J. Korean Soc. Microbiol., Vol. 35, No. 2, 2000

Two-dimensional gel Electrophoresis of *Helicobacter pylori* for Proteomic Analysis

Tae-Sung Jung, Seung-Chul Kang, Yeo-Jeong Choi, Beong-Sam Jeon, Jeong-Won Park, Sun-Ae Jung, Jae-Young Song, Sang-Haeng Choi, Seong-Gyu Park, Mi-Young Choe, Byung-Sang Lee, Eun-Young Byun, Seung-Chul Baik, Woo-Kon Lee, Myung-Je Cho, Hee-Sang Youn*, Gyung-Hyuck Ko and Kwang-Ho Rhee**

Department of Microbiology, Paediatrics and Pathology**, Gyeongsang National University College of Medicine, Chinju, Kyung-Nam 660-751 Korea*

Two-dimensional gel electrophoresis (2-DE) is an essential tool of proteomics to analyse the entire set of proteins of an organism and its variation between organisms. *Helicobacter pylori* was tried to identify differences between strains. As the first step, whole *H. pylori* was lysed using high concentration urea contained lysis buffer [9.5 M Urea, 4% CHAPS, 35 mM Tris, 65 mM DTT, 0.01% SDS and 0.5% Ampholite (Bio-Rad, pH 3-10)]. The extract (10 μg) was rehydrated to commercially available immobilised pH gradient (IPG) strips, then the proteins were separated according to their charges as the first dimensional separation. The IPG strips were placed on Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) to separate according to molecular mass of the proteins as the second dimension. The separated protein spots were visualised by silver staining in order to compare different expression of proteins between strains. Approximately 120 spots were identified in each mini-protein electrophoresised gel, furthermore about 65 to 75 spots were regarded as identical proteins in terms of p*I* value and molecular weight between strains used. In addition, distinct differences were found between strains, such as 219-1, Y7 and Y14, CH150. Two representative strains were examined using strips which had pH range from 4 to 7. This strips showed a number of isoforms which were considered large spots on pH range 3-10. Furthermore, the rest of spots on pH 4-7 IPG strips appeared very distinctive compared to broad range IPG strips. 2-DE seems to be an excellent tool for analysing and identifying variations between *H. pylori* strains.

Key Words: *H. pylori*, 2-D electrophoresis, Strain variation

INTRODUCTION

Helicobater pylori, a microaerophilic, Gram-negative, slow-growing and spiral-shaped bacterium, is the causative agent of type B chronic gastritis and recurrent peptic ulcers (5,38). Moreover, the chronic gastritis will be sequentially followed by chronic atrophic gastritis, intestinal metaplasia, dysplasia and gastric carcinoma in a susceptible population (8). The fact that infection with *H. pylori* is carcinogenic to humans (Group 1) was announced by World Health Organization in 1994, after completing

Received for publication : May 8, 2000, Accepted for Publication : August 7, 2000
Corresponding author: Kwang-Ho Rhee, Department of Microbiology, Gyeongsang National University College of Medicine, Chinju, Kyung-Nam 660-751, Korea, 055-751-8746 (phone) / 055-759-1588 (fax), E-mail address: khrhee@gshp.gsnu.ac.kr

cohort and retrospective case-control studies on the seropositivity of *H. pylori* and the incidence and mortality of stomach cancer.

After the organism was first successfully isolated in 1982 by Warren and Marshall, epidemiological study performed by Marshall *et al.* (24) and Dooley *et al.* (11) in the USA and Australia showed that the prevalence rate of *H. pylori* infection is less than 20% at 20 years of age. However, this rate increases with age, up to approximately 50% at 50 years, and remains at that level after 50 years. The prevalence rate in Korea is already 50% at 5 years old, 80% at 8 years, and 90% at and above 20 years (4,36,37,40). Compared to developed countries where the incidence of stomach cancer is lower, most Koreans carry *H. pylori* in their stomach for at least 40-45 years longer, or even for their entire life, when compared to *H. pylori*-infected persons, or *H. pylori*-non-infected persons (28,38). The annual incidence of gastric cancer in Korea in 1986-1987 was reported approximately 57.9 and 25.1/100,000 persons for males and females, respectively. It is one of the highest values in the world until now (1).

Type B chronic gastritis, peptic ulcer diseases, and gastric cancer may be solved by the direct countermeasures targeting *H. pylori*. Unfortunately, strategies for the control of *H. pylori*-induced gastritis are of limited utility, because of a variety of reasons involved, such as ineffectiveness of antimicrobial drugs *in vivo*, the difficulty of developing vaccine caused by ineffective immune response at stomach surface, the difficulty of blocking transmission of *H. pylori* infection (3,12,25,29). Even though the above barriers make it nearly impossible to rapidly decrease the prevalence rate of *H. pylori* infection by conventional measures in the near future (22,31,38), the control of diseases induced by *H. pylori* infection is highly urgent in Korea.

Many proteins were known to be involved in pathogenecity of the bacteria, such as flagella which help the organism to move in the mucus layer (21), urease complex which may help to maintain a neutral micro pH environment in the face of gastric acidity (13), the VacA proteins which generate vacuoles in eukaryotic epithelial cells (9) and the *cag* pathogenicity island which may be involved in triggering severe inflammatory responses (7). Recently, the complete genomic DNA sequence of *H. pylori* has been reported (2,39). However, many of the proteins inferred from this DNA sequence have no known function, and this DNA sequence does not always predict which open reading frames are likely to encode virulence factors or antigens.

