The Journal of Microbiology, August 2008, p. 448-455 Vol. 46, No. 4
DOI 10.1007/s12275-008-0063-9

Application of Free-Flow Electrophoresis/2-Dimentional Gel Electrophoresis for Fractionation and Characterization of Native Proteome of *Pseudomonas putida* KT2440

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(Received March 5, 2008 / Accepted June 3, 2008)

Free Flow Electrophoresis (FFE) is a liquid-based isoelectric focusing method. Unlike conventional in-gel fractionation of proteins, FFE can resolve proteins in their native forms and fractionation of subcellular compartments of the cell is also possible. To test the efficacy of the FFE method, the native cytosol proteome of a bacterium, *Pseudomonas putida* KT2440 was fractionated by FFE and the spectrum of protein elutes was characterized in association with 2-dimentional gel electrophoresis (2-DE). Major native proteins of *P. putida* KT2440 were eluted in the range of pH 4.8~6.0 in FFE, whereas the denatured proteome of *P. putida* KT2440 was widely distributed in the rage of pH 4~10 in the 2-DE analysis. In addition, one of the three FFE major fractions, which was eluted at pH 5.0, was further analyzed using 2-DE/MS-MS. Then, the pH range of identified proteins eluted in 2-DE/MS-MS was 4.72~5.89, indicating that observed pI values of native cytosolic proteomes in FFE were narrower than those of denatured cytosolic proteome. These results suggest that FFE fractionation and 2-DE/MS analysis may be useful tools for characterization of native proteomes of *P. putida* KT2440 and comparative analysis between denatured and native proteomes.

***Keywords*:** free flow electrophoresis, native proteome fractionation, *Pseudomonas putida* KT2440

Free-Flow Electrophoresis (FFE) is a liquid-based IEF (isoelectric focusing) technology. In this apparatus, protein samples are continuously injected into a carrier ampholyte solution flowing as a thin film between two parallel plates (Fig. 1). Therefore, high throughput analyses of proteins are possible because a large amount of proteins can be applied to an FFE apparatus. In addition, gel-free isoelectric focusing is used under native conditions. Therefore, sample proteins are not denatured during fractionation, rendering it possible to fractionate native proteomes. As a result, proteins that are part of a protein complex can be fractionated together in FFE. Therefore, it is feasible to identify proteins and their partners involved in protein-protein interactions.

Protein samples in FFE are also separated by IEF according to their pI values by applying an electric field perpendicular to the flow direction (Fig. 1; Hoffmann *et al*., 2001). The matrix-free separation system employed in FFE offers several advantages over other electrophoresis systems, including high sample recovery, nondestructive fractionation, and high sample loading capacity per run (Simpson, 2004). FFE can be used in conjunction with other proteomics techniques for protein fractionation and purification. For example, cytosolic proteins from various origins were fractionated and separated by FFE in combination with SDS-PAGE (Hoffmann *et al.*, 2001). In particular, rare proteins or proteins with extreme pI values, which had not been previously identified, were detected by this technology (Cho *et al*., 2005). Recently, reverse-phase high-performance liquid chromatography (RP-HPLC) was coupled with FFE for fractionation of proteins and peptides, resulting in high resolution of fractionation (Mortiz *et al*., 2004; Mortiz *et al*., 2005). Zone electrophoresis in FFE (ZE-FFE), which is one of FFE based-fractionation methods, was applied for fractionation of membrane protein and sub-organelle such as mitochondria (Zischka *et al*., 2006; Braun *et al*., 2007).

In this study, we tested the FFE method for the fractionation of native proteome of *Pseudomonas putida* KT2440. This strain is a common soil bacterium that can utilize various aromatic compounds as an energy and carbon source (Nelson *et al*., 2002). The genome of this bacterium has been fully determined and it has about 5,400 annotated transcripts (Nelson *et al*., 2002). In addition, several proteomic studies using 2-DE/MS-MS were reported for biodegradation by *P. putida* (Benndorf *et al*., 2006; Kim *et al*., 2006; Kurbatov *et al*., 2006). With its genome sequence and previous proteomic studies available, *P. putida* can serve as a model microorganism to investigate the proteome of the bacterium employing FFE. Herein, we report FFE fractionation and subsequent 2-DE/MS analysis may be useful tools for characterization of native proteomes and identification of protein complexes in *P. putida* KT2440.

