chl (60 μg proteins) were used. The gel in the second dimension contained 16% acrylamide and 6 M urea in Tris/MES system. Panel B is the same as panel A, but identities of major spots are shown (see Fig. 4 and Table 1). Asterisks on the left of polypeptide names indicate hydrophobic membrane proteins. For U and L of PsbB, PsbC and PsbO, see Table 1.

forms of membrane proteins, which will reduce the disturbance of resolution in the low molecular mass region in the second dimension.

Fig. 3A shows the separation pattern of PS II complexes in our IEF system using agarose as a supporting matrix and DDM as a surfactant in combination with Tris/MES gel system in the second dimension. The number of appeared spots increased drastically in our novel system compared to that separated by two-dimensional electrophoresis using IPG shown in Fig. 2.

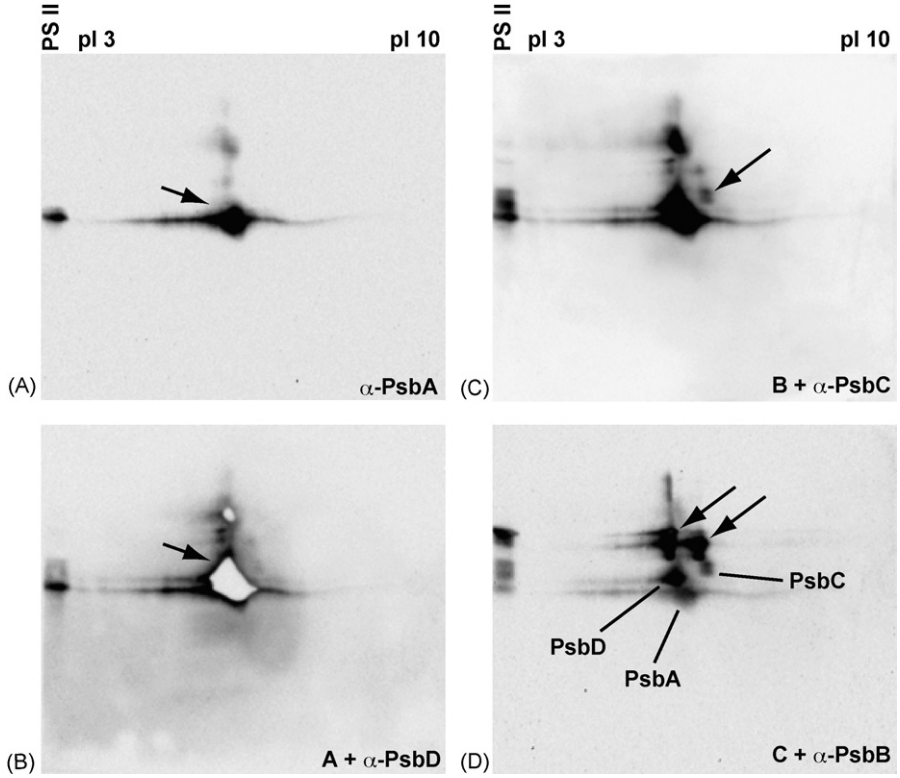


Fig. 4. Identification of polypeptides by immunoblotting. After electroblotting onto PVDF membrane, antibodies against PsbA (α-PsbA, panel A), PsbD (α-PsbD, panel B), PsbC (α-PsbC, panel C) and PsbB (α-PsbB, panel D) were sequentially applied to the single blotted PVDF membrane for the ease in recognition of the relative position of the antigenic polypeptides. After every probing by individual antibodies, polypeptides were detected by chemiluminescence (indicated by arrow), and then the antibody was stripped by use of Restore Western Blot Stripping Buffer (Pierce). Stripping was not performed thoroughly to visualize the relative position of PsbA, PsbB, PsbC and PsbD on the same single PVDF membrane (see panel D). Since the titer of α-PsbD was too high, halation has occurred in panel B. While, the titer of α-PsbC was low compared to the previously used antibody (α-PsbA and α-PsbD), the signal level was quite low for PsbC in panel C.

