

Research Article

The Utilization of Triton X-100 for Enhanced Two-Dimensional Liquid-Phase Proteomics

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One of the main challenges in proteomics lies in obtaining a high level of reproducible fractionation of the protein samples. Automated two-dimensional liquid phase fractionation (PF2D) system manufactured by Beckman Coulter provides a process well suited for proteome studies. However, the protein recovery efficiency of such system is low when a protocol recommended by the manufacturer is used for metaproteome profiling of environmental sample. In search of an alternative method that can overcome existing limitations, this study replaced manufacturer's buffers with Triton X-100 during the PF2D evaluation of *Escherichia coli* K12. Three different Triton X-100 concentrations—0.1%, 0.15%, and 0.2%—were used for the first-dimension protein profiling. As the first-dimension result was at its best in the presence of 0.15% Triton X-100, second-dimension protein fractionation was performed using 0.15% Triton X-100 and the standard buffers. When 0.15% Triton X-100 was used, protein recovery increased as much as tenfold. The elution reliability of 0.15% Triton X-100 determined with ribonuclease A, insulin, α -lactalbumin, trypsin inhibitor, and cholecystokinin (CCK) affirmed Triton X-100 at 15% can outperform the standard buffers without having adverse effects on samples. This novel use of 0.15% Triton X-100 for PF2D can lead to greater research possibilities in the field of proteomics.

1. Introduction

The development of analytical tools for rapid analysis and identification of expressed protein profiles in cell, tissue or organism is currently an important area in biological research [1–3]. Although two-dimensional gel electrophoresis (2DE) is a classical technique that monitors and distinguishes multiple forms of proteins with differences in molecular mass or *pI* values, it can face difficulties with proteins of extreme mass (e.g., >200 kDa or <10 kDa) or *pI* values [4–6]. In addition, 2DE is not readily amenable to automation. Liquid-phase separation methods such as size-exclusion chromatography, affinity chromatography, and ion-exchange chromatography exhibited practical difficulties due to

the lack of the isoelectric (*pI*) information and limited labeling efficiency [7–9]. Alternatively, the ProteomeLab PF2D platform (Beckman Coulter, USA) that can be used for the separation/fractionation, as well as quantitative comparisons of various biological and clinical samples, works in full automation combining chromatofocusing separation and hydrophobic fractionation [10]. During the first-dimension chromatofocusing of PF2D, proteins are separated by their *pI* and separated proteins with a pH gradient are collected using a fraction collector [11, 12]. Subsequently, fractions collected from the first dimension are separated using reversed phase chromatography in the second dimension, which separates on the basis of hydrophobicity [12]. Separated fractions are monitored with UV detection to observe changes in

the proteome [13–15]. Then, the selected peak can be identified by mass spectrometry. Although PF2D system offers high loading capacity and improved detection limit with lower abundance proteins [16, 17], its protein recovery efficiency during the chromatofocusing step will be low when the standard protocol recommended by the manufacturer is used. Sheng et al. [18] reported that the inclusion of 20% isopropanol in the isoelectric focusing (IEF) buffer increased the number of proteins they could identify in the serum. They demonstrated improved recovery of protein, but purified BSA was used instead of complete serum. This buffer's ability to improve the recovery of all proteins thus remains unclear.

The columns used with PF2D require the use of nonionic detergents such as Triton X-100 for the separation of proteins. Triton X-100 is a low-cost mixture of octylphenol ethoxylates, with an average of about 9–10 ethylene oxide units per molecule. In search of an alternative method that can increase the recovery of a wide range of proteins, this study modified the standard protocol using Triton X-100. Buffers recommended by Beckman Coulter's ProteomeLab PF2D protocol was replaced by Triton X-100 during protein profiling of *Escherichia coli* K12, in which its recovery efficiency was determined at various Triton X-100 concentrations. Subsequently, the elution accuracy of Triton X-100 at its optimized condition was confirmed by the control protein mixture of ribonuclease A, insulin, α -lactalbumin, trypsin inhibitor, and cholecystokinin (CCK).

