Molecular mass sorting of proteome using hollow fiber flow field-flow fractionation for proteomics

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**ABSTRACT**  
Hollow fiber flow field-flow fractionation (HF FIFFF) has been demonstrated as a tool for pre-fractionating proteomes by differences in molecular mass (Mr), where the resulting protein fractions are subsequently digested and analyzed by shotgun proteomics using two-dimensional liquid chromatography-electrospray ionization-tandem mass spectrometry (2D-LC-ESI-MS/MS). HF FIFFF is a separation device capable of fractionating proteins or cells by hydrodynamic radius, and protein fraction can be readily collected as intact conditions in aqueous buffer solutions. In this study, HF FIFFF was applied to fractionate the proteome of \textit{Corynebacterium glutamicum}, a well known soil bacterium that has been widely used in biotechnology due to its remarkable ability to secrete high amounts of glutamic acid. The collected HF FIFFF fractions of different MW intervals were enzymatically digested for protein identification by 2D-LC-ESI-MS/MS. Experiments showed improvements in protein identification when HF FIFFF pre-fractionation was applied, due to decreases in the ionization suppression effect and the MS exclusion effect by spectral congestion. Pre-fractionation of \textit{C. glutamicum} proteome allowed us to find 90 additional proteins by 2D-LC-ESI-MS/MS that were not found by a direct shotgun analysis without pre-fractionation. A total of 415 proteins were found overall with 203 proteins commonly found from experiments with and without pre-fractionation.

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**1. Introduction**  
Discovery of the proteins contained in a complicated proteome sample has been an important issue in proteomics research, due to the complexity of protein mixtures and the increased importance of identifying low-abundance proteins for the development of biomarkers [1,2]. The advancement of mass spectrometric methods has accelerated the possibility of characterizing complicated biological species such as proteins and their modified products, with the aid of high-speed tandem mass spectrometric measurements, as well as by ultrahigh-resolution ion detection using Fourier transformation-ion cyclotron mass spectrometry (FT-ICR MS). However, the complexity of proteomes still exceeds the resolution capabilities of mass spectrometry either by bottom-up or top-down approaches; therefore, proteome analysis still requires a suitable separation technique [1] – or a combination of separation techniques – prior to mass spectrometric analysis.

Sample complexity can be reduced at the protein level using one-dimensional or two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) [3-5], free flow electrophoresis (FFE), capillary isoelectric focusing (CIEF) [6], size exclusion chromatography (SEC) [7], and etc. While 2D-PAGE has served as an efficient tool providing a high-resolution separation of protein mixtures in two orthogonal dimensions by their differences in isoelectric point (pI) and their molecular masses (Mr), it requires long processing times and proteins are trapped in the gel matrix which makes it difficult to retrieve...
them intact. It is still difficult to identify low-abundance proteins even with the help of sophisticated nanoflow liquid chromatography-electrospray ionization-tandem mass spectrometry (nLC-ESI-MS/MS) [8,9]. FFE provides an unlimited throughput separation of proteins for preparative usage, however, the carrier ampholine solution must be removed prior to MS analysis. CIEF can be coupled to MS directly, but the removal of ampholine solution is required too. SEC can be used to isolate a target protein, but there are concerns that sample interaction with packing materials during the penetration of proteins through pores may result in the loss or dissociation of protein complexes. Sample complexity can be decreased at digested peptide levels by utilizing two-dimensional LC (2D-LC) in which separation is first carried out by charge difference via strong cation-exchange (SCX) chromatography and followed by reversed-phase LC (RPLC) [10–12]. However, the increase in sample complexity due to protein digestion into peptides, which results in the ionization suppression effect by high-abundance proteins/peptides, still remains in this method.