Table 1
Detected peptides by MALDI MS analysis from individual separated polypeptides after trypsin digestion

| | Detected peptides with an error <0.1% | | Detected peptides with an error <0.2% | |
|---|---|---|---|---|
| | Amino acid sequence (from–to) | Mass (*m*/*z*) | Amino acid sequence (from–to) | Mass (*m*/*z*) |
| Psb27 | 82–88 | 616.67 | 116–119* | 543.71 |
| | 7–11 | 616.67 | 120–129 | 1234.37 |
| | 49–68* | 2266.41 | 119–129* | 1390.56 |
| PsbE | | | 61–81* | 2647.88 |
| PsbU | 68–79 | 1351.57 | 80–100 | 2318.52 |
| | 103–131* | 3095.37 | 45–64* | 2368.59 |
| | | | 101–131* | 3338.63 |
| PsbQ | 84–96* | 1400.71 | 38–52 | 1654.97 |
| | 103–120* | 1969.27 | 59–83 | 2837.19 |
| | | | 58–83* | 2993.38 |
| | | | 59–84* | 2993.38 |
| | | | 58–84* | 3149.56 |
| PsbV | 34–49 | 1616.79 | 150–160* | 1356.50 |
| | 73–91* | 1968.16 | 73–90 | 1811.97 |
| | 131–153* | 2658.90 | 71–90* | 2041.25 |
| | 103–130* | 3338.57 | 131–149 | 2145.35 |
| PsbO (L) | 212–214 | 361.40 | 264–274* | 1241.37 |
| | 212–215* | 489.57 | | |
| | 83–88 | 714.77 | | |
| | 192–211* | 2129.36 | | |
| | 153–181 | 3158.34 | | |
| | 222–261* | 4133.49 | | |
| PsbO (U) | 212–215* | 489.57 | 264–274* | 1241.37 |
| | 83–89* | 870.96 | | |
| | 192–211* | 2129.36 | | |
| | 153–181 | 3158.34 | | |
| | 222–261* | 4133.49 | | |
| PsbA | | | 258–269 | 1459.62 |
| PsbD | 8–12 | 570.65 | | |
| | 295–304 | 1227.34 | | |
| | 13–24* | 1549.75 | | |
| PsbC (L) | 457–460 | 459.50 | 381–390* | 1311.47 |
| | 357–361 | 769.86 | | |
| | 370–378 | 928.01 | | |
| | 362–369 | 967.14 | | |
| | 26–40 | 1664.80 | | |
| | 457-472* | 1849.05 | | |
| PsbC (U) | 457–460 | 459.50 | 323–338 | 1454.65 |
| | 381–389 | 1155.28 | | |
| | 381–390* | 1311.47 | | |
| | 26–40 | 1664.80 | | |
| PsbB (L) | 420–422 | 392.46 | 273–286* | 1890.04 |
| | 278–286 | 1169.26 | 424–444 | 2352.46 |
| | | | 423–444* | 2480.63 |
| | | | 449–472 | 2838.27 |
| PsbB (U) | 278–286 | 1169.26 | | |
| FtsH (Slr1604) | 322–324 | 436.47 | 37–55* | 2270.44 |
| | 146–149* | 460.53 | | |
| | 525–532 | 781.95 | | |
| | 525–541* | 1653.95 | | |
| | 362–384* | 2371.68 | | |
| | 361–383* | 2371.68 | | |
| | 385–407* | 2529.81 | | |
| | 486–510 | 2719.94 | | |
| | 542–571* | 3358.49 | | |

Table 1 (*Continued*)

| | Detected peptides with an error <0.1% | | Detected peptides with an error <0.2% | |
|---|---|---|---|---|
| | Amino acid sequence (from–to) | Mass (*m*/*z*) | Amino acid sequence (from–to) | Mass (*m*/*z*) |
| FtsH (Slr0228) | 333–335 | 436.47 | 84–98* | 1651.88 |
| | 485–492 | 731.87 | | |
| | 48–56 | 1069.18 | | |
| | 57–67 | 1266.37 | | |
| | 204–218* | 1432.77 | | |
| | 68–83* | 1799.01 | | |