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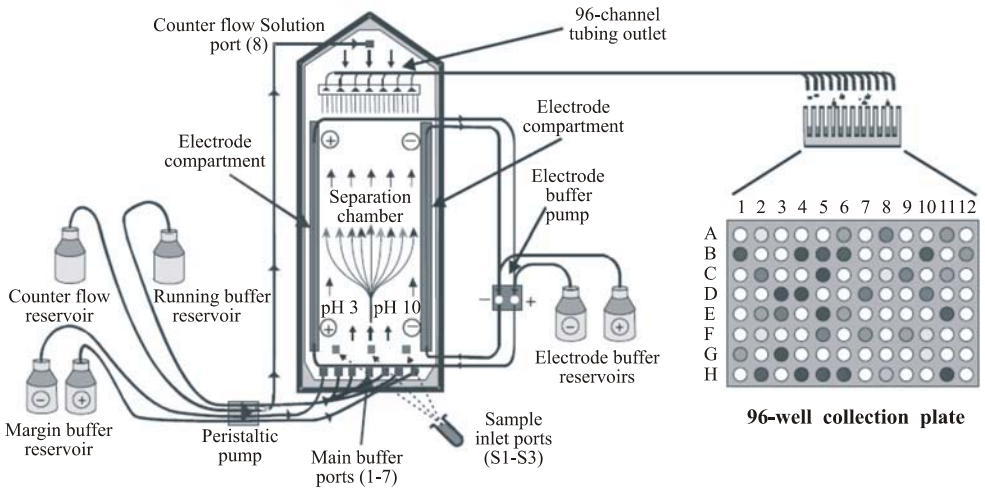

Fig. 1. Schematic diagram of the continuous free-flow electrophoresis (FFE) apparatus (ProMetHEUS). The dimension of the separation chamber is 500×100×0.4 mm (Moritz and Simpson, 2005). The running buffer is introduced into the main buffer ports (1∼7) and a sample solution is injected into the inlet ports (S1∼S3). Protein samples focused at their isoelectric points are collected into a 96-well collection plate. Collection volume is approximately 1.8 ml/well.

Materials and Methods

Cell culture and sample preparation

P. putida KT2440 was cultured and harvested as previously described elsewhere (Kim *et al.*, 2006). Harvested cells were suspended in 20 mM Tris-HCl buffer pH 8.0, and disrupted twice by a French pressure (SLM AMINCO, USA) at 20,000 lb/in^2. The supernatant (crude cell extracts) was collected by centrifugation at 15,000×g for 45 min at 4°C.

Free-Flow Electrophoresis (FFE)

Continuous liquid-based isoelectric focusing was performed using a ProMetHEUS FFE apparatus according to the protocol supplied by the manufacturer (Kirchheim, Germany). The IEF running buffer was aqueous 0.2% (w/v) hydroxypropylmethylcellulose (HPMC) containing 0.2% (w/v) carrier ampholytes (Servalyte pH 3∼10). The electrode solutions were 100 mM H_3PO_4 (anode) and 50 mM NaOH (cathode); the counter flow solution was 0.2% HPMC. Electrophoresis was performed at 4°C with a flow rate of 57 ml/h and 1,000 volts. The system was first equilibrated with the IEF running buffer at 1,000 volts until the current became stable. The performance test was conducted by measuring the pH-gradient and the separation of low-molecular-mass pI markers by isoelectric focusing. For preparative separations, the sample protein solution was diluted to a final concentration of approximately 2 mg/ml. The volume of collected fractions was about 1.8 ml. Fractions were stored at 4°C until use for the SDS-PAGE or 2-DE analysis.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein samples for SDS-PAGE were obtained from 96 well fraction plates. For each sample, 12 μl of sample solution was loaded on 12% SDS-polyacrylamide gels and protein bands were visualized by silver staining using a kit (Pharmacia Biotech, UK).

2-D gel electrophoresis

2-DE was performed by following the procedure previously reported by Park *et al.* (2006). Protein samples were precipitated from the collected solutions by TCA precipitation. TCA stock solution (50%) was added to the collected solution to reach 20% TCA solution that was then incubated at 0°C for 1 h. Precipitated proteins were separated by centrifugation at 15,000×g for 10 min at 4°C. Protein samples were washed 3 times using ice-cold acetone and subjected to 2-D gel electrophoresis. Electro focusing was conduced by IPGphor (Amersham Pharmacia Biotech, Sweden) and then 12% SDS-polyacrylamide electrophoresis was performed using a PROTEAN II xi Electrophoresis Kit (Bio-Rad, USA).

Protein identification using MALDI-TOF/TOF MS

In-gel digestion was conducted in accordance with the previously described methods (Kim *et al.*, 2006). Protein spots on the 2-D gel were excised and digested using 7∼8 μl trypsin (0.1 μg/μl) for 12 to 16 h at 37°C after the reduction and alkylation of cysteine residues in the proteins. The digested peptides were then recovered via two extraction steps using a solution containing 50 mM ammonium bicarbonate, 50% acetonitrile and 5% trifluoroacetic acid (TFA). The digested peptide extracts were used for MS/MS analysis by a 4700 Proteomic Analyzer (Applied Biosystems, USA). For protein identification, the MS/MS spectra were searched using MASCOT software (Matrix science, UK; www.matrixscience.com). Proteins with at least one significant peptide match (≥individual score) were selected from homology search.