Flow field-flow fractionation (FlFFF) is an alternative that is ideally suited for fractionating proteins or other biological macromolecules in intact forms, since separation in FlFFF is carried out with any biological buffer solution in an open channel that is free of packing materials [13,14]. Therefore, it is capable of fractionating proteins of large molecular mass (≥10^5 Da), in which case gel electrophoresis is hardly effective, and of separating or isolating proteins which are associated with other proteins in their intact forms. Hollow fiber FlFFF (HF FlFFF) is a tubular version of FlFFF that is applicable for the separation of biological macromolecules such as proteins, DNA, and cells [15–19]. Separation in HF FlFFF is carried out in a polymeric hollow fiber (HF) membrane by balancing two orthogonal streams: axial and radial flows. The radial flow (through the fiber wall) in the HF plays a role by driving proteins toward the inner wall of the fiber membrane, leading sample components to find radial equilibrium positions where sample diffusion and the driving force of the radial flow countercbalance each other. Due to the differences in diffusion coefficients of proteins according to their sizes, larger proteins find equilibrium positions closer to the wall than smaller ones. Thus, when axial flow with a parabolic velocity profile is applied, proteins of different hydrodynamic diameters migrate with different speeds. Therefore, HF FlFFF separation can be achieved with elution according to increasing hydrodynamic diameter of proteins. The use of HF as a separation device was conceived in 1979 [20] and was first implemented in a form of FlFFF for particle separation [15,21]. HF FlFFF was later improved with the development of retention theory as well as improvement of separation resolution [22–24], and was applied to various biomolecules, such as proteins, cells, and lipoproteins [18,25–27]. Trials were conducted on off-line coupling with MALDI-MS [28], and on-line coupling with ESI-MS [29]. Microbore HF channels were implemented for separating high abundant proteins (HAP) and low abundant protein (LAP) fractions from blood serum [30]. Very recently, the technique has been applied to the development of a non-gel-based 2D (pl-Mr) separation technique, in which CIEF is coupled on-line with HF FlFFF (CIEF-HF FlFFF) for proteomics applications [31].

In this study, we utilized HF FlFFF for the pre-fractionation of a proteome sample prior to shotgun proteomic analysis. Pre-fractionation of complicated proteome mixtures can be effective in reducing sample complexity prior to analysis, which may improve the chances of finding some proteins that cannot be detected when the shotgun method is utilized for digested peptide mixtures. HF FlFFF was applied to the lysate of Corynebacterium glutamicum, a nonhazardous bacterium that is industrially important due to its remarkable ability to excrete not only high amounts of L-glutamic acids, but also diverse compounds from organic acids to proteins, anticancer drugs, and antibiotics [32–34]. Fractions of C. glutamicum proteome during HF FlFFF separation were collected and digested for shotgun identification of proteins/peptides using 2D-LC-ESI-MS/MS. Evaluations were made with and without HF FlFFF pre-fractionation by comparing the number of proteins subsequently found using the shotgun method.

2. Experiments

2.1. Materials and reagents

A sample of a wild-type strain (ATCC13032) of C. glutamicum, cultivated aerobically on a rotary shaker at 30 °C, was obtained from CJ Corp. (Seoul, Korea). Cells were washed twice in ice-cold 0.1 M phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, and 1.8 mM KH2PO4) solution at pH 7.4 and were re-suspended in lysis buffer (20 mM MgCl2, 10 mM MnCl2, and 200 units/mL DNase-I; Sigma, St. Louis, MO, USA) in 0.1 M PBS. The washed bacteria were incubated for 1 h at 37 °C and then were disrupted by ultrasonication using a model CP130 Ultrasonic Processor from Cole Parmer Instrument Co. (Vernon Hills, IL, USA) at a pulse mode with a 0.5 s interval under 15 W. Cell debris and unbroken cells were removed by two rounds of centrifugation at 5000 g for 30 min. The supernatant was diluted to 1 μg/μL in 0.1 M PBS. Protein concentration was measured by the Bradford method. The remaining protein solution was kept at 4 °C. Na2HPO4, MgCl2, MnCl2, and DNase I were purchased from Sigma. Protein standards were carbonic anhydrase (29 kDa), BSA (66 kDa), alcohol dehydrogenase (150 kDa) from yeast, and apoferritin (443 kDa) from Sigma.

2.2. HF FlFFF

The microbore HF FlFFF module (or channel) was made in our laboratory by inserting a polysulfone fiber (MWCO 30KDa) into a glass tubing with 3.2-mm OD and 1.6-mm ID (Fig. 1). The HF had 450-μm ID and 720-μm OD, and was 21 cm in length; its geometrical fiber volume was 40 μL. Both ends of the glass tube were connected to silica capillaries by means of a union at one end and a tee at the other. The detailed plumbing of a microbore HF module is explained in literature [30,31]. The carrier solution used for HF FlFFF separation of the C. glutamicum proteome and of protein standards was a 10 mM NH4HCO3 solution prepared with ultrapure water (≥18 MΩ) which was filtered before use with a membrane filter with...
0.45-μm pore size (Millipore Corp., Bedford, USA). Sample was introduced along with carrier solution to the HF channel by a model SP930D solvent delivery pump from Young-Lin Instrument (Seoul, Korea).

