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Dual-purpose sample trap for on-line strong cation-exchange chromatography/reversed-phase liquid chromatography/tandem mass spectrometry for shotgun proteomics Application to the human Jurkat T-cell proteome

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Abstract

A dual-purpose sample-trapping column is introduced for the capacity enhancement of proteome analysis in on-line two-dimensional nanoflow liquid chromatography (strong cation-exchange chromatography followed by reversed-phase liquid chromatography) and tandem mass spectrometry. A home-made dual trap is prepared by sequentially packing C_{18} reversed-phase (RP) particles and SCX resin in a silica capillary tubing (1.5 cm × 200 μm I.D. for SCX, 0.7 cm × 200 μm for RP) ended with a home-made frit and is connected to a nanoflow column having a pulled tip treated with an end frit. Without having a separate fraction collection and concentration process, digested peptide mixtures were loaded directly in the SCX part of the dual trap, and the SCX separation of peptides was performed with a salt step elution initiated by injecting only 8 μL of NH_4HCO_3 solution from the autosampler to the dual trap. The fractionated peptides at each salt step were directly transferred to the RP trap packed right next to the SCX part for desalting, and a nanoflow LC–MS–MS run was followed. During the sample loading–SCX fractionation–desalting, flow direction was set to bypass the analytical column to prevent contamination. The entire 2D-LC separation and MS–MS analysis were automated. Evaluation of the technique was made with an injection of 15 μg peptide mixtures from human Jurkat T-cell proteome, and the total seven salt step cycles followed by each RPLC run resulted in an identification of 681 proteins.

Keywords: Proteomics; Liquid chromatography–mass spectrometry; Two-dimensional; Dual trap; Nanoflow liquid chromatography; Tandem mass spectrometry; Jurkat T-cells

1. Introduction

Proteome analysis requires a comprehensive use of techniques including protein/peptide separation, mass spectrometric characterization, and bioinformatics for identification. Recent advances in mass spectrometry have boosted the possibility of characterizing complex mixtures of proteins and biomolecules [1,2]. Especially, tandem mass spectrometry (MS) has become a reliable and highly selective method for characterizing peptides/proteins [3,4]. Prior to mass spectrometric analysis, complicated proteins/peptides mixtures are required to be separated or simplified by some means. Among many separation techniques available, two-dimensional electrophoresis (2DE) has been widely used to isolate proteins from mixtures based on the differences in molar mass and p*I* [5–7]. While 2DE has been extensively utilized due to the simplicity and the advantages in isolating thousands of protein spots, it usually requires a large amount of sample loading, has difficulties in handling hydrophobic proteins and in quantitation, and is labor intensive [7–9]. Recently, liquid chromatography has been combined with mass spectrometry

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through an electrospray ionization (ESI) interface to separate and characterize peptide mixtures that are digested from proteome [10–13]. In most cases of LC–ESI-MS technique, a tandem mass spectrometric analysis is used since this provides an unambiguous identification of peptides and proteins by examining a collision-induced dissociation (CID) pattern of a single peptide. However, complexity in peptide mixtures still remains a barrier toward a complete identification. This has led to the utilization of a jointed separation technique which is normally preceded by strong cation-exchange (SCX) chromatography prior to reversed-phase liquid chromatography (RPLC) in order to spread the number of peptides injected to RPLC, so called as two-dimensional LC (2D-LC), either on-line or off-line [14–22]. SCX separation is based on the difference between charges of peptides and RPLC differentiates peptides according to variations in hydrophobicity.

Two-dimensional chromatographic separations have become an effective means of resolving a complicated peptide mixture with an increased loading capacity, and have expanded the dynamic range of protein identification number through a sequential RPLC–MS–MS analysis followed by several salt step gradients to displace peptides from SCX column. The so-called MUDPIT (multidimensional analysis of proteins identification technology) on-line approach was introduced by Yates III and co-workers [15,16], and the two-dimensional separation approach in a single column improved separation resolution with an identification of about 1400 proteins out of *S. cerevisiae*. The schematic illustration of biphasic column used in MUDPIT is shown in Fig. 1a. While an off-line 2D-LC method provides advantages in obtaining a better separation using linear salt gradient, in storing collected fractions for re-examination, and in concentrating peptides of low abundant proteins by handling large amount of sample [21,22], an on-line approach still offers the merit of automation throughout the entire separation and mass analysis process. However, most on-line approaches require using separate pump systems for providing salt gradients, and they must allow for a certain period for re-equilibrium after each solvent conversion and washing.