Results

Optimization of FFE

Operation of FFE was performed according to the manual of the ProMetHeus FFE apparatus (Weber GmbH, Germany)

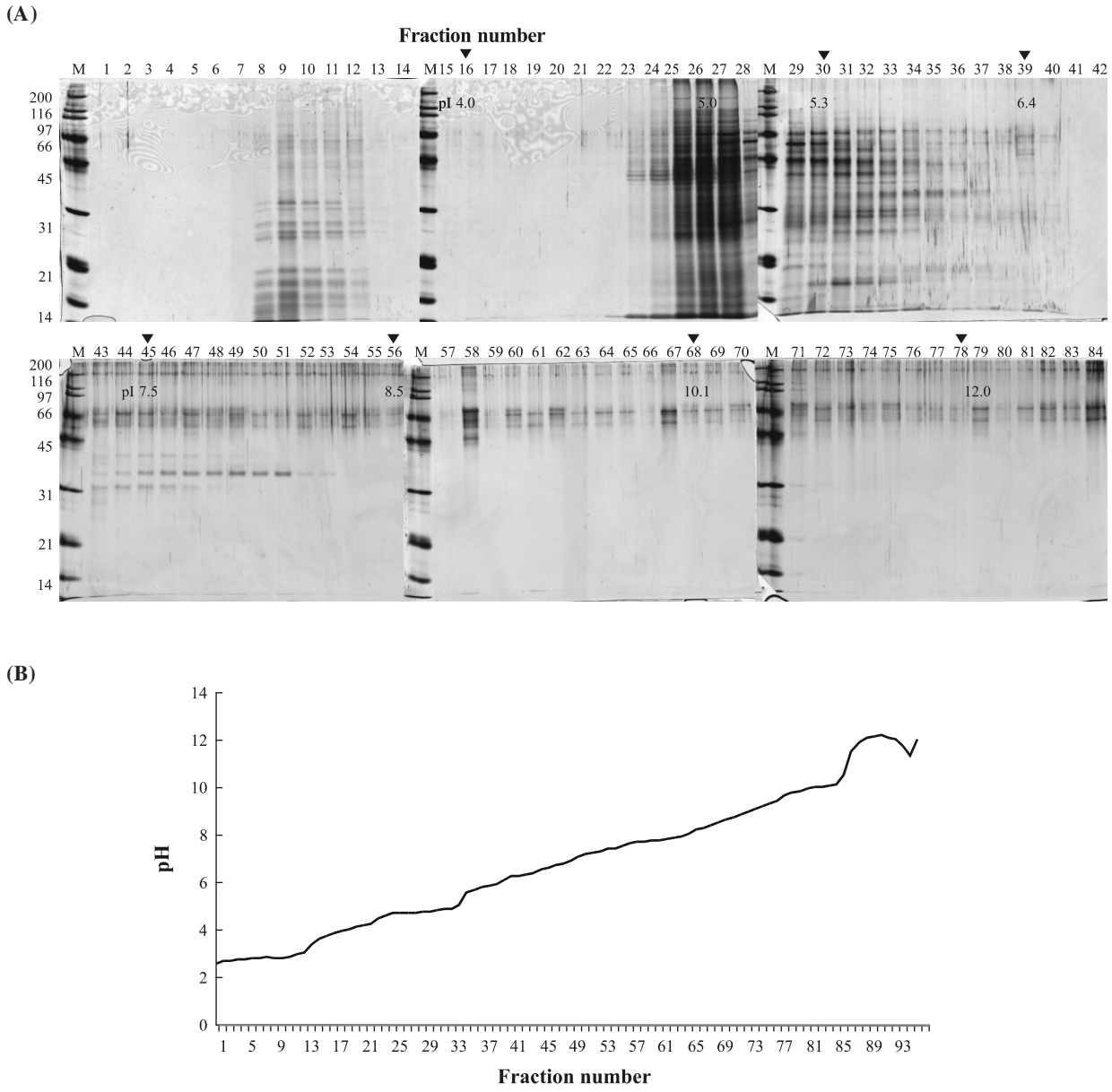

Fig. 2. Analytical SDS-PAGE of FFE fractions (no. 1∼no. 84) of a crude extract of *P. putida* KT2440. Seven pI standard markers were eluted at the indicated fractions (▼; no. 16, 30, 39, 45, 56, 68, 78) (A). A typical pH gradient generated by liquid phase IEF is shown from fractions 1 to 93 (B).

and the protocol suggested by Moritz and Simpson (2005). All the medium inlet tubes and the counterflow tube were filled with Milli-Q water to ensure that all air bubbles were displaced from the chamber. To verify that the system had a consistent laminar flow in the separation chamber, a red dye (SPADNS) was loaded in the inlets I2, I4, and I6. A stripe performance test was carried out using the red dye. After the performance test, FFE running buffer was loaded and a pH gradient was made by setting the voltage at 1,000 V and the current at 50 mA. The resulting fractions were collected in a 96 well plate and used for pH measurement. The pH measurement showed a successful pH gradient that ranged pH 3∼12 in the separation chamber (Fig. 2B). A separation test of pI markers was also performed. The pI marker was a mixture of seven dyes (one red and six yellow) of different pI values (pI 4.0, 5.3, 6.4, 7.5, 8.5, 10.1, and 12.0). These dyes were collected in 16, 30, 39, 45, 56, 68, 78th fraction wells (Fig. 2A), suggesting that each fraction in the 96 well plate was separated by about 0.1 pH unit in our experimental condition.