2. Materials and Methods

2.1. Sample Preparation. The eligibility of Triton X-100 as a replacement for buffers used for ProteomeLab protein liquid chromatography was determined with *Escherichia coli* K12 substrain W3110 and control mixture of proteins (ribonuclease A, insulin, α -lactalbumin, trypsin inhibitor, and cholecystokinin (CCK)). *E. coli* K12 was cultivated at 37°C in 200 mL LB medium (1% Bacto tryptone, 0.5% Bacto yeast extract, 0.5% NaCl, pH 7.2), to an OD₆₀₀ of 1.5. The cells were harvested by centrifugation at 6000 ×g for 10 min at 4°C and washed in 10 mM Tris-HCl buffer (pH 8.0). After being resuspended in 10 mL of ice-cold lysis buffer (7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 65 mM dithiothreitol (DTT), 1.25 mM protease inhibitor), cells were sonicated on ice 30 times for 10 s each. Obtained cell lysates were centrifuged at 6000 ×g for 10 min at 4°C, and the final protein concentration in the supernatant was determined using the Bradford protein assay (BioRad, USA). 3.0 mg protein aliquots were stored at –70°C until the next use. ProteomeLab PF2D Protein Test Mix, which included 5 proteins (ribonuclease A, insulin, α -lactalbumin, trypsin inhibitor, and cholecystokinin (CCK)), was purchased from Beckman Coulter (CA, USA).

2.2. Liquid Chromatography. Before the chromatofocusing, cell extracts were exchanged to various start buffers using a PD-10 column (GE Healthcare Life Sciences, USA), and

the first 3.5 mL fraction was collected. While the start buffer included in the ProteomeLab kit was designated as the “Start Buffer A,” “Start Buffer B, C, and D” were prepared with the following: 0.1%, 0.15%, and 0.2% Triton X-100 in distilled water (EMD Chemicals, Inc., USA) at pH 8.4 for “B,” “C,” and “D,” respectively; 6 M urea; 25 mM Bis-Tris; 1 M ammonium hydroxide. Protein concentration was estimated using Quant-iT Protein Assay Kits (Invitrogen, USA). All samples were diluted with each start buffer to obtain a final protein concentration of 1.5 mg/mL, and 2 mL of *E. coli* protein was injected into the chromatofocusing column. All protein samples were filtered through 0.2 μ m PES Membrane filters (Millipore, USA). The chromatofocusing was performed using the ProteomeLab PF2D (Beckman-Coulter, USA) with an HPCF-1D column (250 mm × 2.1 mm, Eprogen, USA) that was loaded with each starting buffer (pH 8.5 ± 0.1) for 120 min. Each starting buffer was then equilibrated to the initial pH 8.5, and protein sample was loaded at a flow rate of 0.2 mL/min for 45 min. The protein sample elution was initiated with a linear gradient of various elution buffers (pH 4.0 ± 0.1) that took ~60 min to complete. Eluent buffer included in the ProteomeLab kit was labeled as “Eluent Buffer A.” “Eluent Buffer B, C, and D” were prepared with the following: 0.1%, 0.15%, and 0.2% Triton X-100 in distilled water at pH 4.0, respectively; iminodiacetic acid; 6 M urea; 10% v/v polybuffer 74 (GE Healthcare, USA). Proteins were eluted and collected by their isoelectric point (*pI*) in 4.0–8.5 range with 0.2 pH intervals into a 96-deep-plate well using the FC/I module. Remaining protein samples were finally eluted by washing the column with 1 M NaCl for 40 min. The column was then rinsed with 10 column volumes of distilled water before the next sample injection. The entire chromatofocusing step was operated at 20°C with a flow rate of 0.2 mL/min, and elution profiles were monitored at 280 nm by Beckman 166 UV detector (Beckman Coulter, USA). And the second-dimension separation was performed using HPRP column (33 mm × 4.6 mm, 1.5 μ m nonporous ODS-IIIE C18 silica beads, Eprogen, USA) at 50°C with a flow rate of 0.75 mL/min. A 200 μ L from the first chromatofocusing fraction was injected into the column and eluted with a 0–100% linear gradient of solvent A (0.1% w/v TFA in distilled water) and solvent B (0.08% w/v TFA in acetonitrile) for 35 min. At the end of second-dimension run, the column was equilibrated with an initial mobile phase for 10 column volumes. Proteins were detected by a Beckman 166 UV detector (Beckman Coulter, USA) at 214 nm. Protein profiles obtained using UV detection were analyzed by ProteoVue 2D (Beckman Coulter, USA).