In this study, a dual-purpose sample-trapping strategy is introduced for an on-line nanoflow LC–LC–MS–MS by sequentially packing SCX resin and the C_{18} reversed-phase packing materials into a short capillary column connected prior to an analytical column. With this method, ionic solutions of different concentrations can be delivered for salt step elution from an autosampler, and peptide fractions eluted from SCX trap transferred to the C_{18} trap packed right next to the SCX trap. After each salt step, a typical binary gradient elution (water–acetonitrile) for RPLC followed by MS–MS analysis can be carried out. This allowed us to perform multiple RPLC runs with tandem mass spectrometry for a singly injected peptide mixture using full automation. An evaluation was done with human Jurkat T-cell proteome, of which cell is widely used in creating a model system for investigations into T-cell receptor-mediated signaling.

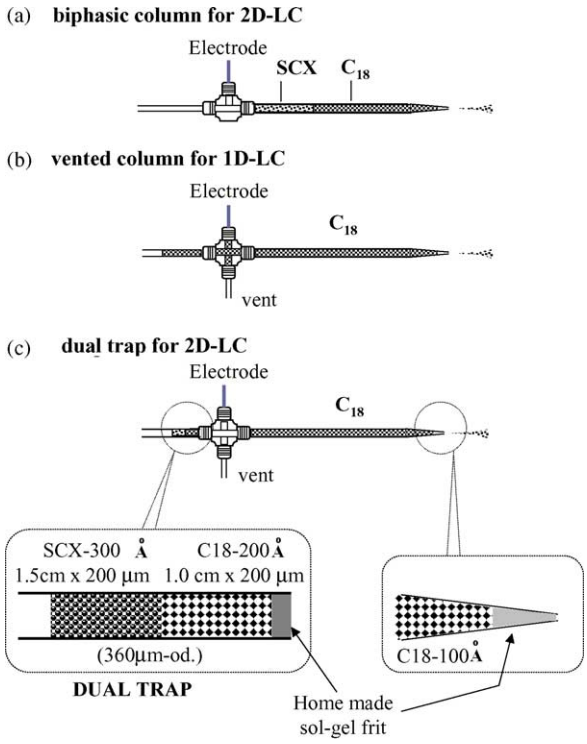


Fig. 1. Schematics of column configurations for microcapillary LC–MS: (a) a biphasic column (SCX and C_{18}) used for 2D-LC in MUDPIT [15,16]; (b) vented column using a microcross of which inner space is packed C_{18} [14] (during the sample loading, vent tubing is opened and on-line desalting of sample is achieved); and (c) the dual-purpose trap for 2D-LC.

2. Experimental

2.1. Materials

Human Jurkat T-cells were cultivated in RPMI 1640, tissue culture medium, supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere containing 5% CO_2. The Jurkat T-cells were washed twice with PBS buffer and were solubilized in the reagent containing 9 M urea. The solution was homogenized for 3 min at room temperature and then was centrifuged for 30 min at 4800 rpm using an Optima LE-80K ultracentrifuge from Beckman (CA, USA), which was kept at 14 °C. The supernatant solution containing Jurkat T-cell lysate was transferred to a lyophilized vial. To remove the detergents which impedes tryptic digestion of proteins, the cell lysate solution was transferred to an Amicon YM-3 centrifugal filter unit (15 mL) having a membrane filter (3000 molecular mass cut-off) from Millipore (Bedford, MA, USA), and 0.1 M phosphate buffer solution was added. The solution was centrifuged at 2000 rpm at 14 °C and the cell lysate was reconstituted with 0.1 M phosphate buffer solution.