In order to overcome such problems as well as to elucidate a function for each proteins, proteomics, the study of the expressed part of genome, is urgently necessary. As the first step of proteomics, 2-dimensional gel electrophoresis (2-DE) is essential. Here, we addressed the conditions of 2-DE and performed comparisons of *H. pylori* strains with 2-DE.

METHODS AND MATERIALAS

Bacteria and culture conditions

H. pylori strains (Table 1) stored at liquid nitrogen tank were thawed to culture onto a Mueller-Hinton agar plate containing 10% bovine serum, vancomycin (6.9 μM/L), and amphotericin B (1.1 μM/L). The plates were incubated at 37℃ under 10% CO_2 and 100% humid atmosphere overnight. The bacteria were washed with washing solution (40 mM Tris-Cl, pH 7.2, 1 mM EDTA) twice with centrifugation, dissolved bacterial pellet to make up the bacteria concentration O.D. 2.5 at 600 nm with spectrophotometer. One microliter of the measured bacteria was taken up and centrifugated at 12,000 rpm, 5 min. The bacterial pellet was kept in ice for further use.

2-Dimetional Gel Electrophoresis

Sample preparation and isoelectric focusing (IEF). Lysis buffer was prepared following O'Farell (30), with slight modification. 9.5 M Urea, 4% CHAPS and 35 mM Tris were dissolved in deionised distilled water (dDW), then deionised using resin (Bio-Rad) for 10 min. The deionised solution was filtrated through disposable 0.45 μm microfilter and measured the volume, finally added 65 mM DTT, 1 mM EDTA, 0.01% SDS and 0.5% ampholite 3/10. The lysis buffer was prepared freshly and kept in the ice slurry. Bacterial pellet was spread on the walls of eppendrof tube with tip, then added 500 μl of lysis buffer. Vortex mixed the bacteria and lysis buffer until pellets were not noticed. The bacteria was incubated for 20 min in ice slurry and then centrifuged at 12,000 rpm, 30 min at 10℃. 150~200 μl of supernatants was taken up and preserved in ice to measure the protein concentration. Twenty microgram of lysised sample was taken out then mixed with rehydration sample buffer (8 M Urea, 4% CHAPS, 10 mM DTT, 0.2% Ampholite 3/10) to make up 260 μl. The mixed solution was centrifuged at 12,000 g, 10 min at 10℃, then 130 μl of the supernatants were carefully pipetted into rehydration trays. Immobilised pH gradient (IPG) strip (Bio-Rad) was taken out from -20℃ freezer, and then carefully removed the IPG cover sheet to expose the gel. The gel side was placed down in the disposable IEF tray, at that time care should be taken not to trap air bubbles under the IPG strips, and then mineral oil was laid over the gel not to evaporate during dehydration. The gel was rehydrated under passive conditions: 0 volts, 20℃, 14-16 hours with Protein IEF Cell (Bio-Rad). The rehydrated gel was washed gently with dDW, and then removed excess water by putting on saturated filter paper with dDW for 2 min. Two wicks were saturated with dDW, blotted excess water on filter paper, then placed wicks over electrodes in IEF focusing tray. Electrode wicks are known to be important as receptacles for salts and other non-amphoteric constituents of the sample. The strip was placed gel side down in the IEF focusing tray such that the acidic (marked +) end was at the anode (red/+) electrode of the IEF Cell. The gels made contacts with the electrode wicks then overlay with mineral oil.

Three preset programs of the Protein IEF Cell was followed with slight modification as focusing conditions composed of conditioning step, voltage ramping and final focusing. The first conditioning step was to remove salt ions and charged contaminants at 250 V for 15 min. When the conditioning step was completed, linear voltage ramping step was followed for 2 hours, the current was limited not to exceed 50 μA/strip. At the final focusing step, a time was programmed to complete the focusing process once the maximum voltage was reached in terms of volt-hours, 30,000 volt-hours. The current limit would not exceed 50 μA/strip as well. After completing focusing, the gels were stored at -70℃ freezer until further use.

Equilibration. Prior to running the second dimension, it was necessary to equilibrate the IPG strips in a buffer which contained SDS (Sodium Dodesyl Sulfate). If the strip was frozen, thawed it at room temperature, then washed it with dDW and dried slightly as the above. The strips were placed in 15 ml conical tube contained first SDS-PAGE (Sodium Dodesy Sulfate-Polyacrylamide Gel Electrophoresis) equilibration buffer (6 M Urea, 0.375 M Tris pH 8.8, 2% SDS, 20% glycerol, 2% (w/v) DTT), then gently shaked on shaker table. After 15 min, the strip was equilibrated with second SDS-PAGE equilibration buffer (6 M Urea, 0.375 M Tris, pH 8.8, 2% SDS, 20% glycerol, 2.5% (w/v) iodoacetamide) as the above.

SDS-PAGE. The equilibrated strip was washed with dDW and dried as the above, then gently placed on the top of separating gel contain-

ing 10% acrylamide. The remained space was filled with 0.5% low melting agarose dissolved in running buffer. Molecular weight marker was inserted at the either side of the strip by digging and sealing with agarose. After filling with running buffer, second dimensional electrophoresis was performed at 15 mA for 15 min, then 30 mA until that the bromophenol marker reached to the bottom. The gel was placed into fixation solution to perform silver staining.