Each Coomassie stained spots were cut out from dried gel, re-hydrated, and digested by trypsin according to the standard method. Since the polypeptide components in PS II complexes are determined (Kashino et al. [6]), the masses of all predicted fragments by trypsin-digestion including partially cleaved fragments are calculated from the deduced amino acid sequence obtained from CyanoBase (http://www.kazusa.or.jp/cyano/Synechocystis/index.html) using a protein sequence analysis software GPMAW version 3.1 (Lihgthouse data, Odense, Denmark). MALDI-TOF data were compared with those masses and the detected fragments with an error below 0.1% or 0.2% are listed in this table. Asterisks indicate fragments of partial digestion. L and U in parentheses indicate lower and upper spots of PsbB, PsbC and PsbO in Fig. 3, respectively.

The identities of these separated polypeptides were determined by immunoblotting (Fig. 4) and MS analysis (Fig. 5 and Table 1). Since single electroblotted PVDF membrane was probed by antibodies against PsbA, PsbB, PsbC and PsbD, the relative position of these hydrophobic polypeptides were easily recognized (Fig. 4D). On the basis of the migration lengths of these polypeptides, identities of other major polypeptides were empirically and tentatively assigned since the polypeptide composition of PS II complexes were already determined [6]. Then, the individual Coomassie-stained polypeptides were excised and digested by trypsin, and subjected to MS analysis. Fig. 5 shows the typical MALDI spectrum obtained from PsbO (see Fig. 6B). Major polypeptides larger than ∼10 kDa in PS II complexes were identified as is listed in Table 1. The identities of individual Coomassie-stained polypeptides are drawn in Fig. 3B.

Hydrophilic extrinsic proteins which had been separated in IPG IEF (Fig. 2) were also clearly separated in this agarose-based system with much more efficient focusing (Fig. 3). Other than these hydrophilic proteins, it is clear that hydrophobic proteins made distinct spots (Fig. 3B). The hydrophobicity of these proteins is very high when GRAVY values are over 0.1 (Table 2). It is especially noteworthy that very hydrophobic PsbD whose GRAVY value reaches 0.361 and PsbA whose GRAVY value reaches 0.308 appeared as distinct spots.

In Fig. 3, PsbB, PsbC and PsbO showed separate spots with different molecular weights as was confirmed in Fig. 4 and Table 1. Empirically, in some purified PS II samples, PsbO split into two forms in different apparent molecular weights. The relative amounts of them vary depending on the sample batch (compare with those in Fig. 9). These two bands were detected in one-dimensional SDS-PAGE [6] and both of them were determined as PsbO (Table 1 and [6]). Because N-terminal amino acid sequences of these two bands are the same [6], the C-terminal part might be degraded. Two bands of PsbB were also detected in one-dimensional SDS-PAGE [6]. Since it is known that phosphorylation in PsbC (CP43 protein) causes a shift of apparent molecular weight [23], the split spots of PsbC may reflect the phosphorylation status of PsbC. Accordingly, the appearance of two different forms of these proteins could not be attributed to the artificial effect in IEF in the first dimension. Additionally, PsbB and PsbC showed other spots of different pIs. It is reported that phosphorylation of core proteins in PS II occurs under high light condition in higher plants [23], which will cause p*I* shift. The appearance of different spots in different pIs might indicate the phosphorylation status of PsbB and PsbC. Or, this may be just caused by the different alkylation status [24].