2.3. Determining Elution Reliability of 0.15% Triton X-100 during PF2D. To investigate the elution accuracy of 0.15% Triton X-100, the mixture of five proteins purchased from Beckman Coulter (USA) was injected into the HPCF-1D column that allows the elution of proteins in pH range of 4.0–8.5. The first- and second-dimension protein separations were achieved following the standard procedure described in Section 2.2 using Start Buffer C (pH 8.4) and Eluent Buffer C (pH 4.0). Protein profile data obtained using UV detection

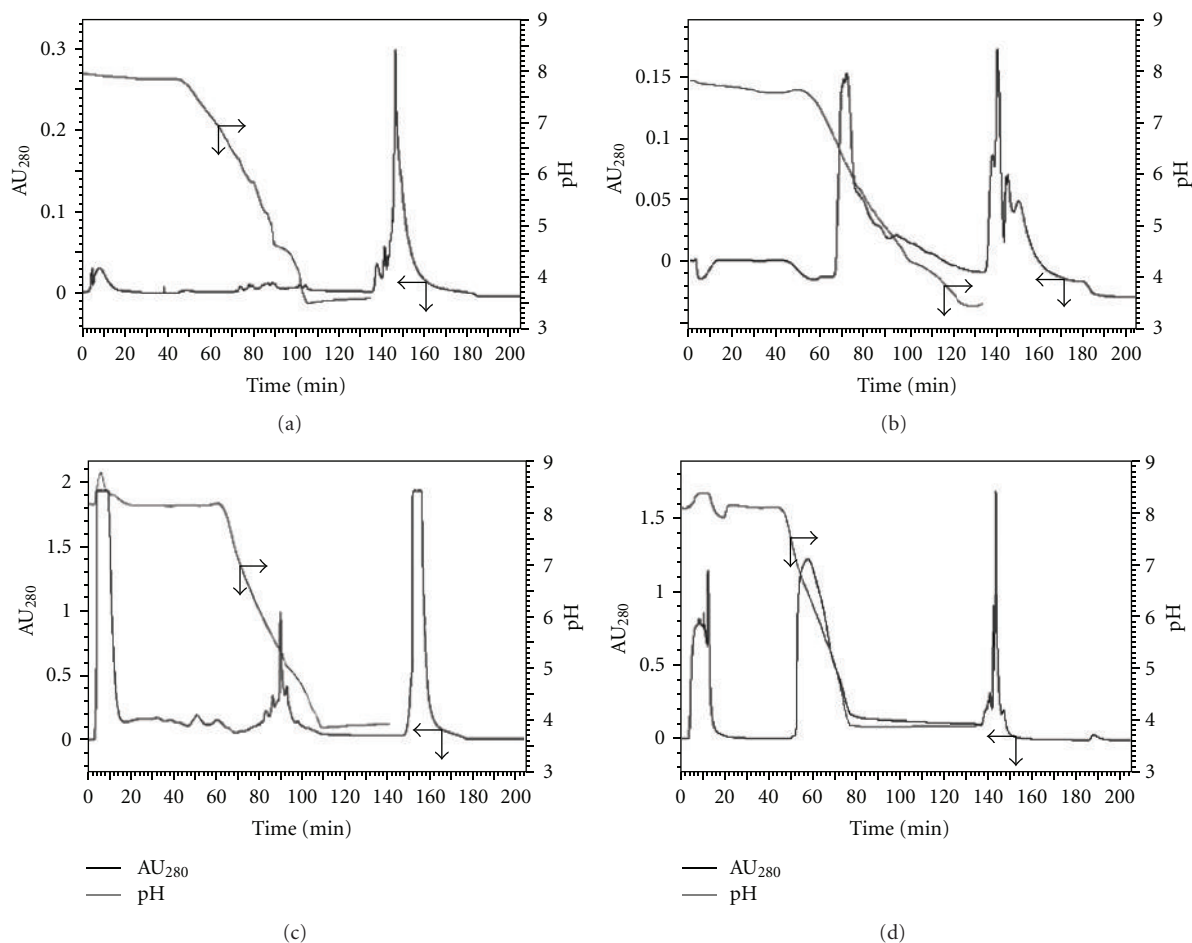


FIGURE 1: First-dimension elution profiles of *E. coli* K12 chromatofocusing analyzed on Beckman Coulter's PF2D system over 200 min using the buffer provided by the manufacture (A) or buffers prepared with Triton X-100 of various concentrations (0.1, 0.15, or 0.2% for B, C, and D, resp.). The elution profiles were monitored at 280 nm.

were collected and analyzed by ProteoVue 2D software (Beckman Coulter, CA, USA).

3. Results and Discussion

3.1. The Use of Triton X-100 for PF2D Chromatofocusing of *E. coli*. The eligibility and efficiency of Triton X-100 as an alternative to the buffers suggested for ProteomeLab (Beckman Coulter, California, USA) PF2D were evaluated with *E. coli*, using the standard ProteomeLab buffer as well as solutions prepared with 0.1%, 0.15%, and 0.2% Triton X-100. The first-dimension chromatofocusing separates proteins to differences in *pI* values, and the absorbance profiles created by each solution are shown in Figure 1. Proteins were eluted in the order of decreasing *pI* values, and all four solutions eluted its first peak