Protein assay

Quantifying protein in 2-DE solubilisation buffers was followed Ramagli (35). The modified Bradford protein assay was characterised by adding dilute acid to neutralise the sample. Briefly, ovalbumin standard (5 mg/ml) aliquots, dissolved in sample lysis buffer, were taken out from -80℃ freezer and left it at room temperature. Once thawed, the ovalbumin solution was clarified with centrifugation at 12,000 g for 3 min. The ovalbumin standard, from 5 μg to 50 μg in five folds, solution was dispensed to eppendrof tubes and made 10 μl with sample buffer without ovalbumin, and the unknown sample was prepared 10 μl in 3 eppendrof tubes as the same way. A mixture of 0.1 *N* HCl (10 μl/tube) and dDW (80 μl/tube) was prepared freshly, then added to the ovalbumin standard solution and the unknown sample. Coomassie Brilliant Blue G-250 dye concentrate (Bio-Rad) was diluted 5 times with dDW then filtered through Whatman Number 1 fiter paper. The filtrates was added to the ovalbumin standard solution and the unknown sample, and then mixed with vortex gently. After 5 min incubation, a standard curve was graphed using ovalbumin standard solution with spectrophotometer, and calculated the concentration of unknown protein samples.

Silver Staining and Gel drying

Silver staining was followed Heukeshoven *et al.* (14) with slight modification. Briefly, the gel was fixed with fixing solution containing 50% methanol, 12% acetic acid and 0.5 ml 37% formaldehyde/L over 1 h 30 min. All incubation was performed on shaker with gentle shaking. After fixing, the gel was washed with 50% ethanol twice for 20 min, and then washed again using dDW for 20 min. The gel was then pre-treated with $Na_2S_2O_3$ $5H_2O$ (0.2 g/L) for 1 min, this step was highly critical for the next steps, then washed again with dDW. Silver nitrate was impregnated by incubating in $AgNO_3$ (2 g/L) and 0.75 ml 37% formaldehyde for 30 min, and then rinsed with dDW three times for 20 second. Visualisation was performed by incubating in developing solution until clear image observation. The solution consisted of Na_2CO_3 (60 g/L), $Na_2S_2O_3$ $5H_2O$ (4 mg/L) and 0.5 ml 37% formaldehyde, which was prepared previously and persevered in ice slurry. When clear spots appeared, the gel was washed

Table 1. *Helicobacter pylori* bacterial strains used for 2-DE analysis

Strains	Source of patient	Nationality of patient
95-335	Chronic active gastritis	Korea
NCTC 11637	Duodenal ulcer	Australia
CJH 53	Gastric ulcer	Korea
HPSS 1	Duodenal ulcer	Australia
Y 7	Chronic active gastritis	Korea
Y 14	Chronic active gastritis	Korea
M 3-1-2	Gastric cancer	Korea
CH 150	Gastric ulcer	Korea
137-1	Chronic atrophic gastritis	Korea
219-1	Chronic active gastritis	Korea
51-1	Duodenal ulcer	Korea
92-489-1	Chronic active gastritis	Korea

