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Microbore liquid chromatography ultraviolet detection for quantification of total peptide amount and its application for assessing sample quality in shotgun proteome analysis of hundreds of cells

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ABSTRACT

Mass spectrometric profiling of the proteome of a small number of cells requires not only a sensitive instrument for protein/peptide separation and detection, but also a robust sample preparation protocol to process a very small amount of proteins (<1 μg) present in few cells. We have developed and evaluated the performance of a microbore liquid chromatography (LC) UV detection system for quantifying the total amount of peptides in a shotgun proteome analysis workflow that is tailored for the analysis of hundreds of cancer cells. Upon the sample injection into a 1-mm-diameter reversed phase column, a step-gradient was used to first remove salts and other impurities and then elute the peptides quickly without much separation. The UV absorbance of eluted peptides at 214 nm was used for peptide quantification with the aid of a calibration curve of a tryptic digest of a mixture of four standard proteins. Two linear calibration regions could be obtained in the peptide amount ranging from 0.03 μg to 0.3 μg and from 0.6 μg to 5 μg. The limit of quantification (LOQ) was determined to be 30 ng (or 39 ng in the linear calibration range). However, the presence of background proteins, mainly keratins, introduced during the sample preparation process was found to be the limiting factor in quantifying a lower amount of peptides from a cell lysate digest. With background absorbance from the digest of contaminant proteins in a solution, the LOQ was found to be 200 ng. This nondestructive microbore LC-UV method should be useful in assessing sample quality during the development and applications of an efficient sample preparation method for proteome analysis of a small number of cells. As an example, this method was used for quantifying the peptides generated from breast cancer MCF-7 cell extracts with a limited number of cells: 250, 500 and 1000 cells. Using capillary LC quadrupole time-of-flight mass spectrometry, 81–126, 122–154 and 256–282 proteins could be identified from 250, 500, and 1000 cells, respectively, in duplicate experiments. This method was also applied for the analysis of biological triplicate samples of MCF-7 cells. The average numbers of peptides and proteins detected from the experimental triplicate analyses of biological triplicate samples were 400 ± 71 (9 datasets) and 124 ± 14, respectively, from 250 cells, and 531 ± 44 and 162 ± 16, respectively, from 500 cells.

1. Introduction

Shotgun proteome analysis workflow based on liquid chromatography–mass spectrometry (LC–MS) has become a powerful tool for proteome profiling of cells, tissue and biofluids. In most proteomics applications, hundreds of micrograms of proteins are often used as the starting material. However, the sample amount can be very limited in some important areas of applications, such as in dealing with single cells [1–5], circulating cancerous cells captured from a blood sample of a patient with early sign of tumor in a specific organ [6–8], stem cells isolated from a large population of other types of cells [9,10] and primary cells procured from tissues [11,12]. In order to ensure maximum proteome coverage for these samples, both the sample preparation procedure and MS analysis method need to be optimized. Prior to LC–MS analysis, the shotgun workflow requires protein extraction from cells, followed by protein digestion. This sample preparation process can potentially lose some proteins. In working with a large quantity of samples, this sample loss may not affect the proteome coverage. However, for handling a limited amount of proteins, sample loss can reduce

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the proteome coverage significantly. For example, protein or peptide adsorption to containers may lose a fraction of the original sample.

There are a variety of sample preparation methods that have been reported for handling a relatively large number of cells [13–15]. These methods and others can be evaluated and further developed for handling few cells with an objective of increasing the efficiencies of cell lysis, protein extraction and digestion while minimizing sample loss [5,16–19]. However, during the course of developing or applying a sample handling protocol for proteome analysis of a small number of cells, it is desirable to use a simple method to quantify the total amount of peptides generated. The total amount can be used as a bench mark for method comparison. In addition, if the quantification method is non-destructive, the peptides can be collected and the amount can be used to assess the sample quality to determine whether the sample was properly prepared for MS analysis and, if so, what the optimal conditions of the LC–MS settings should be. For example, a shortened gradient time is favored in analyzing smaller amounts of peptides [16].

Determination of the total protein amount extracted from the cells can be done using a commercially available kit, such as Bradford or bicinchoninic acid (BCA) assay, although these are destructive methods [20]. However, knowing the protein amount does not allow us to evaluate the entire workflow of the shotgun method where the final product of analysis is the peptides, not proteins. On the other hand, measuring the peptide amounts in nanograms or sub-microgram range is not trivial. In this work, we report a microbore column (1-mm diameter) LC-UV method with a step gradient elution to rapidly quantify the total peptide amount in proteomic digest samples while removing salts and other reagent impurities that may interfere with LC–MS analysis. This method was illustrated to be useful for quantifying peptides and assessing sample quality in the shotgun proteome analysis of 250, 500 and 1000 MCF-7 breast cancer cells.