When sample was injected into the HF channel using a 7125 loop injector with 25-μL loop from Rheodyne (Cotati, CA, USA), the pump flow was divided into two parts with a 1:9 flow ratio using a metering valve (connected as dotted line configuration of the left side of Fig. 1). One stream (1/10 of total flow rate) entered the channel inlet through injector and the other (9/10) was directed into the channel outlet, inducing radial flow. During this process, called focusing/relaxation, sample components were accumulated at a position 1/10 of the way down the fiber. During this relaxation procedure, sample components seek equilibrium between diffusive forces (protruding from the membrane wall) and the force of radial flow (acting toward the membrane wall). After a period of time sufficient for sample delivery and focusing/relaxation, the valves were switched in such a way that the flow was directed to the fiber inlet only, beginning the separation.

During separation, flow rates in both the radial and axial directions were controlled by means of a needle valve placed at the end of the radial flow outlet. Proteins eluting from the HF module were monitored at 280 nm by a model UV730D UV detector from Young-Lin Instrument (Seoul, Korea) and the detector signals were recorded by AutochroWin software from Young-Lin. During elution, fractionated proteins were collected at intervals of 1–3 min. For protein standards, less than 1 μg of each sample was injected to demonstrate protein separation by HF FIFFF. For the C. glutamicum proteome, 15 μg of protein mixture was injected for each run.

2.3. Digestion of C. glutamicum protein fractions

For tryptic digestion, the fractionated protein solution of each fraction was concentrated to 100 μL using a SpeedVac SC110A from ThermoSavant (Holbrook, NY, USA) and re-suspended in a solution of 8 M urea, 10 mM dithiothreitol and 0.1 M PBS. Diluted proteins were incubated at 37 °C for 2 h to reduce the disulfide bond. The solution was then treated with 20 mM of iodoacetamide (IAM) in an ice bath for 2 h in the dark, to alkylate the remaining thiol group. Subsequently, in order to remove remaining IAM, excess cysteine was added to the solution in 40 times excess at room temperature, and allowed to stand for 30 min. Prior to digestion, the solution was diluted with PBS to a final concentration of 1 M urea. Proteomics-grade trypsin was prepared at 1% (w/v) in 1 M HCl and added to the solution; the weight of trypsin added was about 50 times as much as that of the target proteins. After incubation at 37 °C for 24 h, the digested protein solution was treated with TLCK (N-α-para-tosyl-L-lysine chloromethylketone hydrochloride) which was added 10 times excess to trypsin for deactivation.

![Fig. 1](https://example.com/figure1.png)
The digested protein solution was desalted using an Oasis HLB cartridge from Waters (Milford, MA, USA), and dried with SpeedVac for storage.

2.4. 2D-LC-LC-ESI-MS/MS

The nanoflow LC-ESI-MS/MS experiment was carried out using a model 1200 series capillary flow HPLC from Agilent Technologies (Waldbronn, Germany) with a LCQ Deca XP MAX ion trap mass spectrometer (Thermo Finnigan, San Jose, USA). For online salt step elution of SCX followed by RPLC separation, a dual-trap column and an analytical column were prepared in our laboratory with fused silica capillaries from Polymicro Technologies (Phoenix, AZ, USA) and were connected with a microcross (Fig. 1). Detailed procedures to prepare both capillary columns are given in the literature [12,31]. The dual trap column was prepared in-house from a silica capillary (200-μm ID, 360-μm OD) in which one end of the tubing was fitted with a sol-gel frit (prepared from a 1:4 v/v formamide and potassium silicate solution, baked at 100 °C for 3 h). The column was packed first with Magic C18AQ (5-μm, 200-Å, Michrom BioResources Inc. Auburn, CA USA) for 0.7 cm, followed by polysulfoethyl A™ SCX resin from The Nest Group Inc. (Southboro, MA, USA) for 1.8 cm. An analytical column was prepared with a silica capillary (75-μm ID, 360-μm OD) in which one end of the tubing was flame-pulled to a tip diameter of approx. 10 μm in order to be used as a self-emitter for ESI. The pulled tip tubing was packed with a methanol slurry of 5-μm, 100-Å Magic C18AQ RP resins at a constant pressure (1000 psi) of He. The dual trap and analytical column were connected via a PEEK microcross from Upchurch Scientific, Inc. (Oak Harbor, WA, USA) in which one end was equipped with a Pt wire as an electrode for supplying an electrospray ionization voltage of 2.0 kV [12,35].