The first report of membrane proteins from a Gram-positive bacterium which were solubilized on two-dimensional elec-

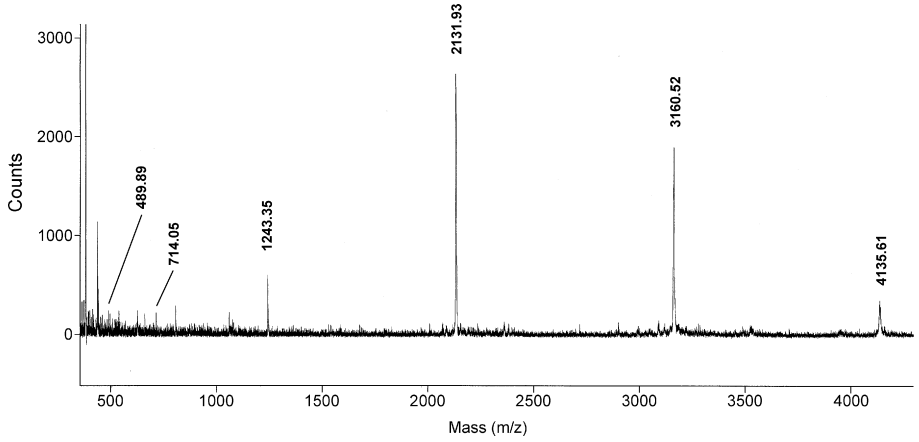


Fig. 5. Mass spectrum obtained from PsbO after trypsin-digestion. Coomassie stained PsbO (Fig. 3) was cut out from dried gel, re-hydrated, and subjected to in-gel digestion with trypsin. The extracted and purified peptides were analyzed by MALDI instrument using α-cyano-4-hydroxycinnamic acid as the matrix.

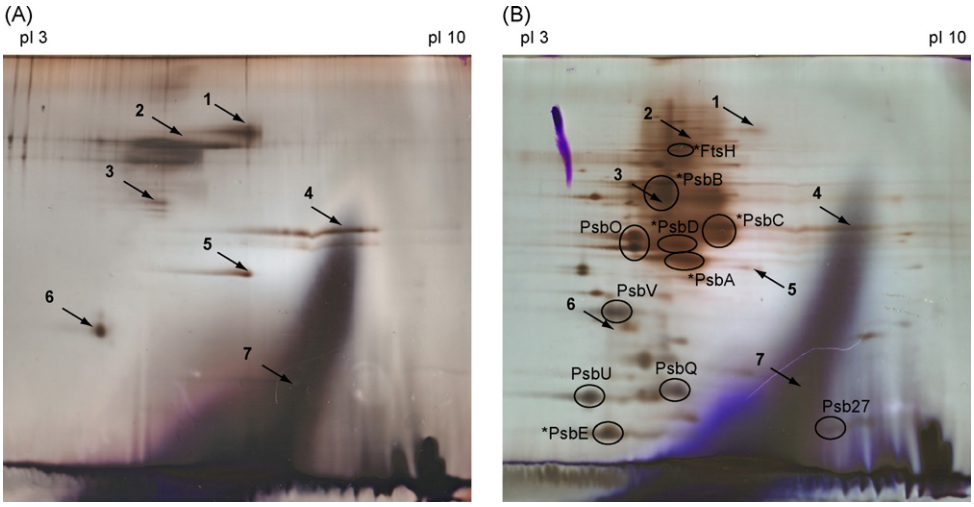


Fig. 6. Distribution of p*I* markers in the two-dimensional electrophoresis using agarose-based IEF. Panel A, silver stained p*I* markers. Panel B, comparison of p*I* between p*I* markers and subunit proteins in PS II complexes on two-dimensional electrophoresis. Proteins were stained with silver. The p*I* markers contained: (1) Hen egg white conalbumin (p*I* = 6.0, 6.3, 6.6, MW = 76,000); (2) Bovine serum albumin (p*I* = 5.4, 5.5, 5.6, MW = 66,200); (3) Bovine muscle actin (p*I* = 5.0, 5.1, MW = 43,000); (4) Rabbit muscle GAPDH (p*I* = 8.3, 8.5, MW = 36,000); (5) Bovine carbonic anhydrase (p*I* = 5.9, 6.0, MW = 31,000); (6) Soybean trypsin inhibitor (p*I* = 4.5, MW = 21,500); (7) Equine myoglobin (p*I* = 7.0, MW = 17,500). Some of these markers show streaks because they contain several isoforms of different pIs. The appearance of them resembles that indicated by the manufacturer.