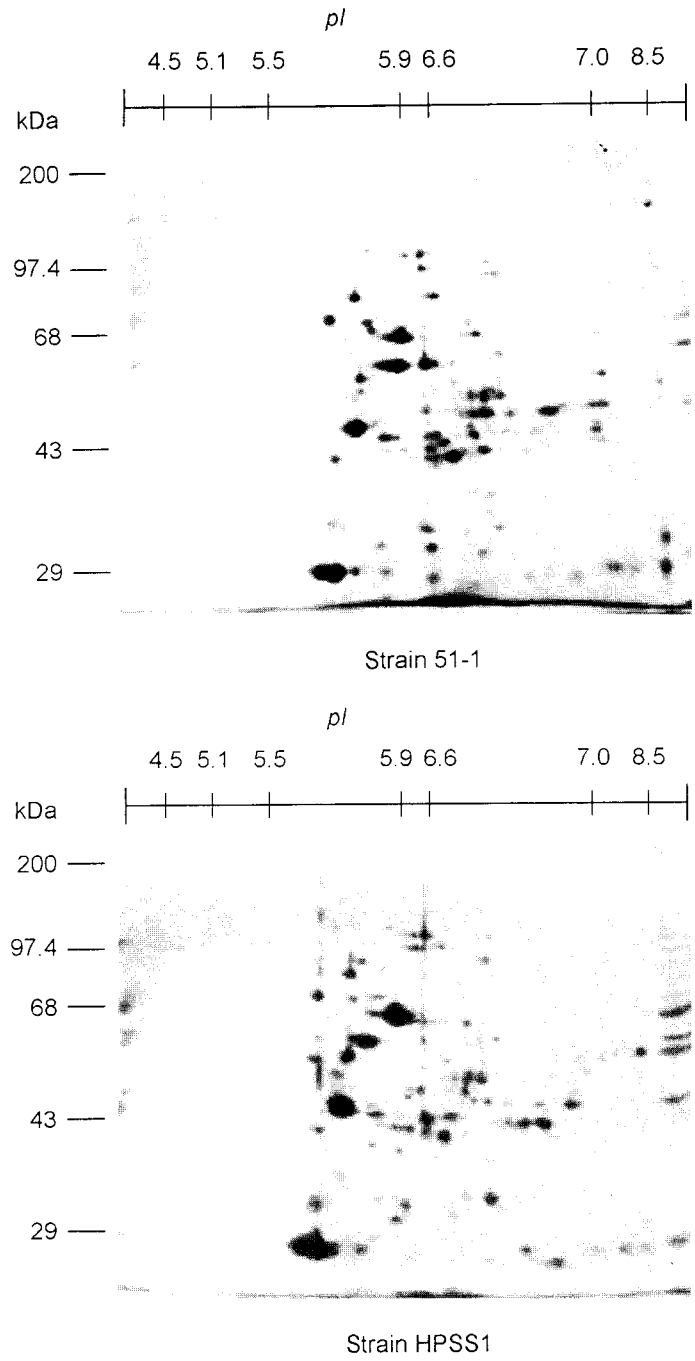

Figure 1. Silver staining results of *H. pylori* strain 51-1 and HPSS1 after separation with 2-dimentional gel electrophoresis. Bacteria were lysed in sample buffer containing 9.5 M Urea, 4% CHAPS, 35 mM Tris, 65 mM DTT, 1 mM EDTA, 0.01% SDS and 0.5% ampholite 3/10 then loaded 10 μg to Immobilised pH gradient (IPG) strips as the first dimensional gel electrophoresis (2-DE). The isoelectric focussed IPG strips were separated by SDS-PAGE.

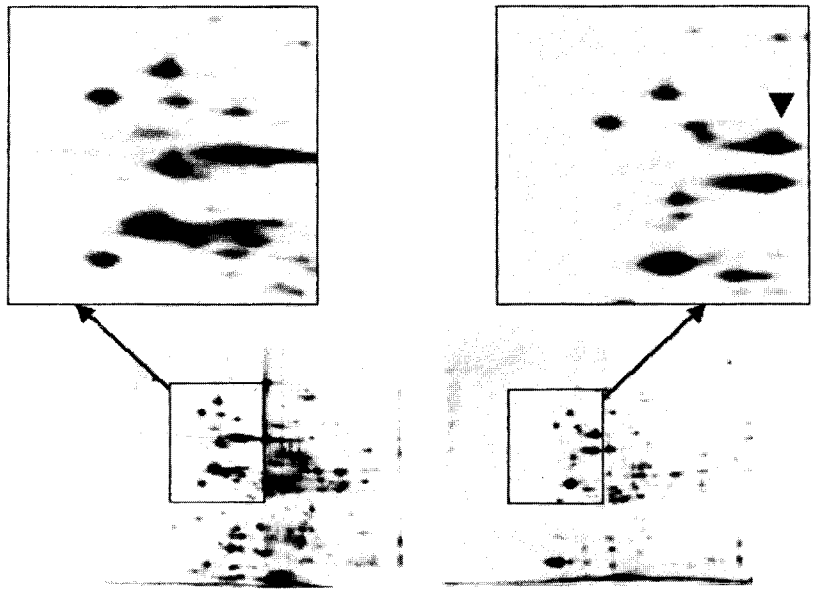

Figure 2. A spot, which had molecular weight 64.6 kDa, p*I* 6.40 value (arrow head), was deleted on 219-1 strain compared with the other strains examined.

with dDW twice for 20 second, then the reaction was stopped by adding 50% methanol and 12% acetic acid for 10 min. The visualised gel was dried with cellophane papers.

Data analysis

PDQUEST program was downloaded from Bio-Rad, analysised dried silver staining gels which were scanned with Adobe Photoshop program.

RESULTS

Fig. 1 showed two represent results of 2-DE using precast IPG strips which had pH range from 3 to 10. All strains (Table 1) studied were lysed at the same conditions, performed 2-DE and stained with silver nitrate. The stained gel showed a number of clear spots. Total spots were counted with PDQUEST program (Bio-Rad), the counted numbers of spots were from 110 to 130 spots in individual gels when used Mini-Protein II Cell (Bio-Rad). In addition, most of spots were placed less than 100 kDa molecular weight (Mr) and over 5.6 as p*I* value. Some of the gels showed vertical streaking arisen from over loading of proteins which were proved by employing different pH range strips, for example pH 4-7. Distinct differences were found between strains, such as a spot which had approximately Mr 64.6, p*I* 6.40 (arrow head) was detected in all strains examined except strain 219-1 (Fig. 2), another large spot which had around Mr 28.7, p*I* 5.70 (arrow head) was observed only two strains, Y7 and Y14 (Fig. 4). Strain CH150 had a characteristic by producing isoforms around 87.9 kDa (arrow head) which was not detected other stains or found very faintly even if existed (Fig. 3).

Two strains, 92-48-1 and 219-1 (data not shown), were examined using strips which had narrow range of pH value, pH 4 to 7 (Fig. 5). The strips, pH 4-7, showed a number of isoforms placed at approximately Mr 43.2, Mr 56.6