The procedure for 2D-LC-ESI-MS/MS runs is as follows. Digested peptide mixtures of each FIFFF fraction were loaded to the dual trap in Fig. 1 from the autosampler connected with the binary pump. When the mobile phase A (CH₃CN/H₂O/HCOOH, 2/97.9/0.1%) delivered the sample to the dual trap at 3 μL/min, the split outlet was blocked with the vent open (the six-port valve configuration shown in the right side of Fig. 1). After sample loading, a breakthrough RPLC run was carried out.

![Fig. 2 - FIFFF separation of protein standards (upper fractogram) and of C. glutamicum proteome (bottom) obtained at the same run condition: outflow/radial flow rate = 0.06/0.54 in mL/min. Protein standards are carbonic anhydrase (29 kDa), BSA (66 kDa), alcohol dehydrogenase (150 kDa), and apoferritin (443 kDa).](image1)

![Fig. 3 - Comparison of nLC-ESI-MS base peak chromatograms of peptides obtained after each breakthrough nLC-ESI-MS/MS run (before the salt step elution began) with/without pre-fractionation of C. glutamicum proteome: a) the digested sample of HF FIFFF fraction 4 and b) the whole digested peptide mixtures.](image2)
by turning the six-port valve 60° so that pump flow was divided at the tee to a desired flow rate (200 nL/min), which was directed to the analytical column. In this configuration, the vent valve at the four-way microcross was closed so that split flow was directed to the main analytical column. A binary gradient began, with an increase to 32% of mobile phase B (95/5/0.1 = CH3CN/H2O/HCOOH) over 105 min, and a ramp to 80% B over 3 min for column washing. After 10 min at 80% B, the gradient was returned to 100% A over 2 min, and held at 100% A for at least 20 min for column re-conditioning. After the breakthrough run, the six-port valve was returned to its original position, and the first salt step elution was carried out by delivering 10 μL of 4 mM NH4HCO3 solution—which was contained in an autosampler microvial—to the dual trap. In this case, the salt solution was delivered only as a plug of solution (10 μL) using the autosampler, and some peptides desorbed from the SCX trap were expected to be trapped at the C18 trap right next to the SCX resins. During this salt delivery step, the valve configuration was adjusted so that salt solution could exit through the microcross without passing through the analytical column. Salt step elution from SCX and continuous loading onto the C18 trap, followed by washing away salt solution with mobile phase A, were

Fig. 4 – Effect of ionization suppression on the detection of peptide ions at precursor scans during each breakthrough nLC-ESI-MS/MS run with/without pre-fractionation: a) precursor scan at 53.1 min of Fig. 4a of digested peptide mixtures from FIFFF fraction 4 and b) at 54.2 min of Fig. 4b of peptide mixtures without pre-fractionation.

Fig. 5 – MS/MS spectrum of the peptide ion ([M+2H]+, m/z = 816.2) detected in Fig. 4a., which was later identified as R.IGVGFTQALRD from porphobilinogen deaminase.
continuously achieved, taking 10 min in total at 3 μL/min. The salt step elution was made by varying concentration of ammonium bicarbonate solutions between 4, 15, and 1000 mM. After the first salt step elution, the six-port valve position was switched to the run mode again, to carry out a gradient RPLC run using the same conditions as used for the breakthrough run. This procedure (salt step elution followed by gradient RPLC run) can be repeated as many times as necessary by changing the salt concentration.

Nanoflow LC effluent was directly fed into the ion trap MS via ESI at a voltage of 2.0 kV; the flow rate was maintained at 200 nL/min. Peptide ions were detected with an MS precursor scan (300–1800 amu) followed by three data dependent MS/MS scans (35% normalized collision energy).