trophoresis with IEF in the first dimension is described in [12]. The GRAVY value of them is −0.178 for LemA and 0.038 for biotin carboxyl carrier protein of acetyl-CoA carboxylase from *Streptococcus mutans*. Without any further solubilization process by surfactants or solvents, we were able to separate much more hydrophobic membrane proteins in a simple method. Outer membrane proteins OmpL1 (GRAVY = 0.036), Qlp42 (GRAVY = −0.235) and Loa22 (GRAVY = −0.493) from *Leptospira interrogans* which had not previously been identified on two-dimensional electrophoresis were reported in [25]. The GRAVY values of hydrophobic proteins which were separated in our system (Fig. 3 and Table 2) are remarkably larger than those in [25] and those detected on two-dimensional electrophoresis with IEF in the first dimension in *S. cerevisiae* (GRAVY = 0.1), *B. subtilis* (GRAVY = 0.15) or *E. coli* (GRAVY = 0.3) [9].

Table 2
Physical and chemical parameters of PS II polypeptides larger than ∼10 kDa

| Polypeptides | MW | p*I* | GRAVY |
|---|---|---|---|
| FtsH | 68496.1 | 5.29 | −0.153 |
| PsbB | 55771.5 | 5.52 | 0.113 |
| PsbC | 51675.6 | 6.50 | 0.268 |
| PsbD | 39492.5 | 5.35 | 0.361 |
| PsbA | 38257.7 | 5.49 | 0.308 |
| PsbO | 26862.0 | 4.69 | −0.377 |
| PsbV | 15119.7 | 4.58 | −0.573 |
| PsbQ | 14181.0 | 5.63 | −0.420 |
| PsbU | 10490.5 | 4.31 | −0.459 |
| Psb27 | 14917.0 | 9.37 | −0.310 |
| PsbE | 9317.4 | 4.49 | −0.239 |

The parameters were computed using ProtParam [34] (http://us.expasy.org/tools/protparam.html). Full sequences of these polypeptides were obtained at CyanoBase (http://www.kazusa.or.jp/cyano/Synechocystis/index.html). Taking the post-transcriptional processing into consideration [6,35], mature parts were applied for computation. In PsbQ, N-terminal 21 amino acids were removed for calculation [36]. In PsbA, C-terminal 16 amino acids were omitted from the sequence of PsbA2 for calculation [37,38]. FtsH has two predicted transmembrane helices [39]. PsbE has one transmembrane helix [3–5].

Judging from the calculated p*I* values of each protein (Table 2), the separation pattern by pH seems to be reasonable. The reliability of the separation by pH in our system is shown in Fig. 6 by using p*I* marker proteins.

Because some important membrane protein complexes contain functional *c*-type cytochrome(s) such as in cytochrome *c* oxidase [26] and PS II complexes [3–5], the detection of *c*-type heme after electrophoresis is desirable. However, it was impossible to detect *c*-type heme after two-dimensional electrophoresis using IPG as a first dimension although PsbV (cytochrome *c*550) was clearly separated (Fig. 7). In contrast, PsbV (cytochrome *c*550) was detected by heme-staining on our two dimensional electrophoresis (Fig. 8), which indicates the advantage of our system for the proteomic analyses of samples containing *c*-type heme. After IPG electrophoresis, cytochrome c550 was not stained on the second dimension while cytochrome c550 in the marker lane which had not experienced IPG electrophoresis was stained on the same gel (Fig. 7). This result implies that the reason for this drastic difference between the IPG system and our novel system could be attributed to the difference in applied voltage. The applied voltage during IEF is quite high in the IPG system, and usually exceeds 2000 V as is performed in Figs. 2 and 7 [1]. Sometimes, a much higher voltage such as 7000 V [12] or 8000 V is applied [25]. Such high voltage may remove the ligated iron. In our novel system, the applied voltage is very low (300 V) compared to that in IPG IEF, which is frequently used in normal SDS-PAGE. This lower voltage during the IEF in our system could enable the detection of *c*-type heme on two-dimensional electrophoresis.