The lyophilized Jurkat T-cell extracts were dissolved in 8 M urea contained in 0.1 M phosphate buffer to denature proteins with the addition of 10 mM dithiothreitol. The protein solution was treated for 2 h at 37 °C to reduce disulfide bonds,

and the reduced thiol groups were alkylated by adding iodoacetamide to a total concentration of 20 mM. The reaction was carried out in the dark at 0 °C for 2 h. After an alkylation reaction, excess cysteine (∼40×) was added to react away the remaining iodoacetamide. The mixture was then diluted to a total concentration of 1.0 M urea by adding phosphate buffer for tryptic digestion. A proteomics grade trypsin from Sigma (St. Louis, MO, USA) was added at a concentration ratio of 1:50 (protein:trypsin), and the mixture was incubated for 24 h at 37 °C. After digestion, TLCK was added to stop digestion at a slight excess to the number of moles of peptides. The digested mixture was finally desalted by using an Oasis HLB cartridge from Waters (Milliford, MA, USA) and dried by an Autospin 314U vacuum centrifuge from BioTron (Seoul, South Korea).

2.2. Two-dimensional LC with MS–MS

Nanoflow LC separation was carried out with an Ultimate Pump system from Dionex (Sunnyvale, CA, USA). The analytical column (150 mm × 75 μm) and the dual trap column shown in Fig. 1c were prepared in-house. The tip at the end of capillary tubing (75 μm I.D., 360 μm O.D.) from Polymicro Technology LLC (Phoenix, AZ, USA) was pulled by flame with a tip diameter of around 10 μm. The empty column tip was filled with a sol–gel frit by the following procedure. The tip of column was immersed very shortly into the 1:4 (v/v) mixture of formamide and potassium silicate solutions obtained from R.S. Chem. (Hwasung, Kyeong-gi, South Korea) and then was baked at 100 °C for 3 h to make a sol–gel frit. Then, the pulled tip capillary with an end frit (∼1 mm in length) was packed with methanol slurry of 5 μm 100 Å Magic C_{18}AQ from Michrom BioResources Inc. (Auburn, CA, USA) at a constant pressure (1000 psi) of He. The dual-purpose trapping column was made with a silica tubing (200 μm I.D., 360 μm O.D.) in which the end frit (2 mm in length) was prepared as the same way explained earlier. It was packed with 5 μm 200 Å Magic C_{18}AQ for 1.0 cm at first, and then packed with 5 μm 300 Å Polysulfoethy A™ strong cation-exchange resin from The Nest Group Inc. (Southboro, MA, USA) for 1.5 cm. The dual-purpose trapping column and the analytical column were connected via a PEEK microcross as shown in Fig. 1c and a gold wire was used as an electrode to supply an electrospray ionization voltage of 2.5 kV as described in the literature [12,24].

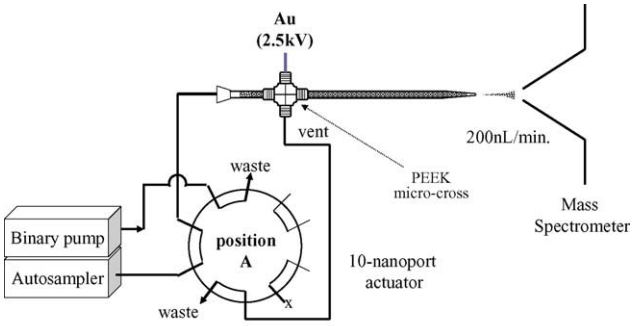
A QSTAR mass spectrometer model from Applied Biosystems (Foster City, CA, USA) was utilized. Peptide ions were detected in a data-dependent analysis mode. The acquisition method involves one MS precursor scan from 300 to 2000 amu followed by three data-dependent MS–MS scans (35% normalized collision energy).

2.3. Data processing

The acquired MS–MS spectra were analyzed by using a Mascot Search program and then compared with a Swiss-Prot human 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. Only those peptides were selected yielding larger than a minimum Mascot score of 29, which indicates identification at the 95% confidence level for this search.