Fractionation by FFE

The concentration of prepared cytosol protein mixtures was about 3.5 mg/ml. Protein solutions were diluted to 2.0 mg/ml using the running buffer and 1.5 ml of sample solutions was injected in FFE and fractionated. Fractionated protein sol-

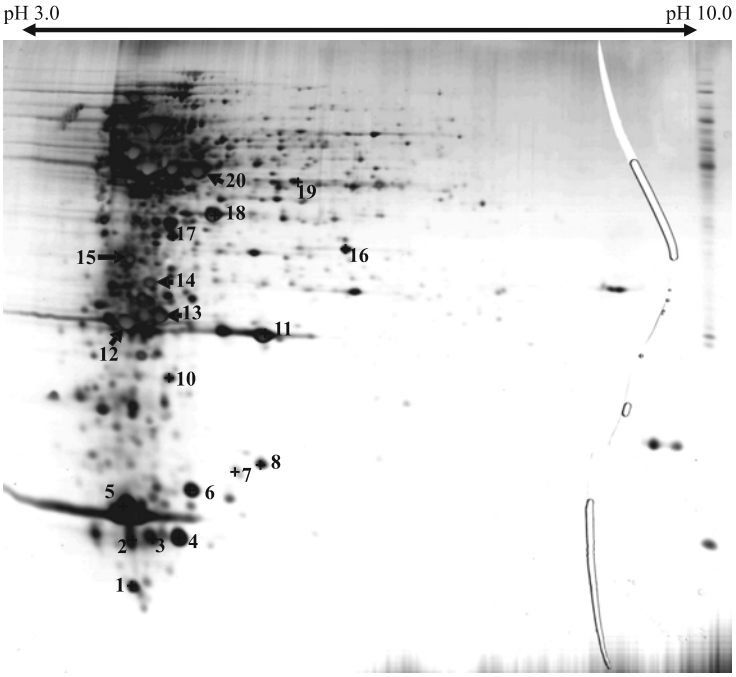

Fig. 3. 2-D gel electrophoresis of FFE fraction 26. Among 20 protein spots designated by the corresponding numbers, 16 proteins are identified by MS/MS analysis.

ution of each 96-well plate was approximately 1.8 ml. A volume of 12 μl of each fraction of the 96-well plate was used for SDS-PAGE. The resulting SDS-PAGE showed that major proteins were eluted between fraction no. 24 and fraction no. 36 (Fig. 2A). The pH range of the running buffer was 4.8∼6.0. Acidic and basic proteins were also detected in the fraction no. 8∼12 and fraction no. 43∼51, respectively.

2-D gel electrophoresis of FFE fraction and protein identification

Fraction no. 26, a major fraction in FFE, was selected for two-dimensional gel electrophoresis (Fig. 3). The pH range of fraction no. 26 was about pH 5.0∼5.1 in FFE according to pH measurement of the running buffer. However, major proteins were displayed on the 2-D gel in the range of pH 4.0∼6.0. Thirteen abundant proteins were identified by MS/MS analysis (Table 1). The pI of most acidic protein (PP_4248, spot no. 2) was 4.72 and the pI of most basic protein (succinyl-CoA synthetase alpha subunit, spot no. 16) 5.89. Among the identified proteins, several protein spots (spot no. 7, 8, 11, 14, 16, 18, and 19) have theoretical pI values that significantly differed from the pH values of the eluted buffer (Fig. 3 and Table 1). However, theoretical pI values were well matched with experimentally calculated pI values on the 2-D gel. Two proteins (spot no. 14, 18) were remarkably biased to the acidic locations and these proteins were identified as periplasmic membrane proteins.