during the first 20 min of sample loading period. As the HPCF-1D column used in this study provides limited elution efficiency with proteins whose *pI* values are in 8.5–4.0 range, the first peak corresponds to protein unbound to HPCF-1D column because of its *pI* value being greater than 8.5. The details of protein separation and pH gradient formation varied among four solutions. At first, *E. coli*

chromatofocusing was performed using the buffer suggested for Beckman Coulter's ProteomeLab platform. As shown in Figure 1(a), first unbound protein was eluted during the first ~20 min, and the pH gradient started forming at 50 min. The pH gradient which started forming at 50 min (from pH 8.1) lasted until 105 min (to pH 3.5), with a slight downward angle created at 92 min. During the pH gradient well-defined, multiple protein peaks were observed. Acidic proteins which remained in HPCF-1D column were eluted after 130 min as a result of washing the column with 1 M NaCl. Under given conditions, Beckman Coulter's standard buffer provided a well-defined protein chromatofocusing results in high resolution.

Alternatively, solutions which included 0.1%, 0.15%, and 0.2% Triton X-100 were prepared and used in place of standard buffers to perform first-dimension protein profiling of *E. coli*. When the solution that included 0.1% Triton X-100 was used the first protein peak created in ~20 min was inverted, giving a negative AU₂₈₀ reading (Figure 1(b)). This peak was followed by another inverted peak that was created during 48–58 min. When pH gradient formed during 50–130 min, proteins of low *pI* values ($5.0 > pI$) were eluted