Accordingly, our novel IEF system is very effective in separating highly hydrophobic membrane proteins as well as detecting *c*-type cytochromes on two-dimensional electrophoresis.

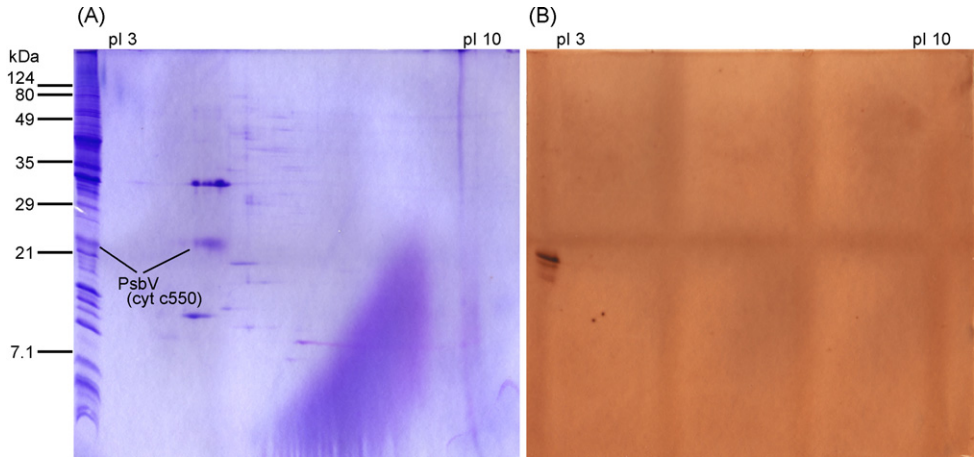


Fig. 7. Heme staining after two-dimensional electrophoresis using IPG. Panel A, polypeptides were stained by Coomassie blue R-250. Panel B, heme was stained using DAB. Two-dimensional electrophoresis was performed as in Fig. 2 using PS II complexes equivalent to 15 μg Chl (~120 μg proteins) for each gel. PS II complexes corresponding to 7 μg Chl were applied to the left end of the gels. PsbV (cytochrome *c*550) is a protein containing *c*-type heme.

Other than these two prominent characteristics, there are several points superior to the prevailing IPG system for proteomics. (1) No special processing of the samples such as de-lipidation and solubilization is necessary. This enables more precise analysis of intact membrane protein samples, and saves valuable samples and time. (2) Salts in the sample do not disturb the IEF since the buffering size of the reservoir is very large compared to the volume of sample in the capillary tube; the salts will move away into the reservoir buffer. Because of this feature, it is not necessary to remove salts like in the standard procedure for IPG before the IEF, which will also save samples and time. Furthermore, it will help to keep the membrane proteins (complexes) in their solubilized form. (3) Membrane proteins are detected in almost the same level as hydrophilic proteins. This enables quantitative comparison between hydrophobic and hydrophilic proteins. (4) The applied voltage is very low (300 V) compared to the IPG in the first-dimensional IEF. This enables the detection of *c*-type cytochromes. It is not necessary to use high-power

