Vol. 10, No. 3, 2019
ISSN 2233-4203/ e-ISSN 2093-8950
ARTICLE www.msletters.org | Mass Spectrometry Letters

Analysis of *Entamoeba histolytica* Membrane *via* LC-MALDI-TOF/TOF

Jorim Anak Ujang, Rahmah Noordin, and Nurulhasanah Othman*

Institute for Research in Molecular Medicine, Universiti Sains Malaysia, 11800, Penang, Malaysia

Received June 25, 2019; Revised July 23, 2019; Accepted July 23, 2019
First published on the web September 30, 2019; DOI: 10.5478/MSL.2019.10.3.84

Abstract : Liquid chromatography mass spectrometry is widely employed in proteomics studies. One of such instruments is the Liquid Chromatography (LC)-Matrix-assisted laser desorption ionisation (MALDI)-Time of flight (TOF) or LC-MALDI-TOF/TOF. In this study, this instrument was used to identify the membrane proteins of a protozoan parasite namely *Entamoeba histolytica*. It causes amoebiasis in human. The *E. histolytica* trophozoites were cultured prior to the membrane protein extraction using the conventional method, ProteoPrep® and ProteoExtract® kits. Then, the membrane protein extracts were tryptic-digested and analysed by LC-MALDI-TOF/TOF. Approximately, 194 proteins were identified and 27.8% (54) were predicted as membrane proteins having 1 to 15 transmembrane regions and signal peptides by combining all three extraction methods. Also, this study has discovered 3 unique proteins as compared to our previous study which merit further investigation.

Key words : LC-MALDI-TOF/TOF, protein identification, *Entamoeba histolytica*, membrane

Introduction

Matrix-assisted laser desorption ionisation (MALDI) was introduced by Michael Karas, Franz Hillenkamp and Koichi Tanaka[1,2] and used for identification of proteins. This ion source, linked to mass analyser i.e. Time-of-Flight (TOF) has become an important tool in proteome research. Using MALDI, the researchers found that organic samples could be ionised more easily by laser if it was mixed with other organic molecules that act as a matrix. Only a few microlitres of the sample-matrix mixture is placed on the target plate and allowed to dry. The drying process forms a crystal lattice incorporated with the sample peptides. The matrix absorbs most of the energy from the laser and transfers the charge to the sample, thus ionising the analyte. The resulting singly charged ions are caused by the protonation and deprotonation in positive and negative ion modes, respectively.[3]

MALDI is often coupled with TOF mass/charge analyser that fits the pulsed nature of MALDI.[4] The analyte accepts a single proton and this results in singly charged ions with large *m/z* values.[5] Therefore, mass analysers with large *m/z* detection range such as the TOF are interfaced with MALDI. The ions generated by laser pulses in the ion source are accelerated in an electric field, then the ions enter a flight tube which has a detector at its end. In tandem MS analysis, selected precursor ions are passed into the collision cell. Here, the fragmented ions are decelerated and reaccelerated for the measurement of *m/z* in the second TOF analyser.[6]

In this study, liquid chromatography (LC) coupled to MALDI-TOF/TOF was utilized to allow the proteome analysis of a complex membrane fraction of a parasite namely *Entamoeba histolytica*. *E. histolytica* causes amoebiasis which is ranked second as death-causing parasitic infection, after malaria.[7] Studies on membrane proteins of this parasite are important as this molecule can further our understanding on the disease pathogenesis and could be explored as vaccine and drug target.[8,9] Only two studies have been conducted to study the membrane and surface membrane *E. histolytica* proteome using LC-ESI-MS/MS.[8,9] However, LC-MALDI-TOF/TOF has never been used to analyse *E. histolytica* membrane proteins. The complementary use of two ionisation systems i.e MALDI and electrospray ionisation (ESI) could enhance the proteome coverage in which some of the identified proteins exclusively being detected using MALDI.[10] Hence, this study was performed to analyse *E. histolytica* membrane proteome using LC-MALDI-TOF/TOF as well as to unravel the unique identified proteins from this instrument.