2.5. Data processing

For protein identification, the collected raw MS/MS spectra were processed by the TurboSEQUEST search program from Thermo Finnigan (San Jose, CA, USA) using NCBI database. The mass tolerance between the measured monoisotopic mass and the calculated mass was 1.0 u for the molar mass of a precursor peptide and 1.0 u for the mass of peptide fragment ions. For screening the data, only peptides yielding minimum delta-

Fig. 6 – Effect of spectral congestions on precursor scan mass spectra obtained from each chromatogram obtained at a same salt step elution condition (1000 mM NH₄HCO₃). Precursor scans were obtained during time intervals of a) 50.0–51.7 min of nLC-ESI-MS/MS run for the HF FIFFF fraction 6 and b) 50.4–51.7 min for the whole mixtures. The peptide ion ([M+2H]+², m/z=1187.3) was detected in both precursor scans (see enlarged MS spectrum in scan b), but CID of the ion was not accomplished in run b.
correlation (ΔCn) scores of 0.1 and cross-correlation (Xcorr) values larger than 2.0, 2.5, and 3.3 for singly, doubly, and triply charged ions, respectively, were selected for extensive homology.

3. Results and discussion

HF FlFFF separation of C. glutamicum proteome was made at a run condition that was established with protein standards. The upper plot in Fig. 2 shows the HF FlFFF separation of proteins standards by the differences of MW. As expected from the retention time scale of protein standards, the major peak (around 2 min) of the lower fractogram (from C. glutamicum proteome) appeared to be smaller than 30 kDa and some large MW species (ca. 150 kDa) eluted until 15 min. During elution, proteins were collected at time intervals marked with broken lines in Fig. 2, and each collected fraction was digested in solution with trypsin for 2D-LC-ESI-MS/MS analysis.

The main purpose of pre-fractionating proteins at different MW regimes in this study was to demonstrate that pre-fractionation by FlFFF helps to reduce the complexity of proteome samples, which can result in identification of additional proteins that are not found by a direct shotgun analysis. For comparison, shotgun analysis of original C. glutamicum proteome digests without pre-fractionation using HF FlFFF was made with 2D-LC-ESI-MS/MS. When 10 μg of the whole peptide mixtures was loaded onto SCX section of the dual trap, salt step fractionations were carried out at three different concentrations (4, 15, and 1000 mM NH4HCO3) by delivering a plug of salt solution from the autosampler. After each salt step elution, a binary gradient capillary RPLC separation was conducted. Including a breakthrough LC run, which was the first LC run to analyze peptides that were not loaded to SCX resin but retained at C18 resin of the dual trap, four LC-ESI-MS/MS runs were carried out for digested peptides from C. glutamicum.

Pre-fractionation of C. glutamicum proteins using HF FlFFF followed by off-line shotgun nLC-ESI-MS/MS resulted in successful identification of some peptides where direct shotgun analysis failed to identify the same peptide. The two BPCs (base peak chromatograms) shown at Fig. 3 were obtained by nLC-ESI-MS/MS of peptide mixtures with and without pre-fractionation of proteins; a) the digests of HF FlFFF fraction 4 and b) the whole digested mixtures, both were obtained at each breakthrough nLC-ESI-MS/MS run. Both chromatograms in Fig. 3 showed complexity of sample mixtures, but the BPC b appeared to be crowded more than BPC a in terms of population of peaks. When MS precursor scan results were compared, a serious difference in ionization of the same ions was observed. An example showing an ionization suppression effect due to a highly abundant species is demonstrated in Fig. 4 with a peptide ion m/z = 816.2 ([M+2H]+2). The same ions were detected at 54.2 min from the digests of fraction 4 in Fig. 3a and at 53.1 min from the whole digested mixtures in Fig. 3b. The retention time difference between the two runs was due to the difference in capillary columns used. While the ion intensity (7.04 × 107) of the peptide ion m/z = 816.2 in Fig. 4a was second highest among other ions in the specific precursor run of the digests of pre-fractionated sample, MS intensity (4.20 × 107) of the same ion in Fig. 4b was not sufficiently strong to be selected for the MS/MS experiment compared to the other ions detected in the same scan. Due to the ion suppression effect from more abundant ions, such as m/z 1223.3 (peak intensity of 8.30 × 108), m/z 956.0 (7.50 × 108) and etc, ionization of m/z 816.2 was suppressed to levels similar to the peak intensities of numerous ions detected together. The peptide ion m/z = 816.2 in Fig. 4a appears with a peak intensity that is 67% (7.4 × 107) larger than that observed in Fig. 4b, though the intensity of the m/z 1223.3 ion in Fig. 4a was reduced as low as 40% of the ion intensity in Fig. 4b. It can be thought that ionization of the peptide (m/z = 816.2) in Fig. 4a was not strongly interfered with other ions due to the simplification of sample complexity by pre-fractionation with HF FlFFF, while it was strongly influenced in Fig. 4b by other abundant ions (m/z = 956.0, 1141.4, 668.8, etc) having larger peak intensities than the peptide ion. Thus, collision induced dissociation (CID) experiment of m/z 816.2 was successfully carried out in Fig. 4a, but it was not in Fig. 4b. Fig. 5 showed the CID spectra of the peptide ion m/z = 816.2 ([M+2H]+2) which was identified as R.IGVGVTQAL.R from porphobilinogen deaminase. While 5 other peptides belong to the same protein were found from the nLC-ESI-MS/MS runs of HF FlFFF fractions 3 and 4, the same protein (porphobilinogen deaminase) was not identified at all from the peptide mixtures without pre-fractionation. The high-abundance peptide ions of m/z 1223.3 and 956.0 were identified as K.ENNADVQVYTVEPL.A from cysteine synthase and K.SLGSNAINVHATVDGLK.Q from ribosomal protein S5, respectively. While the protein cysteine synthase was found in both sample mixtures, the latter protein was only found in the peptide mixtures without pre-fractionation.