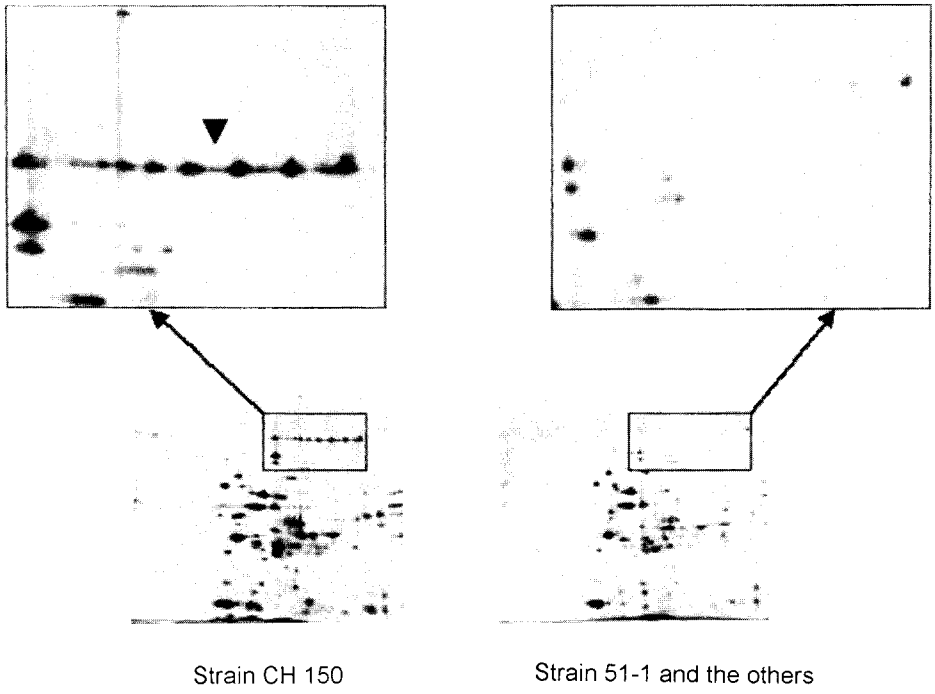

Figure 3. Isoforms appeared characteristically on CH 150 strain at 87.9 kDa (arrow head) after 2-DE.

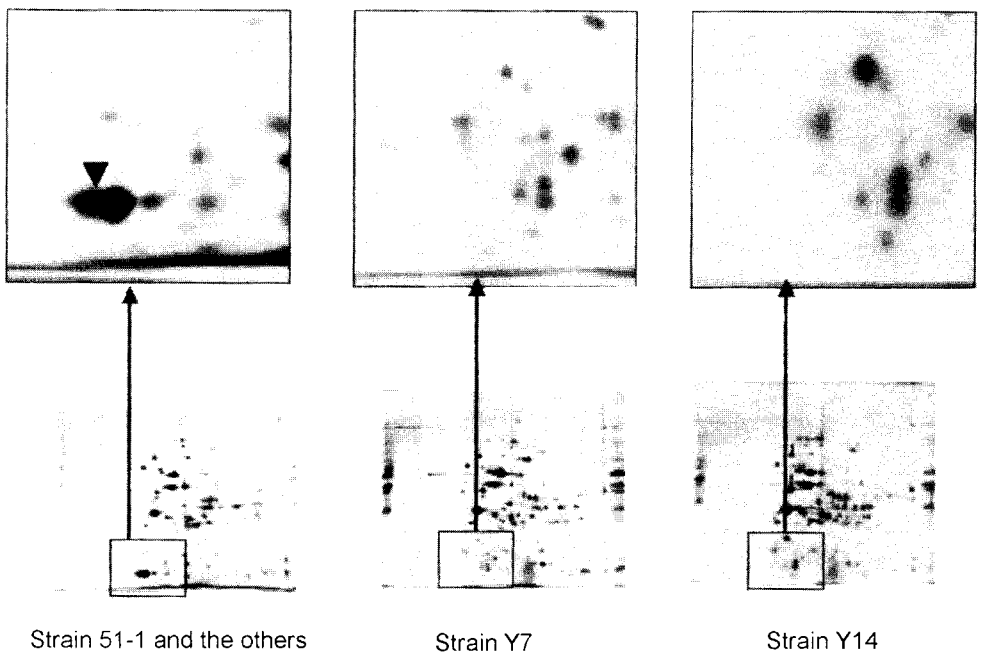

Figure 4. A spot (Mr 28.7, p*I* 5.70: arrow head) disappeared in Y 7 and Y 14, but the other strains studied showed large spot on silver stained gel after 2-DE.

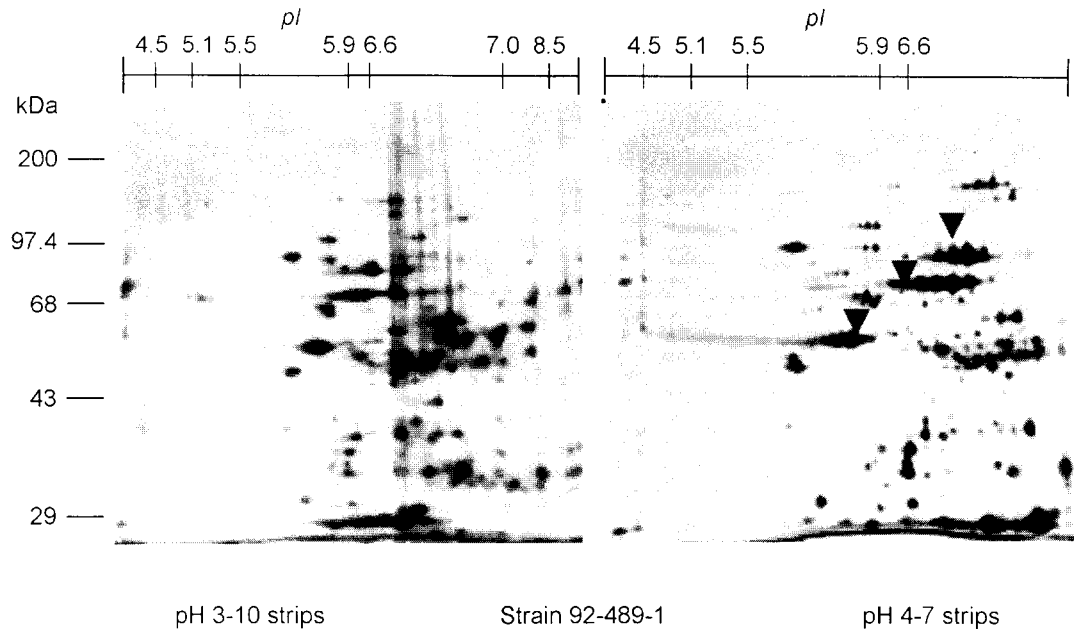

Figure 5. Comparison of two different pH ranges IPG strips with 2-DE for *H. pylori* strain 92-489-1. pH 4-7 IPG strip showed several isoforms (arrow head) which was observed as large spots on pH 3-10 IPG strip.

and Mr 64.6 (arrow head) as considered large spots with wide pH range strips. In addition, the rest of spots appeared very distinctive compared to broad range pH values.