Experimental

Axenic culture of *E. histolytica* trophozoites

Before performing the maintenance of the culture, the growth of the trophozoites and the presence of

*Reprint requests to Nurulhasanah Othman
E-mail: nurulhasanah@usm.my

contamination were observed using an inverted microscope. Then, a culture tube was gently tapped throughout and inverted a few times until sediment cells were unsettled from the bottom of the tube. Under a sterile environment, the old medium was discarded. Then, 9 mL of fresh TYI-S-33 medium was refilled into the tube. The tube was incubated at 36°C for 48-72 hours before subsequent maintenance.

Membrane protein extraction

The conventional method was performed according to Teixeira et al..[11] Initially, 10 mM sodium phosphate buffer, pH 8 was kept on ice for the subsequent extraction steps. 10×10^6 *E. histolytica* trophozoites cell pellet were suspended in 10 µL protease inhibitor cocktail and 1 mL of 10 mM sodium phosphate buffer. The suspension was then sonicated on ice for 1 min using 0.5 s pulse on and off with power level 2. Next, the supernatant was discarded and the cell pellet was resuspended in 1 mL ice-cold 10 mM sodium phosphate buffer and centrifuged at 100, 000 × *g* and 4°C for 1 h. Subsequently, the supernatant was discarded and the resulting pellet containing membrane fraction was resuspended with 200 µL of 50 mM ammonium bicarbonate. On the other hand, ProteoPrep® (Sigma Aldrich, USA) and ProteoExtract® kits (Calbiochem, Germany) were also used to extract the membrane protein by following the manufacturer's instructions. Then, the concentration of proteins was measured using Bio-Rad RC DC™ protein assay kit (Bio Rad, USA) and the quality of the protein extract was assessed by running 10% SDS-PAGE.

In-solution digestion

One hundred micrograms of protein was top-up to 100 µL of 50 mM ammonium bicarbonate to achieve a concentration of 1 µg/µL. Next, 100 µL of 0.05% RapiGest was added to the mixture and mixed well by vortexing. The sample was then incubated at 80°C for 15 min. Subsequently, the sample was allowed to cool at room temperature for approximately 5 min before 5 µL of 100 mM DTT was added. The sample was mixed and incubated at 60°C for 15 min. Next, the sample was cooled to room temperature again for 5 min and 5 µL of 200 mM IAA was added. The sample was mixed well and then incubated at room temperature in the dark for 30 min. Then, 2 µL of 1 µg/µL trypsin was added. The mixture was incubated at 37°C for 16 h. Subsequently, 2 µL TFA was added and the mixture was incubated at 37°C for 20 min to stop the digestion and remove the RapiGest. The aggregated RapiGest was then pelleted down at 14,462 × *g* for 15 min. The supernatant containing the peptide mixtures was then collected and filtered with 0.45 µm minisart syringe filter. The peptide samples were then stored at -80°C until further analysis.

LC-MALDI-TOF/TOF

Fractionation of the peptides was performed using Eksigent nanoLC ultra 1D plus linked to an automated MALDI spotter (Eksigent, Netherlands). To achieve spatial discrimination of the peptide mixtures, 2-5 µl of peptide samples was auto-loaded and packed into a C18 column. The gradient pump was set to elute the peptides with 20 to 80% acetonitrile for a duration of 165 min and at a flow rate of 0.3 µL/min. Mobile phase buffer A consisted of 0.1% TFA in 2% ACN and 97.9% water while mobile phase buffer B consisted of 0.1% TFA in 98% ACN and 1.9% water. The system was linked on-line to an automated MALDI spotter in which eluted peptides were spotted between the 30 and 160 min of gradient phase, with CHCA matrix flow of 1.8 µL/min for a duration of 25 s for each spot. Mass spectrometry analyses were performed in an automated (LC mode) on the AB Sciex TOF/TOFTM 5800 system. Data were obtained in the MALDI reflector mode using at least 6 spots of the internal calibration standard (TOF/TOF calibration mixture). Mass spectra from each spot were obtained in the *m/z* range from 800 to 4000, whereby up to 500 laser shots were accumulated per spectrum. The signal-to-noise (S/N) ratio was set to a minimum of 10, and the spots with the highest intensity of precursor ion were subjected to MS/MS analysis. A maximum of ten precursors were allowed for the MS/MS analysis; for each spectrum, up to 2000 laser shots were accumulated per spectrum, and the S/N were set to a minimum ratio of 15. The mass spectrometry data were analysed using ProteinPilot™ Software 4.5 and searched using Paragon against a combined AmoebaDB 4.1 and cRAP ('protein contaminants database') which was set to search with the following parameters: false discovery rate of < 1%, detected protein threshold of > 0.47 (66%), and competitor error margin of 2.00. The cRAP includes the possible contaminant proteins in this study such as BSA and keratin.