Discussion

Liquid-based isoelectric focusing by FFE uses HPMC (hydroxypropylmethylcellulose) and carrier ampholytes as a running buffer for protein separation. In this running buffer condition, proteome can maintain native forms, namely native proteome. Since native proteome has biologically active proteins, we applied FFE for fractionation of native proteome of *P. putida* KT2440. In this study, we compared the native proteome fractionated by FFE with the denatured proteome using 2-D gel electrophoresis. The theoretical pI values of proteins, which were deduced from the genome of *P. putida* KT2440, showed that the proteins are well distributed on the pI range of 3.6∼12.0 and the average pI value of total proteins was estimated to be 6.96 (Fig. 4). The 2-DE result showed that the induced proteins under succinate medium were also widely distributed on the 2-D gel with a pI range of 3∼10 (Fig 5). Overall, the pI values of identified proteins on the 2-D gel were well correlated with their theoretical pI values. Therefore, it may be inferred that 2-D gel electrophoresis is reliable for measuring pI values of denatured proteome of *P. putida* KT2440. In addition, FFE was used for the fractionation of native proteome of *P. putida* KT2440. The pI values of the eluted buffer and standard dyes showed pH gradients ranging from pI 4.0 to pI 12.4 on the FFE separation chamber (Fig. 2). Unlike the denatured 2-DE result, however, native proteins were mainly eluted in a narrow range of pH 4.8 to 6.0. Particularly, major native proteome was eluted in the three fractions (No. 25, 26, and 27). Among these, a major fraction, No. 26 (pH 5.0∼5.1), was subjected to a 2-D gel under a denatured condition (Fig. 3) and this denatured proteome was distributed between pH 4.7∼5.9. However, two proteins (spot no. 16 and spot no. 18) had significant pI deviations from their theoretical pI values (Table 1).

Table 1. Proteins identified from the 2-D gel of the FFE fraction number 26

Spot no.	Gene ID	Identified protein	MW (Da) / pI	Score (MS/MS)	Amino acid sequences by MS/MS analysis
2	26990939	hypothetical protein PP_4248	10342 / 4.72	71	VDTAYLCEYRD LPGSDNWETFNAGDK
6	26991979	endoribonuclease	13499 / 5.15	123	AGNTVYMSGQIPLDPK YFEQPYPAR
7	3913226	10 kDa chaperonin (Protein Cpn10) (groES protein) (Heat shock protein 10)	10261 / 5.40	151	TAGGIVLPGSAAEKPNRGEVVAVGTGR VLDNGEVR VVFGPYSGSNTVK
8	26987585	nucleoside diphosphate kinase	15035 / 5.45	525	TFSIIKPDAVAK AEAEGFYAEHSER ADFAESIDANAVHGSDSEAAAAR EIAYFFAATEVTTR
10	26990298	thiol peroxidase	17398 / 4.99	72	VLNIFPSVDTPTCATSVR FCGAEGLDNVK
11	12084342	Chain A, Cloning, Sequence, and Crystallographic Structure Of Recombinant Iron Superoxide Dismutase	21876 / 5.55	260	AFELPPLPYAHDALQPHISK HHNTYVVNLNNLVPGTEFEGK FKEEFTK TSVGTFGSGWGWLVK
12	26987276	inorganic pyrophosphatase	19177/4.77	180	YEIDKDSDTLFVDR ARPVGVLNMTDDGGGDAK IEGWEGADAAR
13	26987820	antioxidant, AhpC/Tsa family	21716 / 5.06	328	IPDFQAR AYDVESEGGVAFR SQIVNDLPLGR NMDELLR
14	26987024	amino acid ABC transporter, periplasmic amino acid-binding protein	27851 / 5.46	303	MGIEAAYPPFNNK QAVDFTNPYYSNK LYDTQENAYLDLVSGR YVQYEWLK
15	26988588	elongation factor P	21290 / 4.73	280	IDNDPWLVQK TETVYGADDKLDDVILDR KVVYTENAAR
16	26990877	succinyl-CoA synthetase alpha subunit	30092 / 5.89	174	GGTTHLGLPVFNTVK DSILEAAFGGIK ALAELTGWEAK
17	26988324	elongation factor Ts	30413 / 5.14	75	NFVAESLEEAFAQK
18	26988032	general amino acid ABC transporter, periplasmic binding protein	36402 / 5.84	261	IVGIDADVCR FTALQSGEVDVLSR NTTWTSSR SQLFAQR
19	26991362	ketol-acid reductoisomerase	36347 / 5.48	218	GGGIPDLIAIYQDASGNAK NNAAHGIEIIGEQLR
20	26987193	elongation factor Tu	43451 / 5.22	527	GITINTAHVEYNSNIR DLLSTYDFPGDDTPIIIGSAR LVETLDAYIPEPVR VQDPLEIVGLR AGENCGVLLR