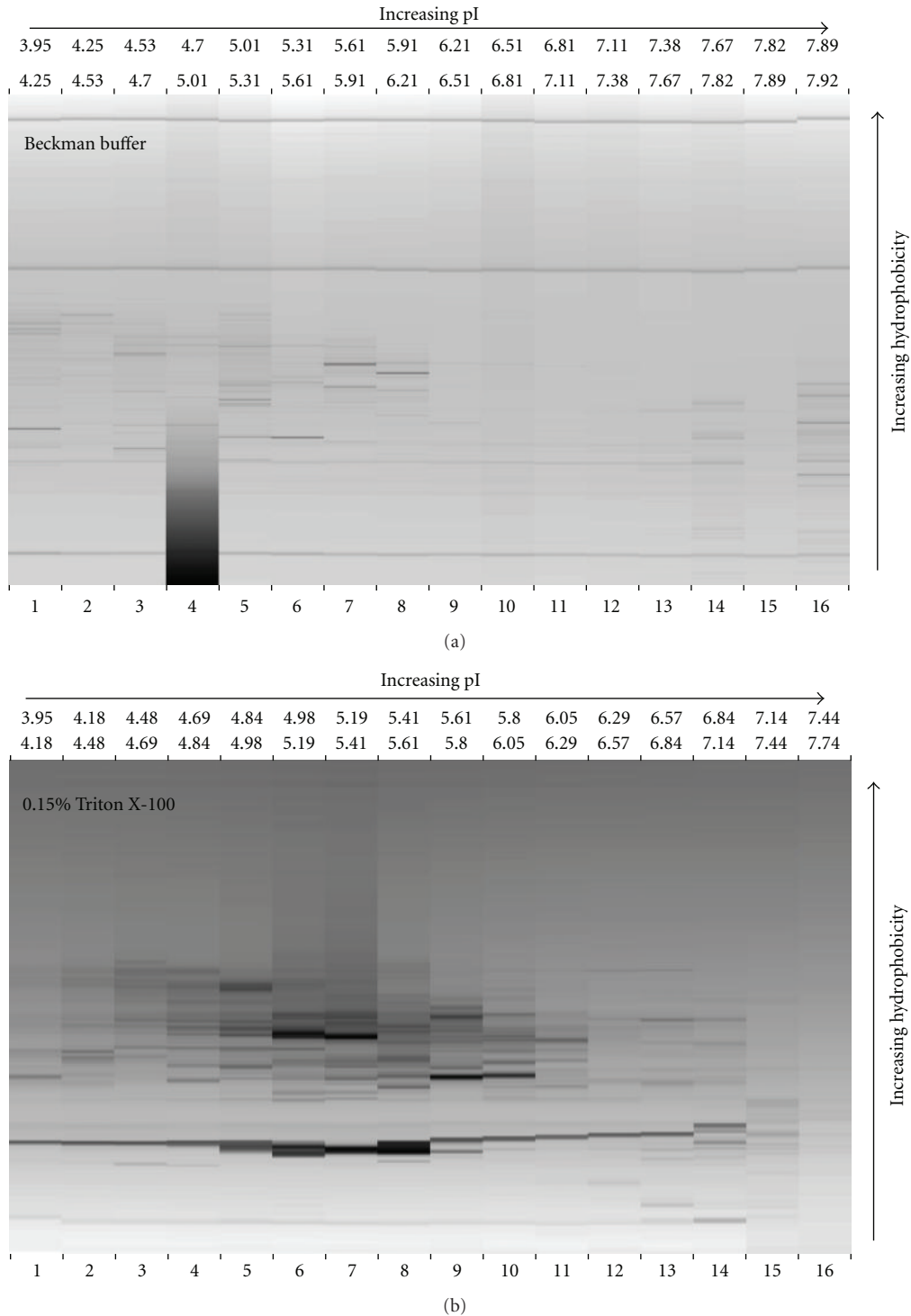


FIGURE 2: Second-dimension separations of 16 first-dimension fractions which were generated with Beckman Coulter buffer over pH 7.92–3.95 (a) and 0.15% Triton X-100 over pH 7.74–3.95 (b). The 2D fraction profiles were monitored at 214 nm and eluted in the order of increasing hydrophobicity.

indistinctively. After 130 min, remnant protein in HPCF-1D column was eluted with column washing and created a large peak area. Subsequently, the concentration of Triton X-100 was increased to 0.15%. Figure 1(c) of 0.15% Triton X-100 showed significantly improved protein profiling results:

an unbound protein was eluted at <20 min, small amounts of proteins were eluted during 20–60 min (pH 8.5–7.74) while the sample was being loaded, and a pH gradient was created during 60–110 min (pH 7.74–3.95). Unlike 0.1% Triton X-100, 0.15% Triton X-100 created a linear pH gradient,