Prediction of membrane protein

Membrane protein prediction was analysed by TOPCONS 2.0 server at http://www.topcons.net/pred/. In this server, amino acid sequences of the identified proteins were analysed for protein topology using five sub-methods (OCTOPUS, Philius, PolyPhobius, SCAMPI and SPOCTOPUS). The final results were decided from the consensus prediction of all the sub-methods based on the presence of signal peptides and the number of transmembrane regions of ≥ 1.

Results

In total, combined all three extraction methods, LC-MALDI-TOF/TOF identified 194 protein hits with 27.8% (54) were predicted as membrane proteins having 1 to 15 transmembrane regions and signal peptides (Figure 1). LC-MALDI-TOF/TOF identified 156 proteins from the membrane fractions by the conventional method. TOPCONS predicted 32% (50) of the identified proteins as membrane proteins. Meanwhile, LC-MALDI-TOF/TOF identified 69

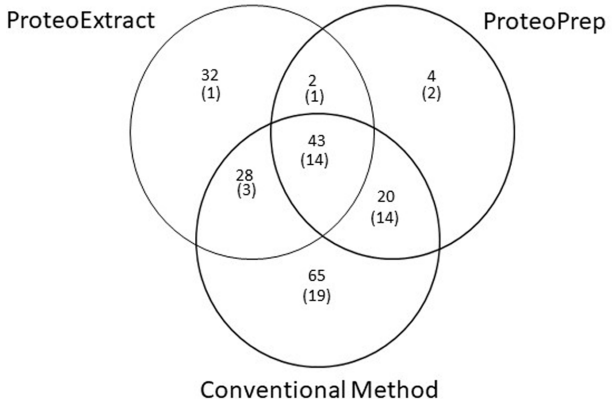

Figure 1.

Table 1. Common proteins that were identified by LC-MALDI-TOF/TOF from three extraction methods.

No.	No. TM	Accession no.	Sequence name
1	1	EHI_012270	Gal/GalNAc lectin heavy subunit
2	13	EHI_014030	NAD(P) transhydrogenase subunit alpha, putative
3	1	EHI_015380	immuno-dominant variable surface antigen
4	1	EHI_024530	hypothetical protein
5	1	EHI_042370	galactose-specific adhesin 170kD subunit, putative
6	1	EHI_047800	hypothetical protein
7	13	EHI_055400	NAD(P) transhydrogenase (AB-specific), alpha subunit, putative
8	1	EHI_059830	hypothetical protein
9	1	EHI_069560	hypothetical protein
10	1	EHI_077500	galactose-specific adhesin 170kD subunit
11	1	EHI_133900	galactose-inhibitable lectin 170 kDa subunit, putative
12	2	EHI_158240	fatty acid elongase, putative
13	2	EHI_165070	estradiol 17-beta-dehydrogenase, putative
14	1	EHI_178470	hypothetical protein

and 105 proteins by ProteoPrep® and ProteoExtract kits®, respectively. Furthermore, ProteoPrep® and ProteoExtract kits membrane extracts comprised of 44.9% (31) and 18% (19) predicted membrane proteins, respectively. The 14 identified and predicted membrane proteins by three extraction methods is shown in Table 1. All protein identification data of this study is available at AmoebaDB database.[21] This study has identified 3 exclusive proteins when compared to our previous study using LC-ESI-MS/MS.[8,22] The proteins are dolychil-diphosphooligosaccharide--protein glycosyltransferase subunit, putative (EHI_042580), NAD(P) transhydrogenase beta subunit, putative (EHI_060020) and signal peptidase complex subunit, putative (EHI_200720).