3. Results and discussion

The on-line LC–LC–MS–MS using a dual-purpose trap method is based on the combination of the biphasic column method [15,16] in Fig. 1a and the vented column method [14] as shown in Fig. 1b. While the biphasic column provides a merit of 2D-LC–LC separation on-line, it requires a series of column clean up process after each salt step. In the case of using the vented column method, sample can be enriched and purified prior to separation in analytical column for 1D-LC–MS–MS. With the dual trap method shown in Fig. 1c, ionic solutions of different concentrations can be delivered for salt step elution from an autosampler with the vent valve open, and peptide fractions eluted from SCX trap transferred to the C_{18} trap packed right next to the SCX trap. After each salt step, a typical binary gradient elution (water–acetonitrile) for RPLC can be carried out. Fig. 2 shows the schematic view of the on-line LC–LC system used in this study. The performance of on-line LC–LC–MS–MS using a dual trap was tested with human Jurkat T-cell proteome. A digested human Jurkat T-cell protein mixture (15 μg) was loaded onto the SCX part of the dual trap from an autosampler. During the sample loading (valve position A of Fig. 2) onto the SCX trap, a flow rate of 4 μL/min was applied from the sample delivery pump with the vent valve open, located at the end of the waste line connected to the PEEK microcross, so that any non-retaining impurities were removed via the vent. The valve configuration A shown in Fig. 2 was used for sample loading and also for the delivery of salt solution to the SCX trap from the autosampler for salt step gradient elution. At the valve configuration A (with the vent valve open), all the flow is expected to direct to the vent due to the high pressure exerted from the analytical column. After sample loading for 8 min, the 10-port valve was turned to block the end of the vent tubing (position B: 36° turn from position A in Fig. 2) and was then ready for RPLC gradient separation. Before the salt step elution began, a breakthrough run was carried out to resolve some peptides that were not expected to be retained in the SCX trap during the sample loading, but were retained in the C_{18} part of the dual trap. The binary gradient RPLC separation was carried out by varying the mobile phase composition of: (A) 3% acetonitrile (ACN) in water; and (B) ACN. Both mobile phases contained 0.1% (v/v) formic acid. The gradient began with an increase to 5% B (from 2% B at default) over 4 min and it ramped to 20% for 60 min, and to 32% B for 20 min. Then, it was raised to 80% B for 2 min, maintained for 20 min, down to 2% B during 3 min, and maintained



Mode	valve position
1. Sample loading (8 min, 4 μL/min)	A
2. Breakthrough run (RPLC gradient) 200 nL/min.	B*
3. Salt step elution (8 μL of salt sol.) delivery rate: 4μL/min	A
4. Gradient RPLC run (200 nL/min.)	B
5. Repeat 3 and 4 for desired salt step	

*Position B: 36 degree turn to Position A

Fig. 2. Schematic representation of the dual trap two-dimensional nanoflow LC setup. Salt step elution was made by delivering salt solution to the dual trap using autosampler and each RPLC run was followed. Sample was initially loaded to the SCX part of the dual trap with the valve position A followed by a breakthrough run (RPLC gradient) with the position B. At position B, the vent tubing connection to the valve is blocked (expressed with x). After the breakthrough run, valve position was turned back to position A for salt step elution. Eight microliters of 3 mM NH_4HCO_3 solution was delivered to the dual trap from autosampler for 8 min and during the salt elution, peptides desorbed from the SCX trap were transferred to RP part of the dual trap. Then, the valve position was changed to B and an RPLC gradient run was performed. For additional salt step elution and RPLC run, steps 3 and 4 shown in Fig. 2 were repeated.