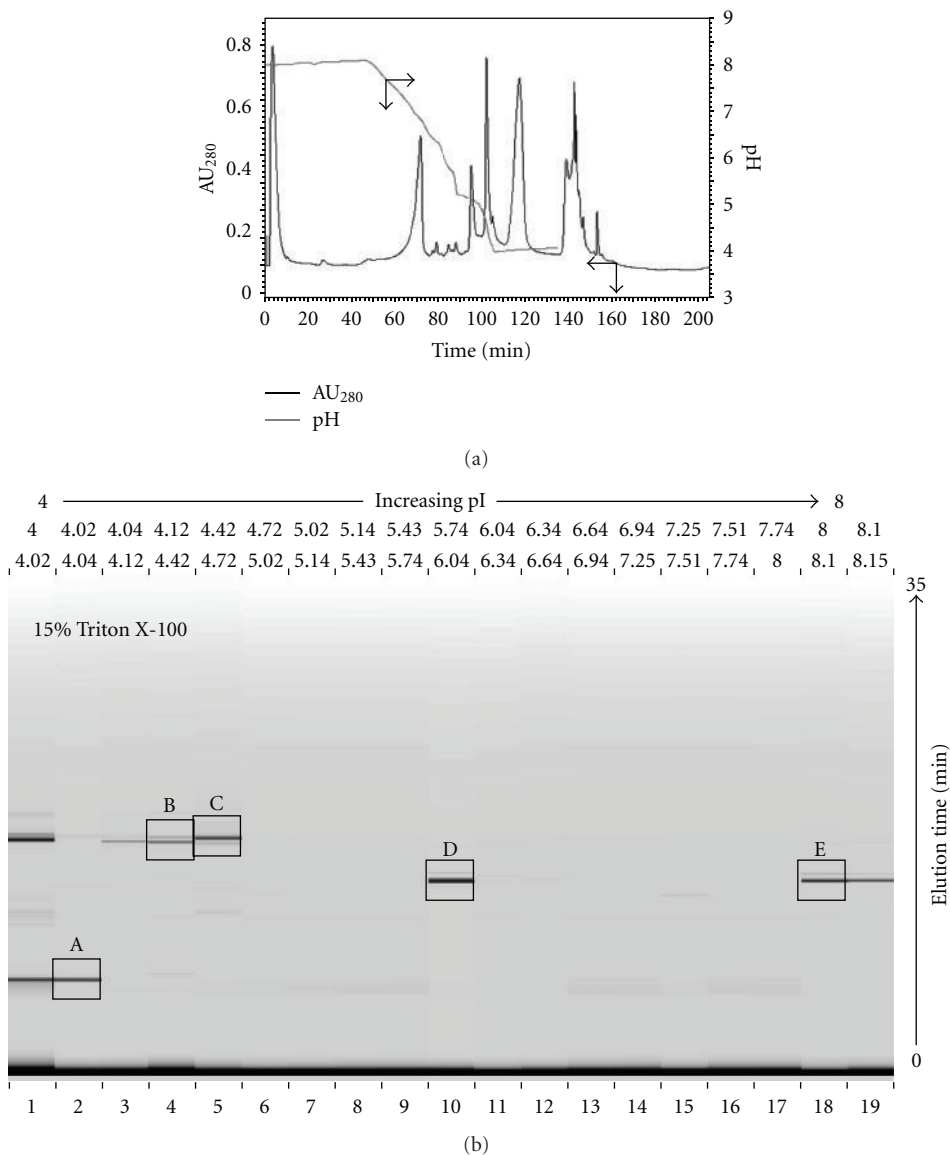


FIGURE 3: PF2D results of Beckman Coulter’s protein test mixture containing five proteins (Ribonuclease A, insulin, α -lactalbumin, trypsin inhibitor, cholecystokinin (CCK)). (A) First-dimension elution profile of five proteins generated using 0.15% Triton X-100 over 200 min. The elution profiles were monitored at 280 nm. (B) ProteoVue 2D map of five proteins having pI 8.15–4.00 eluted in the order of increasing hydrophobicity. The fraction profiles were monitored at 214 nm.

an indication that the capacity is even throughout its pH range. Chromatofocusing that took place over the pH gradient is also sharp. As with other solutions, a large protein peak appeared at 160 min. Finally, 0.2% Triton X-100 was used to determine whether increasing the concentration of Triton X-100 would bring further improvements for chromatofocusing of *E. coli*. Interestingly, after the first unbound protein was eluted at <20 min, no significant protein elution was observed until the start of the pH gradient at 50 min (Figure 1(d)). As the pH gradient for 0.2% Triton X-100 was created over the shortest time frame (during 50–80 min, pH 7.11–3.89), a high volume of proteins were eluted abruptly during pH gradient thus creating unreliable profiling data (Figure 1(d)).