DISCSSION

Two-dimensional gel electrophoresis was established with commercially available IPG strips as a part of proteomics, and it was proved to be reproducible at this study. Even though Mini-Protein Cell system was employed at present study, differences were evident between strains examined. A few number of proteomic works were reported for *H. pylori* (17,26,27) with the aiming of identifying a particular interest proteins which might be an important antigens and/or immunogens, and tried to connect the amino acid information with genome sequence of the pathogen.

Two-DE has become the method of choice for the micropreparative purification of proteins for subsequent amino acid characterisation expressed by the genome of an organism (15,20, 23). Two-DE was established to carry out *H. pylori* protein analysis.

At initial stage to establish 2-DE, sample preparation was paramount to obtain clear spots at silver staining gels. The role of sample buffers is involved in solubilisation, denaturation and reduction to completely break up the interaction between proteins (34). In historical, the principle of an isoelectric focusing separation followed by polyacrylamide electrophoresis was first published in 1969 by Righetti *et al.* however, a truly successful 2-DE method required the development of an effective sample preparation procedure by Klose (19) and O'Farrell (30) in 1975. Urea solubilizes and denatures proteins, unfolding them to expose internal ionizable amino acids. Detergent solubilizes hydrophobic proteins and minimizes protein aggregation. The detergent must have zero net charge-use only non-ionic and zwitterionic detergents

(33). Reductant cleaves disulphide bonds to allow proteins to unfold completely. DTT or DTE (20 to 100 mM) is commonly used (25). IPG Buffer (carrier ampholyte mixture) can improve separations and sample solubility, particularly with high sample loads. IPG Buffers for each pH range are a mixture of carrier ampholytes that enhances sample solubility and produces more-uniform conductivity across the pH gradient during IEF without affecting the shape of the gradient (Bio-Rad recommendation). IPG Buffers are also specially formulated not to interfere with silver staining. Tracking dye (bromophenol blue) provides a monitor for IEF progress at the beginning of the protocol. If the tracking dye does not migrate toward the anode, no current is flowing. The solubilised sample should be subjected to as minimum handling as possible and kept cold at all times (10). Many trial and errors were made to develop an appropriate sample buffer at this laboratory, and the sample buffer was aimed to get as many spots as possible. The sample buffer contained high molar urea and reductant concentration, because *H. pylori* was suspected to have rather strong bacteria membrane.

The other important steps in 2-DE were isoelectric focusing (IEF) and visualisation stages by silver staining of the gel. The conditions of IEF, especially on voltage of time, the recommendation of Protein IEF Cell (Bio-Rad), was adjusted by changing from 20,000 to 30,000 in order to obtain distinct spots. As a visualisation method, commercial kit was purchased and tried, but it was identified not to be compatible. Another advantage of the present silver staining method was that this procedure was compatible with following steps in proteomics.

2-DE was able to evaluate both molecular weight and p*I* at the same time. In the first dimension, the proteins are separated according to their charges and in the second dimension, according to their molecular masses. Moreover, either dimension is capable of resolving 100-200 protein species. Up to approximately 10,000 individual protein species have been resolved in a single gel (19), which is similar magnitude to the estimated number of expressed proteins in a eukaryotic cell (6) or bacterium (32). However, the limits of small size IPG strips (7 cm) and Mini Portein Cell were not allowed to separate such large numbers of proteins. The small scale was made to separate approximately 110-130 spots for *H. pylori* which was counted with PDQUEST program (Bio-Rad). It is difficult to understand why most of spots were placed less than 100 kDa molecular weight and over 5.6 as p*I* value. This seemed to be similar patterns of other reports (17,26,27) and was regarded as one of *H. pylori* characteristic.

The vertical streaking was, at first, observed on silver stained gel used pH 3-10 IPG strips, a variety of components in the sample buffer was suspected as the causative chemicals, such as salt concentration, denaturants, detergents, DNA, reductant and buffer or carrier ampholytes. Later, different pH range strips (pH 4-7) were employed, the streakings disappeared. So the vertical streaking was proved to be arisen from over loading of proteins to the pH 3-10 IPG strips, because the p*I* value of most *H. pylori* proteins were accumulated between p*I* 5.5 to p*I* 7.0 range, on the contrary the p*I* range of the strip was very narrow compared with pH 4-7 IPG strips.

In order to evaluate the accumulation of protein profile patterns at p*I* 5.5 to 7.0, two strains, 92-48-1 and 219-1 (data not shown), were examined using strips which had narrow range of pH value, pH 4 to 7. The narrow range pH value strips were produced several horizontal streakings which were not found on broad range strips. These vertical streakings were only followed more or less large isoform spots, Mr 43.2, Mr 56.6 and Mr 64.6, the streakings might in turn be related with either breakdown of the large spots or over loading of sample proteins. The rest of spots in narrow pH range

appeared very distinctive spots compared to broad range pH values, these facts supported that the proteins were placed at the appropriate pH compared with broad range strips.