In addition to the common ion suppression effect, there is a possibility of missing CID experiment due to the spectral congestion. Fig. 6 showed an evidence of missing CID

| Table 1 - a) Number of proteins of C. glutamicum found from each salt step elution followed by nanoflow LC-ESI-MS/MS and b) number of proteins found from each FlFFF fraction followed by 2D shotgun analysis |
|---------------|-------------------------------|-------------------------|-----------------|---------------------------|
|                | a) Without FlFFF              | b) With FlFFF            |                |                           |
|                | Concentration of salt step    | Fraction # of HF FlFFF   |                |                           |
|                | 0 mM  4 mM  15 mM  1000 mM    | Total                    | No. of proteins found | No. of unique proteins   |
| No. of proteins found | 270  127  89  164            | 325                      | 157            | 92  135  130  136  76    |
| No. of unique proteins | 270  22  4  29              |                           | 157            | 31  55  22  17  11  293  |
| No. of unique proteins not identified by direct shotgun analysis | 10  17  30  14  11  8  90 |                           |                           |

The complete protein list is available as supplementary information on the journal website.
peptide ion m/z=1187.3 ([M+2H \]^2+\) was detected in both 51.7 min of the whole mixtures in Fig. 6b. For example, the scan was extracted from each BPC at 50.0 the whole sample without pre-fractionation. Each precursor precursor scans of the peptide digests of HF FlFFF fraction and the whole digested mixture sample in Fig. 6b. The MS/MS Fig. 6b); however, CID of the same ion was not achieved with the peptide sequence from the database as R. GDFALTVGEN-
VVHGSDSPESAER.E, from nucleoside diphosphate kinase. In both precursor scans of Fig. 6 (as shown with enlarged view at Fig. 6b); however, CID of the same ion was not achieved with the whole digested mixture sample in Fig. 6b. The MS/MS experiment of the m/z 1187.3 resulted in an identification of the peptide sequence from the database as R. GDFALT-VGEN-VVVHGSDSPESAER.E, from nucleoside diphosphate kinase. Since the intensities of observed peptide ions (m/z=1187.3) in both precursor runs are not significantly different from each other (4.7×10^6 and 3.9×10^6 for MS spectra of a and b of Fig. 6, respectively), the peptide ion in Fig. 6b is thought to be excluded for CID due to spectral congestion.