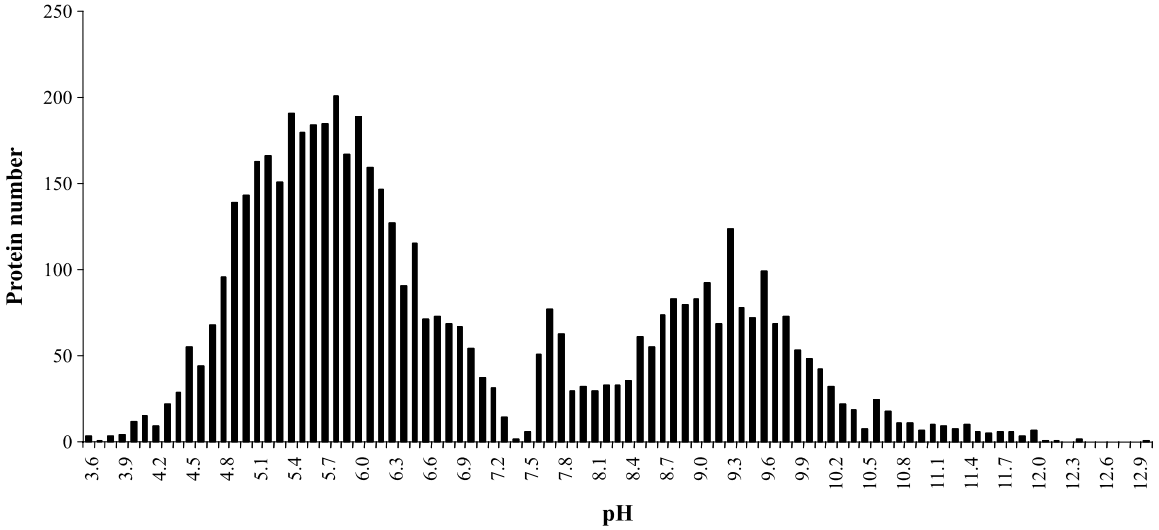

Fig. 4. Theoretical pI distribution of total proteome of *P. putida* KT2440. Calculation of pI values was performed using the pI/Mw tool available at ExPASy (http://www.expasy.org/tools/pi_tool.html).

This result indicates that the pI values of native proteome differ from those of denatured proteome. There may be several factors contributing to the discrepancy between pI values of native and denatured proteome. For example, native proteins can maintain their secondary and tertiary structures during FFE analysis. Therefore, hydrophobic amino acid residues may be buried in the core of the proteins, causing pIs to be different from those of denatured proteins. In addition, post-translational modification, truncation, formation of multi-protein complexes or sub-cellular localization can also explain the disagreement of pI values between FFE and denatured protein fractionations. For instance, spot no. 18 (pI 5.89) is a periplasmic binding protein with one transmembrane domain. Because the transmembrane domain of protein spot no. 18 is most likely to be in the membrane under the native condition during FFE, we tried to calculate the pI value of this protein after removing the sequence of the transmembrane domain and cytosolic re-

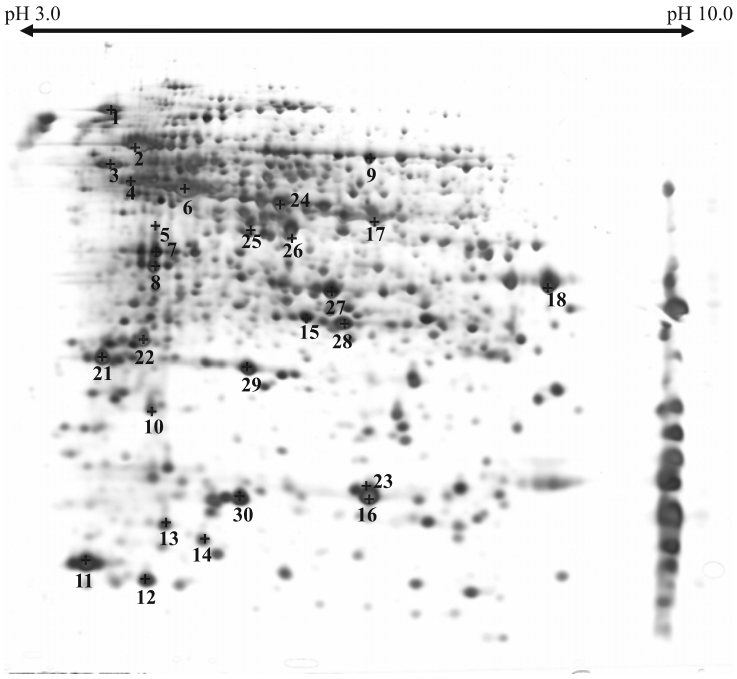

Fig. 5. 2-DE (pH 3~10) of a crude extract of *P. putida* KT2440. Proteins marked with the numbers were previously reported (Kim *et al*., 2006).