2-DE in combination with protein spot analysis with mass spectrophotometer (MS) has been known a powerful tool in studying proteomics. As a complete proteomics, a spot analysis at the level of amino acid will enable a protein to explore the relation between genome and proteome. At present study, even though 7 cm IPG strips and Mini-Pprotein Cell system was employed, it was sufficient to prove the value of proteomics. This study was performed as the first step to employ large size strips and gels. Further study is carrying on analysing peptide fingerprinting for individual spots from 2-DE results using matrix-assisted laser desorption/ ionisation-time of flight MS (MALDI-TOF MS).

Acknowledgements

This study was supported by research grants (96-, 97- and 98-NF-04-03-A-01) from National R&D Program of the Ministry of Science and Technology and in part by Grant from Brain Korea 21 project of Education (Tae-Sung Jung, Jae-Young Song, Beong-Sam Jeon, Yeo-Jeong Choi, Sun-Ae Jung, Sang-Haeng Choi, Seong-Gyu Park, Mi-Young Choi, Jeong-Won Park, Byung-Sang Lee, Eun-Young Byun) Korea.

REFERENES

1) Ahn YO, Park BJ, Yoo KY, Kim NK, Heo DS, Lee JK, Ahn HS, Kang DH, Kim H, Lee MS, Park TS: Incidence estimation of stomach cancer among Koreans. *J Kor Med Sci* **6:** 7-14, 1991.
2) Alm RA, Ling LS, Moir DT, King BL, Brown ED, Doig PC, Smith DR, Noonan B, Guild BC, deJonge BL, Carmel G, Tummino PJ, Caruso A, Uria-Nickelsen M, Mills DM, Ives C, Gibson R, Merberg D, Mills SD, Jiang Q, Tayler DE, Vovis GF, Trust TJ: Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen *Helicobacter pylori. Nature* **397:** 176-180, 1997.
3) Axon AT: *Helicobacter pylori* therapy: Effects on peptic ulcer disease. *J Gastroenterol Hepatol* **6:** 131-137, 1991.
4) Baik SC, Kim JB, Cho MJ, Kim YC, Park CK, Ryou HH, Choi HJ, Rhee KH: Prevalence of *Helicobacter pylori* infection among normal Korean adults. *J Kor Soc Microbiol* **25:** 455-462, 1990.
5) Blaser MJ: *Helicobacter pylori*: its role in disease. *J Infect Dis* **15:** 386-391, 1992.
6) Celis JE, Rasmussen HH, Gromov P, Olsen E, Madsen P, Leffers H, Honoré B, Dejgaard K, Vorum H, Kristensen DB: The human keratinocyte two-dimensional gel protein database (update 1995): mapping components of signal transduction pathways. *Electrophoresis* **16:** 2177-2240, 1995.
7) Censini S, Lange C, Xiang Z, Crabtree JE, Ghiara P, Borodovsky M, Rappuoli R, Covacci A: *cag*, a pathogenicity island of *Helicobacter* encodes type I-specific and disease-associated virulence factor. *Proc Natl Acad Sci USA* **92:** 14648-14653, 1996.
8) Correa P: Human gastric carcinogenesis: A multistep and multifactorial process - First American Cancer Society Award Lecture on Cancer Epidemiology and Prevention. *Cancer Res* **52:** 6735-6740, 1992.
9) Cover TL, Blaser MJ: Purification and characterization of the vacuolating toxin from *Helicobacter pylori. J Biol Chem.* **267:** 10570-10575, 1992.
10) Dunn MJ: *Gel electrophoresis of proteins.* BIOS Scientific Publishers Ltd. Alden Press, Oxford 1993.
11) Dooley CP, Cohen H, Fitzgibbons PL, Bauer M, Appleman MD, Perez-Perez GI, Blaser MJ: Prevlaence of *Helicobacter pylori* infection and histologic gastritis in asymptomatic persons. *N Engl J Med* **321:** 1562-

1566, 1989.

12) Goodwin CS, Marshall BJ, Blincow ED, Wilson DH, Blackbourn S, Phillips M: Prevention of nitroimidazole resistance in *Campylobacter pylori* by coadministration of colloidal bismuth subcitrate: clinical and in vitro studies. *J Clin Pathol* **41:** 207-210, 1988.

13) Graham DY, Go MF, Evans DJJr: Urease, gastric ammonium/ammonia, and *Helicobacter pylori*-the past, the present, and recommendations for future research. *Aliment Pharmacol Ther* **6:** 659-669, 1992.

14) Heukeshoven J, Dernick R: Improved silver staining procedure for fast staining in PhastSystem Development Unit. I. Staining of sodium dodecyl sulfate gels. *Electrophoresis* **9:** 28-32, 1988.

15) Hochstrasser DF: Proteome in perspective. *Clin Chem Lab Med* **36:** 825-836, 1998.

16) IARC Monographs on the Evaluation of Carcinogenic Risks to Human, Vol 61. Lyon: World Health Organization, pp177-240, 1994.

17) Kimmel B, Bosserhoff A, Frank R, Gross Roy, Goebel W, Beier D: Identification of immunodominant antigens from *Helicobacter pylori* and evaluation of their reactivities with sera from patients with different gastroduodenal pathologies. *Infect Immun* **68:** 915-920, 2000.

18) Klose J, Kobalz U: Two-dimensional electrophoresis of proteins: an updated protocol and implications for a functional analysis of the genome. *Electrophoresis* **16:** 1034-1059, 1995.