for at least 20 min for column re-conditioning. The flow rate during the gradient separation was kept at 200 nL/min and the eluted peptides were directly electrosprayed into a mass spectrometer. After the breakthrough run, the 10-port valve was changed to position A with the vent tubing open for the salt step elution. The first salt step fractionation was accomplished by delivering 8 μL of 3 mM ammonium bicarbonate (NH_4HCO_3) solution contained in a microvial from the autosampler to the SCX trap at 4 μL/min. Desorbed peptides from the first salt step elution were readily trapped in the C_{18} trap right next to the SCX trap. By delivering only a small amount of salt solution from the autosampler, salt delivery and desalting were made in sequence by the sample delivery pump over the 8 min of salt step elution. After the first salt step elution, the valve position was turned to carry out a gradient RPLC run (position B). The pump flow delivered to the analytical column was set at 200 nL/min and the binary gradient separation in RPLC was performed. After the first salt step elution followed by a gradient RPLC run, the procedure was repeated for consecutive salt steps by increasing the concentration of NH_4HCO_3 solution to 6, 10, 15, 20, 50, and 500 mM. The last salt step was to deplete all peptides from SCX trap. In this experiment, a total of eight RPLC runs were performed. With the use of a dual-purpose sample trap directly connected to the microcross, the entire system operation is automated and the current setup minimizes the use of a complicated valve operation as well as a separate column washing process after each SCX run needed for on-line two-dimensional LC systems. During RPLC separation, a home-made pulled tip column having a frit at the tip was utilized, as shown in Fig. 1c. By installing a 1 mm long sol–gel frit at the end of the pulled tip, the column blocking that occasionally formed during electrospray ionization due to insoluble solids at the open tip disappeared. Formation of bubble can be observed at the end of a pulled tip column without frit in some cases (at a very low flow rate) and this can interfere with MS analysis. It can be removed when the in-line degassing and the high pressure mixing of mobile phases are integrated to HPLC system. Another remedy is to fill the microcross with packing materials. However, at the current experiment using 200 nL/min., bubble formation was not observed to interfere MS analysis.

Fig. 3 shows the base peak chromatograms of a 7-cycle on-line 2-D-LC–MS analysis of the peptide mixtures of human Jurkat T-cell proteome along with an initial breakthrough run. The first RPLC run in Fig. 3a was obtained right after the sample loading in the SCX fraction. It demonstrated that there was a considerable amount of components which were not retained in the SCX trap but were trapped in the RP part of the dual trap. However, 57 unique peptides were identified from the numerous peaks in Fig. 3a through the database search for the corresponding MS–MS spectra. Most of the peaks observed in Fig. 3a are thought to be due to some non-ionic small molecules or peptide fragments.

The first salt step began by delivering 8 μL of 3 mM NH_4HCO_3 solution from the autosampler to displace weakly bound peptides from the SCX trap to the RP region, and the carrier liquid (the same as solvent A) delivering the salt solution band washed the dual trap to remove any remaining salts. The RPLC run (Fig. 3b) immediately following the first salt step elution did not show a significant peptide peak except for the huge transient peak at the end of gradient; however, the database search did yield 12 unique proteins, although these are single peptide hit. When the salt concentration was increased to 6 mM, peptides began desorbing from the SCX trap as shown by RPLC separation in Fig. 3c. The additional salt steps were carried out by increasing the salt concentration, as shown in Fig. 3d–g, and the final step was made at 500 mM in order to detach all remaining peptides in the SCX trap. Most of the peptides were shown to elute within the concentration range of 6–50 mM ammonium bicarbonate solution.

Fig. 4 shows an example of two tandem mass spectra obtained for the peptide K.DLYANTVLSGGTTMYPGIADR.M (*m/z* 1107.98, doubly charged) from actin (cytoplasmic 1) eluted at the salt cycle of 6 mM. The mass spectra at the top of Fig. 4 was obtained at the parent ion peak eluted at 76.14 min in Fig. 3c, and the bottom was obtained from a chromatographic peak at 75.89 min in the