2. Experimental

2.1. Chemicals and reagents

All chemicals and reagents were purchased from Sigma-Aldrich (Oakville, ON, Canada) unless stated otherwise. Acetonitrile (ACN) and HPLC grade water were purchased from Fisher Scientific Canada (Edmonton, AB, Canada).

2.2. Cell culture and cell counting by flow cytometer or haemocytometer

The MCF-7 breast cancer cells (ATCC® number: HTB-22™) were cultured in 15 cm diameter plates at 37 °C in DMEM Gibco medium supplemented with 10% fetal bovine serum. The cells were harvested by scraping from the plates into the PBS^{++} buffer (0.68 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1.4 mM KH_2PO_4, 4.3 mM Na_2HPO_4, 2.7 mM KCl, and 137 mM NaCl) and centrifugation at 100 *g* for 8 min at 4 °C. The harvested cells were then fluorescently stained by incubating with a FITC-conjugated mouse anti-human HEA antibody (Miltenyi Biotec number: 130-080-301) in a 1:100 (v:v) ratio on ice for 15 min. The stained MCF-7 cells were introduced into the flow cytometer for counting, according to the cell size and their fluorescence response. Then 250, 500, 1000, 2500 or 5000 MCF-7 cells were collected into 0.6 mL low retention microcentrifuge vials.

For the biological triplicate experiments of 250 and 500 cells, the MCF-7 cells were grown and harvested under the same conditions as above. The harvested cells were counted by using a haemocytometer under a microscope. Aliquots of cells containing either 250 or 500 cells were taken from each biological sample for experimental triplicate runs.

2.3. Cell lysis and in-solution digestion

The process used for cell lysis and in-solution digestion of a small number of cells was similar to that reported previously [16]. Briefly, the cells in each vial were mixed with 5–10 μl Nonidet-P40 (NP40) lysis buffer (1%) and sonicated in ice-water ultrasonic bath for 5 min. The protein solutions were reduced with dithiothreitol (DTT) and alkylated with iodoacetamide (IAA). Acetone (pre-cooled to −80 °C) was added gradually (with intermittent vortexing) to the protein extract to a final concentration of 80% (v/v). The solution was then incubated at −80 °C for 4 hr and centrifuged at 14,000 rpm for 10 min. The supernatant was decanted. The pellet was carefully washed once using cold acetone to ensure the efficient removal of NP40 detergent. After the residual acetone was evaporated, 50 mM ammonium bicarbonate was used to sufficiently redissolve the pellet in the vial. Trypsin digestion was then carried out in a final enzyme concentration of 8 ng/μL (5–20 μL) at 37 °C for 4 h.

2.4. Preparation of calibration standards

A four-protein mixture solution containing equal moles of myoglobin (16.7 kDa), cytochrome C (11.6 kDa), lysozyme (14.3 kDa), and β-casein (23.6 kDa) was prepared by dissolving intended amount of protein standards in 50 mM ammonium bicarbonate. After reduction with 20 mM DTT and alkylation with the same volume of 40 mM IAA, the protein mixture was then digested by trypsin at a final enzyme concentration of 8 ng/μl at 37 °C for 8 h.

2.5. RPLC for peptide quantification and desalting

The tryptic peptides were desalted and quantified on an Agilent 1100 HPLC system (Agilent, Mississauga, ON) with a 1 mm × 50 mm Polaris C18 A column (3 μm particle and 300 Å pore). The flow rate used was 100 μL/min. Peptides were eluted from the column by a step gradient: flushing column with 97.5% mobile phase A (0.1% TFA in water) for 5 min and then 85% of mobile phase B (0.1% TFA in acetonitrile) for 5 min to completely elute the peptide fractions. The UV absorbance of eluted peptides was detected at 214 nm. Peak areas of the eluted peptides were determined from the chromatogram by baseline-to-baseline integration between 9.8 min and 11 min.

2.6. MS analysis

After desalting and quantification, the digests were analyzed using a quadrupole time-of-flight (QTOF) Premier mass spectrometer equipped with a nanoACQUITY Ultra Performance LC (UPLC) system. Briefly, the desalted digests were concentrated on Speed-vac and reconstituted with 0.1% formic acid. Then a digest solution was injected onto a 75 μm × 100 mm Atlantis dC18 column. Solvent A consisted of 0.1% formic acid in water, and Solvent B consisted of 0.1% formic acid in ACN. Peptides were separated using their optimal lengths of solvent gradients ranging from 90 min to 150 min and electrosprayed into the mass spectrometer fitted with a nanoLockSpray source at a flow rate of 350 nL/min. One MS scan was acquired from *m*/*z* 350–1600 for 0.8 s, followed by 4 MS/MS scans from *m*/*z* 50–1900 for 0.8 s each. A mixture of leucine enkephalin and (Glu1)-fibrinopeptide B, used as mass calibrants (i.e., lock-mass), was infused at a flow rate of 250 nL/min, and a 1 s MS scan was acquired every 1 min throughout the run.