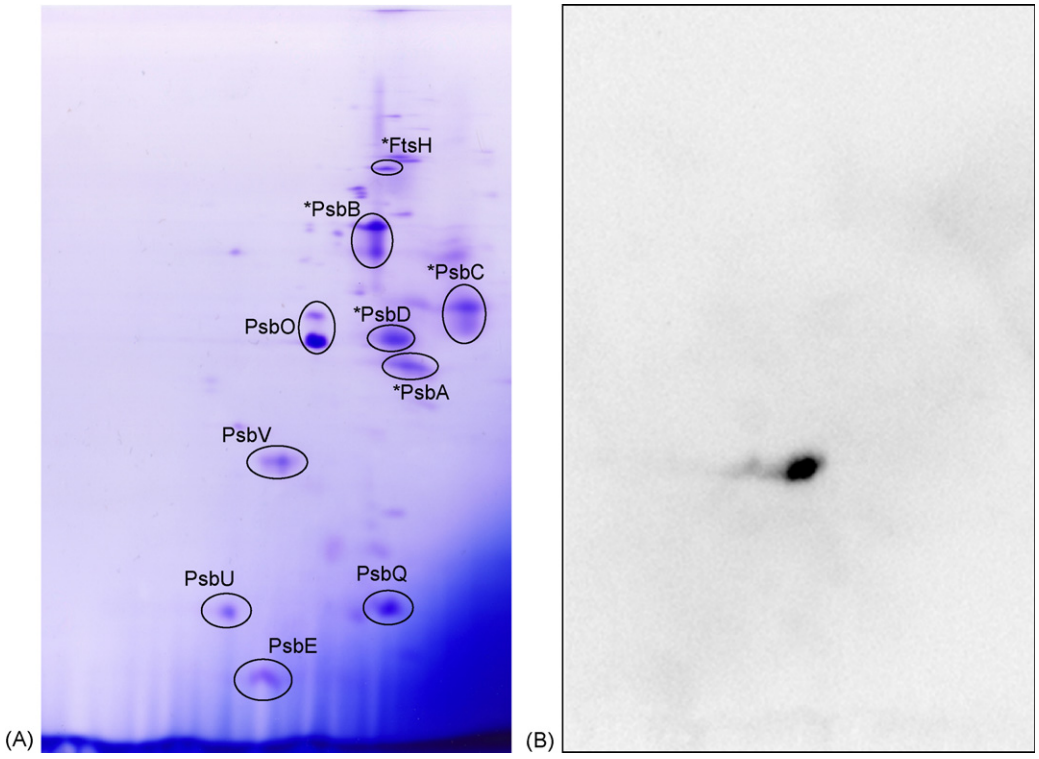


Fig. 8. Heme staining after two-dimensional electrophoresis using agarose-based IEF. Panel A, distribution pattern of polypeptides after two-dimensional electrophoresis performed as in Fig. 3. Polypeptides were stained by Coomassie blue. Panel B, heme was detected by chemiluminescence after electroblotting of polypeptides separated by two-dimensional electrophoresis. For the ease of recognition, only the acidic half of the gels is shown. Asterisks on the left of polypeptide names indicate hydrophobic membrane proteins.

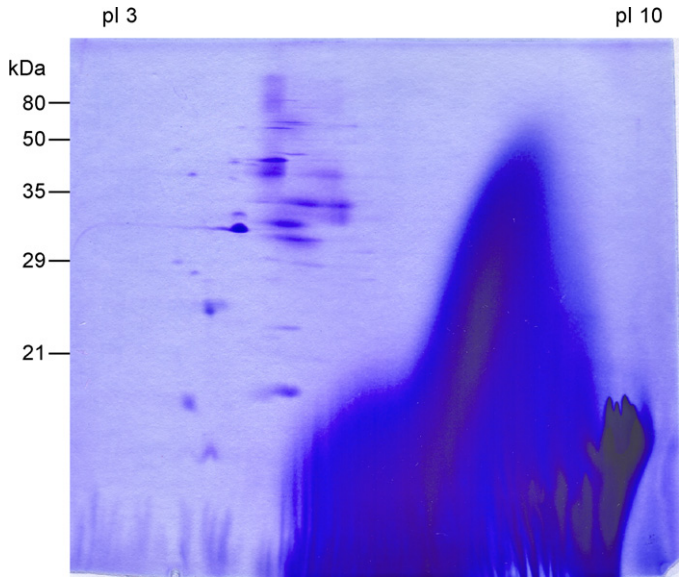


Fig. 9. Two-dimensional separation of PS II complexes using agarose-based IEF and 0.5% DDM. The agarose gel in the first dimension contained 0.5% DDM. Other conditions for IEF is the same as in Fig. 3. PS II complexes equivalent to ~7.5 μg Chl (60 μg proteins) were used. The gel in the second dimension contained 18–24% gradient acrylamide and 6 M urea in Tris/MES system.

electric power supply, which is usually quite expensive. Lower operating voltage is also much safer than high voltage.