Table 2. Abundant protein spots from succinate-grown *P. putida* KT2440 (Kim *et al*., 2006)

Spot no.	Gene ID	Identified protein	MW (kDa) / pI
1	26991410	DnaK protein DnaK	68.8 / 4.83
2	26988095	Chaperonin 60 kDa GroEL	56.7 / 4.97
3	26992088	ATP synthase F1, b subunit AtpD	49.4 / 4.88
4	26988344	Enolase Eno	45.4 / 4.92
5	26990740	Dihydroorotate dehydrogenase family protein	46.1 / 5.25
6	26987193	Translation elongation factor Tu Tuf-2	43.5 / 5.22
7	26988324	Translation elongation factor Ts Tsf	30.4 / 5.14
8	26990893	Electron transfer flavoprotein, a subunit	31.2 / 5.07
9	26990879	2-Oxoglutarate dehydroge-nase, lipoamide dehydrogenase component LpdG	49.9 / 5.93
10	26990298	Thiol peroxidase Tpx	17.4 / 4.99
11	26987187	Ribosomal protein L7/L12 RplL	12.6 / 4.74
12	26991891	Thioredoxin Trx-2	11.7 / 5.02
13	26991979	Endoribonuclease	13.5 / 5.15
14	26988094	Chaperonin 10 kDa GroES	10.2 / 5.40
15	26986970	Cysteine ABC transporter, periplasmic cysteine-binding protein, putative	28.8 / 7.69
16	26991550	Azurin	16.0 / 6.41
17	26987736	Ornithine carbamoyltransferase, catabolic ArgI	37.9 / 5.92
18	26987807	Amino acid ABC transporter	33.4 / 8.61
19	26990526	UTP-glucose-1-phosphate uridylyltransferase GalU	30.9 / 5.46
20	26989367	Universal stress protein family	33.8 / 6.84
21	26987276	Inorganic pyrophosphatase Ppa	19.1 / 4.77
22	26987820	Antioxidant, AhpC/Tsa family	21.7 / 5.06
23	26991658	Hypothetical protein	22.1 / 7.89
24	26987877	Branched-chain amino acid ABC transporter periplasmic amino acid binding protein BraC	39.4 / 6.02
25	26991857	Putrescine ABC transporter PotF-2	40.1 / 6.04
26	26991857	Putrescine ABC transporter PotF-2	40.1 / 6.04
27	26990877	Succinyl-CoA synthetase, a subunit SucD	30.1 / 5.89
28	26987457	Ribosomal 5S rRNA E-loop binding protein	23.2 / 6.00
29	26987651	Superoxide dismutase SodB	21.9 / 5.55
30	26987585	Nucleoside diphosphate kinase Ndk	15.0 / 5.45

gion (residue 1∼29). For this putative extracellular region of the peritrophic protein, the pI value was decreased to 5.36. This result indicates that the intact transmembrane domain can affect the observed pI values of native proteins during FFE. Protein spot no. 16 was identified as a α-subunit of succinyl-CoA synthetase (Table 1, accession number 26990877). This protein is known to form a heterodimer with a β-subunit of succinyl-CoA synthetase in the cytosol in *E. coli* (Kapatral *et al*., 2000). It is believed that two subunit of succinyl-CoA synthetase of *P. putida* KT2440 form a heterodimer and co-eluted in FFE fractionation. However, β-subunit of succinyl-CoA synthetase was not detected in our 2-DE experiment. In addition, succinyl-CoA synthetase complex was co-purified with nucleoside diphosphate kinase in *Pseudomonas aeruginosa* (Kavanaugh-Black *et al*., 1994; Kapatral *et al*., 2000). This result suggests potential protein-protein interactions between succinyl-CoA synthetase and nucleoside diphosphate kinase in *P. aeruginosa*. In the current study, same types of proteins (spot no. 8 and 16) from *P. putida* KT2440 were identified in the same FFE fractionation (Table 1). This result supports the possibility of the interaction between these proteins.

Further studies of native protein complex of succinyl-CoA synthetase and nucleoside diphosphate kinase may lead us to better understanding of differential pI values between denatured and native proteins. Yet, another example of protein complex is ketol-acid reductoisomerase/thiol peroxidase complex. This complex was identified from the cytosolic protein complexes of *Helicobacter pylori* using two-dimensional blue native/SDS-PAGE (Pyndiah *et al*., 2007). Ketol-acid reductoisomerase (spot no. 19) and thiol peroxidase (spot no. 10) of *P. putida* KT2440 were also identified in the same FFE fractionation. Therefore, it is concluded that

fractionation of native proteome using FFE combined with 2-DE/MS-MS can give us a valuable tool for characterization of native proteome and their complex.

Acknowledgements

This work was supported by the Marine and Extreme Genome Research Center Program of the Ministry of Land, Transportation and Maritime Affairs, Republic of Korea.

References

Benndorf, D., M. Thiersch, N. Loffhagen, C. Kunath, and H. Harms. 2006. *Pseudomonas putida* KT2440 responds specifically to chlorophenoxy herbicides and their initial metabolites. *Proteomics* 6, 3319-3329.

Braun, R.J., N. Kinkl, M. Beer, and M. Ueffing. 2007. Two-dimensional electrophoresis of membrane proteins. *Anal. Bioanal. Chem*. 389, 1033-1045.