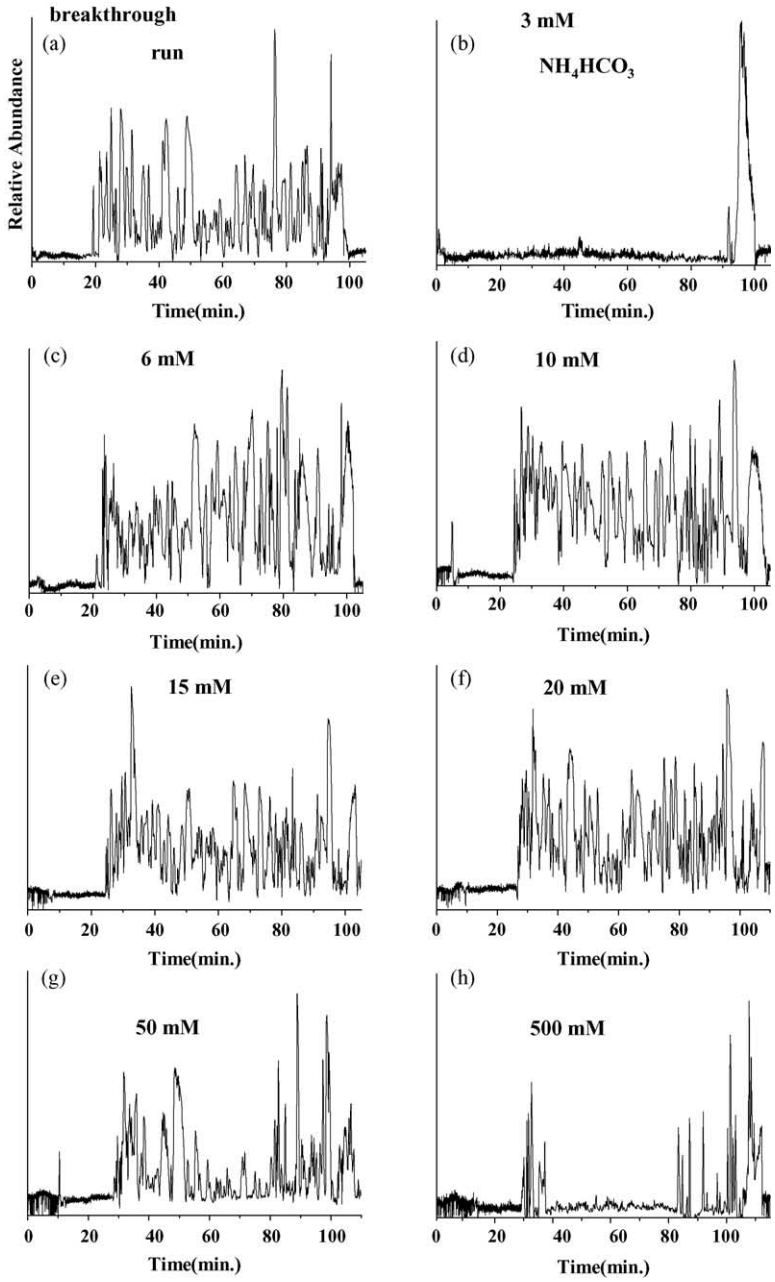


Fig. 3. Base peak chromatograms of digested Jurkat T-cell proteome: (a) at the breakthrough run; and (b–h) after each salt step elution at seven different salt concentrations marked inside the figure. Eight microliters of NH_4HCO_3 solution was delivered to the dual trap for SCX separation by increasing concentration (3–500 mM). RPLC condition for all runs is identical and is described in the text.

same salt cycle (this chromatogram is not shown here) after the repeated injection. The difference in the retention times of the two peaks is only 15 s, about 0.3% in difference, and the patterns of both tandem mass spectra appear to be nearly the same. The of search result Mascot scores were 108 and 105, which were much higher than the threshold value of 29. This shows reproducibility in the separation and the MS–MS characteristics of the proposed method.

Some of the peptides identified at the breakthrough RPLC run were identified with the different peptides at salt step cycles, but as belonging to the same protein. For example, while a peptide with a sequence of ITIADCGQLE (*m*/*z* 560.28, M^{2+}, 43.12 min of top spectrum of Fig. 4) was identified as peptidyl-prolyl *cis–trans* isomerase A from a tandem mass spectra with a Mascot score of 39, the same peptide was not identified at all from the succeeding salt cycles. However, 10 other peptides (from the identification of total 21 peptides) originated from the same protein were identified in the following four salt cycles (6, 10, 15, and 20 mM). For SIYGEKFEDENFILK and VNPTVFFDIAVDGEPLGR, they were eluted at 66.48 min (*m*/*z* 916.40,