Table 1 shows a comparison of the number of proteins of C. glutamicum found with and without pre-fractionation. When the direct shotgun 2D-LC analysis was used, the total number of proteins found from each salt step was 325 (Table 1a). When pre-fractionation of proteome sample was applied using FFIF and the 2D-LC-ESI-MS/MS analysis was carried out for the digested peptides of FFIF fractions, a total of 293 proteins were found from the six fractions (see Table 1b). Among 293 proteins found, only 203 proteins matched with those found with direct 2D-LC-ESI-MS/MS analysis; the other 90 proteins (approximately 30%) were unique. The protein numbers found in the off-line combination of HF FlFFF separation takes place in most buffer solutions, con-
formation of proteins that are associated with other proteins can be preserved while the separation is carried out.

experiment due to the spectral congestion during precursor scan. The corresponding BPCs (not shown here) were obtained at a same salt step elution condition (1000 mM NH₄HCO₃) with precursor scans of the peptide digests of HF FFIF fraction and the whole sample without pre-fractionation. Each precursor scan was extracted from each BPC at 50.0–51.7 min of the peptide digests of HF FFIF fraction 6 in Fig. 6a and at 50.4–51.7 min of the whole mixtures in Fig. 6b. For example, the peptide ion m/z=1187.3 ([M+2H]^2+) was detected in both precursor scans of Fig. 6 (as shown with enlarged view at Fig. 6b); however, CID of the same ion was not achieved with the whole digested mixture sample in Fig. 6b. The MS/MS experiment of the m/z 1187.3 resulted in an identification of the peptide sequence from the database as R. GDFALT-VGEN-VVVHGSDSPESAER.E, from nucleoside diphosphate kinase. Since the intensities of observed peptide ions (m/z=1187.3) in both precursor runs are not significantly different from each other (4.7×10^6 and 3.9×10^6 for MS spectra of a and b of Fig. 6, respectively), the peptide ion in Fig. 6b is thought to be excluded for CID due to spectral congestion.

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formation of proteins that are associated with other proteins can be preserved while the separation is carried out.

Table 2 – Comparison of number of proteins found from 2D-LC-ESI-MS/MS with and without HF FlFFF pre-fractionation; number of unique proteins that are not commonly found in results from both methods

<table>
<thead>
<tr>
<th></th>
<th>Without HF FlFFF</th>
<th>with HF FlFFF</th>
<th>Total</th>
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<tbody>
<tr>
<td>Total number of proteins found</td>
<td>325</td>
<td>293</td>
<td>415</td>
</tr>
<tr>
<td>Number of proteins found commonly from both methods</td>
<td>203</td>
<td>203</td>
<td>203</td>
</tr>
<tr>
<td>Number of unique proteins found</td>
<td>122 (38%)</td>
<td>90 (31%)</td>
<td>212</td>
</tr>
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</table>

m/z=1187.3 ([M+2H]^2+) was detected in both 51.7 min of the whole mixtures in Fig. 6b. For example, the scan was extracted from each BPC at 50.0 the whole sample without pre-fractionation. Each precursor precursor scans of the peptide digests of HF FlFFF fraction and the whole digested mixture sample in Fig. 6b. The MS/MS Fig. 6b); however, CID of the same ion was not achieved with the peptide sequence from the database as R. GDFALT-VGEN-VVVHGSDSPESAER.E, from nucleoside diphosphate kinase. Since the intensities of observed peptide ions (m/z=1187.3) in both precursor runs are not significantly different from each other (4.7×10^6 and 3.9×10^6 for MS spectra of a and b of Fig. 6, respectively), the peptide ion in Fig. 6b is thought to be excluded for CID due to spectral congestion.

### 4. Concluding remarks

In this report, it is demonstrated that HF FlFFF can be employed in proteomics research as a tool for sorting proteins by MW prior to shotgun analysis of digested peptides. Size sorting of C. glutamicum proteome by HF FFIF provides an opportunity to find peptides/proteins which are not found by direct shotgun 2D-LC-ESI-MS/MS, due to its reduction of the ionic suppression effect from co-eluting high-abundance peptide species or of exclusion in MS/MS experiments due to spectral congestion. For C. glutamicum, the test species in this study, pre-fractionation by HF FFIF allowed us to identify 90 additional proteins that were not found by 2D-LC-ESI-MS/MS analysis of the whole peptide digests. MW fractionation of proteins by HF FFIF can be effectively employed when a selective isolation of specific target proteins is needed. Since FFIF separation takes place in most buffer solutions, con-
formation of proteins that are associated with other proteins can be preserved while the separation is carried out.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jprot.2008.02.001.

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