Cho, S.Y., E.Y. Lee, J.S. Lee, H.Y. Kim, J.M. Park, M.S. Kwon, Y.K. Park, H.J. Lee, M.J. Kang, J.Y. Kim, J.S. Yoo, S.J. Park, J.W. Cho, H.S. Kim, and Y.K. Paik. 2005. Efficient prefractionation of low-abundance proteins in human plasma and construction of a two-dimensional map. *Proteomics* 5, 3386-3396.

Hoffmann, P., H. Ji, R.L. Moritz, L.M. Connolly, D.F. Frecklington, M.J. Layton, J.S. Eddes, and R.J. Simpson. 2001. Continuous free-flow electrophoresis separation of cytosolic proteins from the human colon carcinoma cell line LIM 1215: A non two-dimensional gel electrophoresis-based proteome analysis strategy. *Proteomics* 1, 807-818.

Kapatral, V., X. Bina, and A.M. Charkrabarty. 2000. Succinyl coenzyme A synthetase of *Pseudomonas aeruginosa* with a broad specificity for nucleoside triphosphate (NTP) synthesis modulates specificity for NTP synthesis by the 12-kilodalton form of nucleoside diphosphate kinase. *J. Bacteriol.* 182, 1333-1339.

Kavanaugh-Black, A., D.M. Connolly, S.A. Chugani, and A.M. Charkrabarty. 1994. Characterization of nucleoside-diphosphate kinase from *Pseudomonas aeruginosa*: complex formation with succinyl-CoA synthetase. *Proc. Natl. Acad. Sci. USA* 91, 5883-5887.

Kim, Y.H., K. Cho, S.H. Yun, J.Y. Kim, K.H. Kwon, J.S. Yoo, and S.I. Kim. 2006. Analysis of aromatic catabolic pathways in *Pseudomonas putida* KT 2440 by combined proteomic approach: 2-DE/MS and cleavable ICAT analysis. *Proteomics* 6, 1301-1318.

Kurbatov, L., D. Albrecht, H. Herrmann, and L. Petruschka. 2006. Analysis of the proteome of *Pseudomonas putida* KT2440 grown on different sources of carbon and energy. *Environ. Microbiol*. 8, 466-478.

Moritz, R.L., A.B. Clippingdale, E.A. Kapp, J.S. Eddes, H. Ji, S. Gilbert, L.M. Connolly, and R.J. Simpson. 2005. Application of 2-D free-flow electrophoresis/RP-HPLC for proteomic analysis of human plasma depleted of multi high-abundance proteins. *Proteomics* 5, 3402-3413.

Moritz, R.L., H. Ji, F. Schötz, L.M. Connolly, E.A. Kapp, T.P. Speed, and R.J. Simpson. 2004. A proteome strategy for fractionating proteins and peptides using continuous free-flow electrophoresis coupled off-line to reversed-phase high-performance liquid chromatography. *Anal. Chem.* 76, 4811-4824.

Moritz, R.L. and R.J. Simpson. 2005. Liquid-based free-flow electrophoresis-reversed-phase HPLC: a proteomic tool. *Nat. Methods* 2, 863-873.

Nelson, K.E., C. Weinel, I.T. Paulsen, R.J. Dodson, H. Hilbert, V.A. Martins dos Santos, D.E. Fouts, S.R. Gill, M. Pop, M. Holmes, L. Brinkac, M. Beanan, R.T. DeBoy, S. Daugherty, J. Kolonay, R. Madupu, W. Nelson, O. White, J. Peterson, H. Khouri, *et al*. 2002. Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. *Environ. Microbiol.* 4, 799-808.

Park, S.H., J.W. Kim, S.H. Yun, S.H. Leem, H.Y. Kahng, and S.I. Kim. 2006. Characterization of β-ketoadipate pathway from multi-drug resistance bacterium, *Acinetobacter baumanni* DU202 by proteomic approach. *J. Microbiol.* 44, 632-640.

Pyndiah, S., J.P. Lasserre, A. Ménard, S. Claverol, V. Prouzet-Mauléon, F. Mégraud, F. Zerbib, and M. Bonneu. 2007. Two-dimensional blue native/SDS gel electrophoresis of multiprotein complexes from *Helicobacter pylori*. *Mol. Cell. Proteomics* 6, 193-206.

Simpson, R.J. 2004. Purifying proteins for proteomics, p. 463-478. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, N.Y., USA.

Zischka, H., R.J. Braun, E.P. Marantidis, D. Büringer, C. Bornhövd, S.M. Hauck, O. Demmer, C.J. Gloeckner, A.S. Reichert, F. Madeo, and M. Ueffing. 2006. Differential analysis of *Saccharomyces cerevisiae* mitochondria by free flow electrophoresis. *Mol. Cell. Proteomics* 5, 2185-2200.