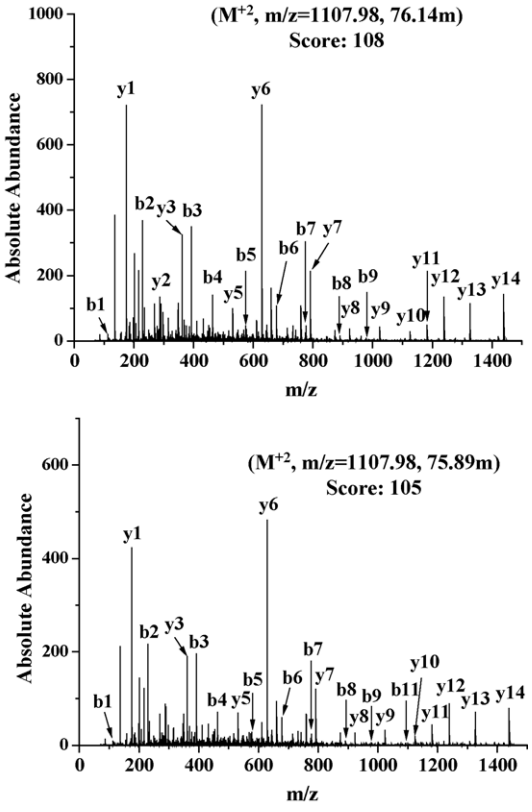


Fig. 4. MS–MS spectra of the peptide K.DLYANTVLSGGTTMYPGIADR.M (*m/z* 1107.98, doubly charged) from actin (cytoplasmic 1) obtained from repeated experiments. The peptide was eluted at the salt cycle of 6 mM NH_4HCO_3.

M^{2+}) and 92.13 min (*m/z* 973.52, M^{2+}), respectively, in the 10 mM salt step LC run. Both were identified to be from the same peptidyl-prolyl *cis–trans* isomerase A with search scores of 94 and 75, respectively. The same peptides appeared in the next salt step (15 mM) at 66.01 min (*m/z* 611.34, M^{3+}), and 91.44 min (*m/z* 973.51, M^{2+}), respectively. The difference in the retention times for the nearby runs was about 0.7%. This showed that same peptides were eluted at nearby salt steps. It also suggested that there was a possibility of a decreased chance of detecting other peptide ions due to the repeated detection of identified peptides in the previous salt step. Moreover, the peptide ITIADCGQLE did not at all appear throughout the entire salt steps except in the breakthrough run, but it was nonetheless identified with a similar retention time (44.39 min, *m/z* 560.26, M^{2+}, Mascot score = 43) when a single-dimensional LC–MS–MS analysis was performed. However, those 10 peptides identified from the 2D-LC–LC–MS–MS experiments were not even identified at all in the single-dimensional LC–MS–MS analysis.

The MS–MS spectra obtained from each RPLC run yielded an identification of 441 different peptides, and the number of proteins was found to be 234 listed in Table 1. Among these numbers, 94 proteins were identified as multiple peptides. The difference between the number of proteins and the number of new proteins in each cycle represents the number of proteins eluted in nearby salt step runs. The number of proteins identified in each run was the highest at the first salt step, and it decreased to a level of 102 in the final cycle, as listed in Table 1. The total number of proteins identified from a 15 μg injection of digested human Jurkat T-cell proteome was 681. When a single dimension LC–MS–MS run is used, the number of proteins identified from a 3 μg injection was only 222, as listed in Table 1. For the single-dimensional LC–MS–MS analysis, a trapping column packed with C_{18} only was used instead of the dual column. Since the on-line 2D-LC technique provides the advantage of handling a smaller amount of protein digests than that required for off-line SCX fractionation, the dual trap method still has the merit of being able to deal with small amount of proteome sample.

Table 2 lists the number of identified proteins that are classified according to their molecular function and types. Only 452 proteins were classified from the total 681 proteins resulting from 15 μg injection using the on-line LC–LC–MS–MS approach, and 229 proteins were not clearly identified with their origin or function. The number of proteins from human Jurkat T-cell obtained by this approach is much larger than that identified by a conventional two-dimensional

Table 1
Coverage of peptide/protein identification from single-dimensional LC–MS–MS and on-line LC–LC–MS–MS using a dual trap from 15 μg injection of digested proteins from Jurkat T-cell

	1D LC–MS–MS (3 μg)	On-line LC–LC–MS–MS							
		0 mM[a]	3 mM[a]	6 mM[a]	10 mM[a]	15 mM[a]	20 mM[a]	50 mM[a]	500 mM[a]
Number of peptides	650	57	12	441	418	429	412	329	167
Number of proteins	222	57	12	234	203	210	204	197	102
(Multiple hits)[b]	126	0	0	(94)	(92)	(87)	(89)	(78)	(30)
Number of new proteins		57	11	221	109	78	85	76	44
Number of proteins (cumulative)		57	68	289	398	476	561	637	681
Total	222	681							

[a] Concentration of NH_4HCO_3.
[b] Number of identified proteins with multiple peptides.