19) Klose J: Protein mapping by combined isoelectric focusing and electrophoresis of mouse tissues. A novel approach to testing for induced point mutations in mammals. *Humangenetik* **26:** 231-243, 1975.

20) Kostrzynska M, Betts JD, Austin JW, Trust TJ: Identification, characterization, and spatial localization of two flagellin species in *Helicobacter pylori* flagella. *J Bacteriol* **173:** 937-946, 1991.

21) Langen H, Takacs B, Berndt P, Lahm HW, Wipf B, Gray C, Fountoulakis M: Two-dimensional map of the proteome of *Haemophilus influenzae. Electrophoresis* **21:** 411-429, 2000.

22) Lee WK, Cho MJ, Choi HJ, Rhee KH: Development of epidemiological method for the *Helicobacter pylori* by polymerase chain reaction. *J Kor Med Sci* **6:** 338-347, 1991.

23) Fichmann J, Westermeier R: 2-D Protein Gel Electrophoresis: An overview. *In* Link AJ(ed), 2-D proteome analysis protocols, Humana Press, Totowa, New Jersey, pp1-8, 1999.

24) Marshall T, Williams KM: Artifacts associated with 2-mercaptoethanol upon highresolution two-dimensional electrophoresis. *Anal Biochem* **139:** 502-505, 1984.

25) Marshall J, Armstrong JA, Francis GJ, Nokes NT, Wee SH: Antibacterial action of bismuth in relation to *Campylobacter pyloridis* colonization and gastritis. *Digestion* **37(suppl2):** 16-30, 1987.

26) McAtee CP, Lim MY, Fung K, Velligan M, Fry K, Chow T, Berg DE: Characterization of a *Helicobacter pylori* vaccine candidate by proteome techniques. *J Chromat* **714:** 325-333, 1998.

27) McAtee CP, Lim MY, Fung K, Velligan M, Fry K, Chow T, Berg DE: Identifi-cation of potential diagnostic and vaccine candidates of *Helicobacter pylori* by Two-Dimensional gel electrophoresis, sequence analysis, and serum profiling. *Infect Immun* **68:** 915-920, 1998.

28) McNulty CAM: The treatment of *Campylobacter*-associated gastritis. *Am J Gastroenterol* **82:** 245-247, 1987.

29) Megraud F, Brassens-Rabbe MP, Denis F, Belbouri A, Hoa DQ: Seroepidemiology of *Campylobacter pylori* infection in vari-

ous populations. *J Clin Microbiol* **27:** 1870-1873, 1989.

30) O'Farrell PH: High resolution two-dimensional electrophoresis of proteins. *J Bio Chem* **250:** 4007-4021, 1975.

31) Park CK, Lee WK, Doh YM, Choi HJ, Cho MJ, Youn HS, Rhee KH, Chang WH: Development of a diagnostic method of *Helicobacter pylori* infection: II. Detection of *Helicobacter pylori* using polymerase chain reaction. *J Kor Soc Microbiol* **27:** 45-58, 1992.

32) Pasquali C, Frutiger S, Wilkins MR, Hughes GJ, Appel RD, Bairoch A, Schaller D, Sanchez JC, Hochstrasser DF: Two-dimensional gel electrophoresis of *Escherichia coli* homogenates: the *Escherichia coli* SWISS-2DPAGE database. *Electrophoresis* **17:** 547-555, 1996.

33) Perdew GH, Schaup HW, Selivonchick DP: The use of a zwitterionic detergent in two-dimensional gel electrophoresis of trout liver microsomes. *Anal Biochem* **135:** 453-455, 1983.

34) Rabilloud T: Solubilization of proteins for electrophoretic analysis. *Electrophoresis* **17:** 813-829, 1996.

35) Ramagli LS: Quantifying protein in 2-D PAGE solubilization buffers. *In* Link AJ (ed), 2-D proteome analysis protocols. Humana Press, pp99-104, 1999.

36) Rhee KH, Youn HS, Baik SC, Lee WK, Cho MJ, Choi HJ, Maeng KY, Ko KW: Prevalence of *Helicobacter pylori* infection in Korea. *J Kor Soc Microbiol* **125:** 475-490, 1990.

37) Rhee KH, Cho MJ, Lee WK, Kim KH, Baik SC, Ryou HH, Jeon YS, Kang KH, Choi HJ, Park PS, Lee SY, Youn HS: Diagnosis of *Helicobacter pylori* infection by polymerase chain reaction using the gene of 120 KD protein of *Helicobacter pylori* as target gene. *J Kor Soc Microbiol* **29:** 41-56, 1994.

38) Rhee KH: *Campylobacter pylori* as a major determinant in hypothetical carcinogenesis of stomach cancer. *J Kor Soc Microbiol* **23:** 550-552, 1988.

39) Tomb JF, White O, Kerlavage AR, Clayton RA, Sutton GG, Fleischmann RD, Ketchum KA, Klenk HP, Gill S, Dougherty BA, Nelson K, Quackenbush J, Zhou L, Kirkness EF, Peterson S, Loftus B, Richardson D, Dodson R, Khalak HG, Glodek A, McKenney K, Fitzegerald LM, Lee N, Adams MD, Venter JC: The complete genome sequence of the gastric pathogen *Helicobacter pylori. Nature* **388:** 539-547. 1997.

40) Youn HS, Baik SC, Lee WK, Cho MJ, Ryou HH, Choi HJ, Rhee KH: Serodiagnosis of *Helicobacter pylori* infection. *J Kor Soc Microbiol* **25:** 463-474, 1990.