The performance of each buffer was judged upon its ability to achieve a well-defined pH gradient as well as an accurate pI -based protein separation at a given pH gradient range. Indistinct chromatofocusing results and negative AU_{280} readings made 0.1% Triton X-100 inadequate for PF2D. While the performance of 0.15% Triton X-100 was comparable to that of standard buffers suggested for ProteomeLab PF2D, 0.2% Triton X-100 created a protein profile whose pI values were dubious as it happened in a shorter time frame. However, AU_{280} readings of 0.15% and 0.2% Triton X-100 were as much as 9–10 times higher than those of Beckman Coulter’s. Such high AU_{280} readings obtained using 0.15% Triton X-100 were investigated further during the second-dimension separation that was performed on 16

TABLE 1: The summary of experimental and theoretical pI intervals of five proteins included in protein test mixture purchased from Beckman Coulter.

Lane no. ^a	Protein band ^b	Protein	MW (Da)	Theoretical pI interval	Experimental pI interval ^c
2	A	CCK (cholecystokinin)	1055	<4.00	4.02–4.04
4	B	Trypsin inhibitor	20100	4.00–4.60	4.12–4.42
5	C	α -Lactalbumin	14200	4.50–5.20	4.42–4.72
10	D	Insulin	5808	5.90–6.60	5.74–6.04
18	E	Ribonuclease A	5500	>8.50	8.00–8.10

^{a,b,c} Refer to Figure 3(b).

first-dimension protein fractions generated using the standard and 0.15% Triton X-100 solution. Second-dimension of PF2D fractionates in the order of increasing hydrophobicity. As shown in Figure 2(a), 16 protein fractions in pH 7.92–3.95 obtained using Beckman Coulter buffers were fractionized, and a thick protein band indicating a large quantity of proteins was seen around pH 5.01–4.70. The second-dimension result of 0.15% Triton X-100 that began from 16 first-dimension fractions (pH 7.74–3.95) showed distinctive bands for wider pI ranges. This novel methodology that utilizes 0.15% Triton X-100 enhances protein recovery efficiency by at least tenfold.

3.2. Reliability Test Results of 0.15% Triton X-100. First- and second-dimension chromatography results shown in this study confirmed protein recovery can be increased in the presence of 15% Triton X-100 during PF2D analysis of *E. coli*, but its reliability with regard to accurate pI separation is yet to be judged without comparing the results with the standard proteins whose pI values are known. Once again, the elution profiles of five-protein test mixture (Ribonuclease A, insulin, α -lactalbumin, trypsin inhibitor, cholecystokinin (CCK)) were generated using 0.15% Triton X-100 (Figure 3(a)). The linear pH gradient was observed during 52–108 min, from pH 8.2–4.1. Multiple peaks were eluted in high resolution during its pH gradient. The accuracy of such protein elution was to be determined using the second-dimension protein chromatogram with a mixture of 5 proteins whose theoretical pI values are known (Figure 3(b)). The theoretical pI value of ribonuclease A is $pI > 8.5$ but the actual elution took over pI 8.1–8.0 (shown with the protein band E in Figure 3(b)), possibly due to a limited pH elution range set by the HPCF-1D column. The elution intervals of insulin, α -lac-talbumin, trypsin inhibitor, and CCK (shown by protein bands D, C, B, and A, resp.) were similar to their theoretical ranges. The detailed comparison of the experimental elution intervals of five control proteins with regard to theoretical values is summarized in Table 1.

4. Conclusion

Triton X-100 is a common nonionic surfactant, and the experimental results of this study affirmed that 0.15% Triton X-100 can be applied towards PF2D of a protein. Not only can 0.15% Triton X-100 greatly increase amount of protein recovery from chromatofocusing column, but it also enables

PF2D analysis of protein with low pI . Combining the beneficial qualities mentioned thus far, 0.15% Triton X-100 for PF2D system can be exploited for further analyses of metaproteome originating from various sources.

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