Table 2
Number of identified proteins of human Jurkat T-cell obtained at two different injection amounts

Categories	Number of proteins classified
Cytoskeleton	66
Heat shock proteins	32
HnRNP	21
Membrane proteins	25
Metabolic enzyme	55
Nucleosome	37
Nuclear proteins	32
Proteosome	20
Ribosome	84
Signaling molecules	26
Spliceosome	24
T-complex proteins	10
Translation factor	20
Others	229
Total	681

electrophoresis-matrix assisted laser desorption ionisation-mass spectrometry (2DE-MALDI-MS) [23].

4. Conclusions

In this article, a fully automated on-line two-dimensional LC system with a dual-purpose sample trap was introduced for the separation of peptide mixtures from human Jurkat T-cell and for the improvement of ESI-tandem MS analysis. It has been demonstrated that the dual trap on-line 2D-LC–LC–MS–MS method described in this study can be applicable to the proteome analysis in an unattended way. Especially, the current method has several advantages over other multidimensional chromatographic methods since complicated valve systems are avoided along with a minimized plumbing and thus, a very small amount of peptides can be efficiently separated into two dimensions with the reduced fear of a possible loss during the passage through the tubing connections. With the use of a dual trap directly connected to a home-made nanoflow RPLC column with a pull tip, the on-line fractionation of peptides by charge in the SCX trap can be simply obtained prior to the analytical column with a minimization of dead volume. The dual trap 2D-LC setup practically obviates the necessity of feeding salt solution to MS since it removes a separate process for column washing after each use of salt solution. This can be achieved by injecting a small volume (only 8 μL) of salt solution from an autosampler, and the delivery liquid following the salt solution band washes remaining salts in the dual trap. This will remove a column re-equilibrium period after each salt elution to column when an integrated biphasic (SCX–RP) column is used as in MUDPIT. In addition, it is advantageous to minimize a chance of deteriorating analytical column caused by impurities or cell debris left in protein digests even after purification since peptide mixtures are loaded to a separate SCX trap initially and injected to analytical column after SCX.

One difficulty in the use of a dual trap system came from a build-up of system pressure in the dual trap during the initial sample loading and the delivery of salt solution at a high speed. In this study, the SCX trap was packed in 200 μm I.D. capillary tubing for 1.5 cm long in order to reduce the system pressure during sample loading. Since the volume of SCX packing in this dual trap was about 50% larger than that of a typical SCX packed in an integrated (SCX and RP) column (4 cm of SCX part, 100 μm I.D.) in literature [16], sample loading capacity of the dual trap was expected to accommodate a sufficient amount of protein digests (more than 100 μg) based on the volume of SCX trap. However, when salt solution was delivered to the SCX trap at an increased rate (∼4 μL/min), the maximum loading capacity was 50 μg of protein digests used in this study. When it is needed for loading sample and salt step elution at an increased rate, the subsequent increase of backpressure needs to be decreased by incorporating a larger inner diameter tubing in the preparation of dual trap or a special device. When a large diameter tubing is used for a dual trap, length of the total trap device must be minimized to reduce inaccurate gradient condition of RPLC mobile phase. In this work, it was focused to implement the dual trap system for the possible use of on-line 2D-LC/tandem MS toward proteomics analysis.

The idea utilized in this study can be expanded to incorporate an affinity based stationary phase such as may be used for the immobilized metal affinity chromatography (IMAC) into the dual trap for the on-line screening of phosphopeptides and for the simultaneous washing of metal solution, which is an essential eluent for affinity separation but is not desirable for an MS analysis. Further studies are needed to expand peptide throughput and to increase the delivery rate of salt solution to the dual trap by reducing system pressure.

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