Discussion

In this study,the use of three different membrane protein extraction methods have allowed to exploit the unique properties of each method, thus resulting in a wider coverage of proteins by mass-spectrometry analysis. The ProteoExtract® kit is a mild differential extraction method whereby membrane proteins were extracted based on their association with the cellular membranes instead of their intrinsic hydrophobicity.[12,13] This might explain why the number of identified proteins and predicted membrane proteins extracted by the ProteoExtract® were less than the conventional method.

The use of high pH phosphate buffer in the conventional method has been known to extract more hydrophilic proteins than hydrophobic membrane proteins.[14] The buffer also allows the disruption of non-covalent interaction of peripheral membrane proteins but is inefficient in solubilising integral membrane proteins.[13,14] In addition, the inclusion of a sonication step further allowed disruption of membrane vesicles, hence allowing the release of soluble and membrane-associated proteins.[15]

The ProteoPrep® extraction kit on the other hand uses differential and sequential extraction strategies for the enrichment of membrane proteins. This was performed by partitioning the membrane proteins to allow differential solubilisation of cytosolic and membrane proteins. After the removal of the cytosolic proteins, the resultant membrane pellet was washed and partially solubilised, which was advantageuous in the reduction of carryover soluble proteins as compared to a single step solubilisation strategy.[16] This method was previously reported as the most sensitive and selective on extracting *E. histolytica* membrane proteins by our group.[8] However, this method extracted moderate number of predicted membrane proteins in this study.

The application of LC-MALDI-TOF/TOF system for large-scale proteomic analysis is not as popular as its counterpart, LC-ESI-MS/MS. However, the LC-MALDI-TOF/TOF has the advantage such that the eluted LC fractions can be kept and archived even after the analysis has been performed. Furthermore, as peptide ions by MALDI are predominantly singly charged, the MS spectra are less complex, thus redundant acquisitions are minimised compared to ESI.[17] Due to the high throughput capabilities of LC-ESI-MS/MS in comparison to LC-MALDI-TOF/TOF, many proteome analysis studies favoured more towards the application of LC-ESI-MS/MS.

However, as demonstrated in this study, the application of LC-MALDI-TOF/TOF is still valid as the technology has been shown to identify proteins that was not identified through LC-ESI-MS/MS. We found that LC-MALDI-TOF/TOF was able to identify proteins that were not identified through LC-ESI-MS/MS when we compared data obtained from our previous study on *E. histolytica* membrane proteome analysis using LC-ESI-MS/MS, by Ujang et al..[8,22]

Important proteins that were exclusively identified through LC-MALDI-TOF/TOF include dolychil-diphosphooligosaccharide-protein glycosyltransferase subunit, putative (EHI_042580), NAD(P) transhydrogenase beta subunit, putative (EHI_060020) and signal peptidase complex subunit, putative (EHI_200720). A study in human cell line described the involvement of dolychil-diphosphooligosaccharide-protein glycosyltransferase in N-glycosylation.[18] This family of proteins might play a part in the synthesis of Gal/Gal-NAc lectin which is an important component in trophozoite adherence to host tissues. Fewer studies have been performed to study these proteins but their role still unclear. The other interesting protein identified was the signal peptidases. This protein subunit is important for normal cell function as they are needed to release translocated pre-proteins from the membrane to extracellular environment.[19] These proteins cleave the N-terminal signal peptides of secretory proteins at the membrane and also the rudiment precursor protein molecules that are under elongation by a ribosome that is bound to the endoplasmic reticulum translocation site.[20] The disruption of the signal peptidase activity has proved to cause significant effects. For example, the replication of Zika and Dengue viruses were inhibited with the inhibition of the signal peptidase in the human cell line.[21]

Conclusions

In conclusion, this study revealed 54 *E. histolytica* predicted membrane proteins and 3 of them were uniquely identified by LC-MALDI-TOF/TOF. Hence, those potential identified membrane proteins merit further investigation.

Acknowledgments

We would like to acknowledge Universiti Sains Malaysia for funding; Research Individual Grant (1001/CIPPM/812118). We would like to thanks Mr Muhammad Hafiznur Yunus for the technical support on LC-MALDI-TOF/TOF.

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