In spite of these superior characteristics, there are several points to be improved or resolved. (1) It is not so convenient compared to a system using ready-made IPG gel strips. However, thinking back the development of the IPG system, it will not take long until the agarose-based IPG will become available. (2) Because Pharmalyte or equivalent carrier ampholytes are used, the visibility is not good in the high p*I*/low molecular mass region. Decreasing the concentration of carrier ampholytes to 1% to 3% [27,28] could resolve this problem. This problem will also be resolved by agarose-based IPG in the future. Otherwise, silver staining is somewhat better than Coomassie staining. But, for the sample preparation for MS analysis, silver staining should be carried out using the proper process to avoid insensitivity in MS [29–31]. (3) Since high concentrations of detergent (1%) is used, the resolution of low molecular mass regions is disturbed even with the use of Tris/MES system [16] or Tris/Tricine system [32]. The low CMC (0.0086% or 0.17 mM) of DDM will enable lower concentrations of detergent in the first dimension. Actually, 0.5% DDM gave similar resolution as 1% DDM in the case of PS II complexes (Fig. 9). Decreasing the amount of LDS (or SDS) that was used to fix the agarose gel onto the top of stacking gel of the second dimension may help the resolution of low molecular mass regions. (4) The agarose gel is easily broken; this problem can be overcome by experience. Siliconisation of the inner surface of the glass capillary tube aids removal of the gel from the capillary tube. Also, reducing the voltage for ~30 min in the final stage in the first dimension ensures the tight gelatinization. When agarose-based IPG is available, it will also resolve this problem. (5) The gel should be kept at a low temperature to stabilise the gel during the electrophoresis. However, keeping the gel at a low temperature is also necessary using the IPG IEF.

There are several points to be considered in preparing hydrophobic membrane proteins for proteomics. (1) Usually, de-lipidation is not good for the sample preparation for IEF. De-lipidation causes heavy aggregation of hydrophobic membrane proteins and the aggregation difficult to resolve [1]. In our system, samples with lipids can be used for analysis. (2) High temperatures should be avoided. High temperature treatment, which is frequently used for sample preparation for electrophoresis, also causes heavy aggregation of hydrophobic membrane proteins [1]. In our novel system, PS II samples were added after the temperature of the gel solution decreased to room temperature. Alternatively, protein samples could be applied on top of the capillary gel which is made in advance without the sample. This may avoid plausible exposure of samples to high temperatures and will keep the proteins intact.

## 4. Conclusion

Our novel, simple IEF system in combination with the Tris/MES electrophoresis system enables easy detection of highly hydrophobic membrane proteins whose GRAVY values exceed 0.3. Although several high-throughput proteomic systems exist (e.g., [33]), this kind of IEF two-dimensional electrophoresis with IEF is still worthwhile since protein spots are directly visible. The protein spots can be cut out and subjected to MS analysis to identify the proteins (Fig. 5, Table 1 and [6]). This system can also detect *c*-type cytochromes. This feature is very important since many kinds of *c*-type cytochromes function on the biological membranes. The high resolution of highly hydrophobic membrane proteins without any laborious solubilization process as well as low cost of this system will promote the proteomics of membrane proteins. The development of IPG using agarose as a supporting matrix will further contribute to the functional analysis of membrane proteins. Using this system, complex I and IV from bovine heart mitochondria, which contain more hydrophobic membrane proteins, are now under investigation.

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