Sample Preparation Methodology for Detection of Bacteriophage Structural Proteins using MALDI-TOF-MS

Directed Studies Proposal

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Abstract

This research aims to develop a methodology for bacteriophage capsid protein sample preparation for MALDI-TOF MS identification using two different phages strains (*EC1KELCTY* and *EC3KAMCTY*) previously isolated. The Bacteriophages were propagated on plate lysates using their respective bacterial host, the lysates were titred using small drop plaque assay system and stored in 1XPBS and 0.1 magnesium sulfate. Finally, for further lysates concentration high general speed centrifugation is performed (41,600 x g for 3h at 4 ºC (ThermoScientific, Solvall Lynx 4000) using F20-12x50LEX rotor (FIBERLITE^{TM}, (ThermoScientific)) the resulting phages were titred and stored at 4ºC. The capsid proteins of the bacteriophage lysates will be digest using thermocycle-assisted protein digestion for MALDI-TOF MS analysis, an SDS-PHAGE gel will be also run to ensure the precise and efficient protein digestion, facilitating subsequent mass spectrometry analysis. If the protein digestion through thermocycle method is successful, we should be able to identify peptide fragments from capsid protein sample using FlexControl software within the range of 2 kDa to 15 kDa.

Introduction

Today some of the most common and best techniques often used for microorganism identification are 16S rRNA and 18S rRNA gene sequencing (Singhal et al., 2015). However, this can be expensive and time consuming, as these require a considerable amount of lab work, time and is not suitable for routine clinical used. Matrix Assisted Laser Desorption/Ionization –

Time-of-Flight Mass Spectrometry (MALDI-TOF MS) has emerged as potential tool for microbial identification and diagnosis (Singhal et al., 2015).

Fingerprinting by MALDI-TOF MS is based on the ionization of compounds after minimal treatment, compared to other techniques used for microorganism identification like gene sequencing, this method is fast, accurate and cheaper, as the main expense is the initial cost MALDI-TOF equipment (Singhal et al., 2015).

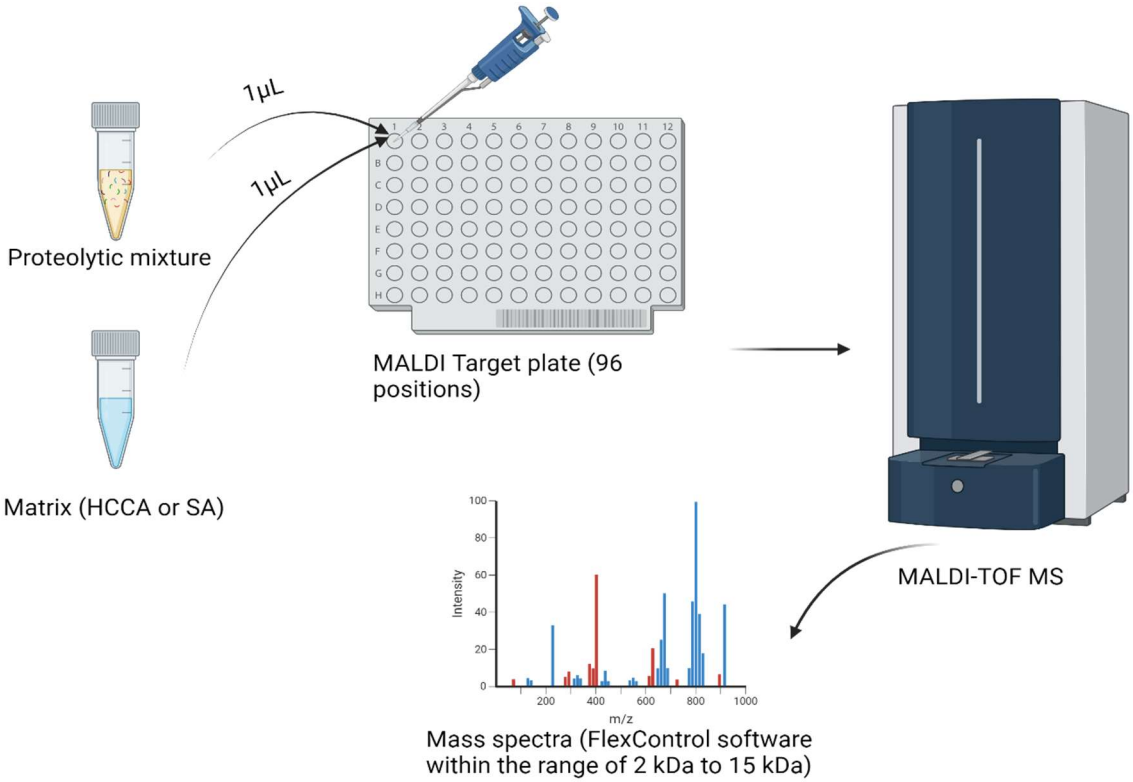

Figure 1. Schematic diagram showing MALDI-TOF MS fingerprinting method and sample preparation. Created with BioRender.com

Nonetheless, it is still not used as a routine identification of phages as stablishing a sample preparation methodology is necessary for the optimization and reproductivity for MALDI-TOF mass spectral quality (Štveráková et al., 2018). Bruker, a leading manufacturer of MALDI-TOF mass spectrometers, has been actively involved in advancing microbial identification technologies, including the development of databases for phage identification. They have been working on expanding their MALDI Biotyper® system to include databases that

encompass phage profiles, aiming to enhance the capability of MALDI-TOF for microbial and viral identification. Their library ensures that a broad range of microorganisms can be identified easily, it includes 4,320 species of 712 microorganism genera this in their last version from 2023 (Bruker , 2024)

Digestion of proteins using a thermocycler is a promising technique for sample preparation has proved to be faster than conventional methods that require chemical denaturants and cannot be analyzed directly by MALDI-TOF as these require purification due to the presence of urea and salts not compatible with Mass Spectrometry (Turapov et al., 2008).

Objectives

The project's key objectives include establishing a MALDI-TOF-MS-based method for bacteriophage capsid protein identification using thermocycler-assisted protein digestion (Objective 1) and determining the proper digestion of proteins through SDS-Phage gel electrophoresis to separate phage proteins and excise bands for individual protein digestion with trypsin. (Objective 2), offering a more efficient alternative to current time-consuming and costly methods in clinical microbiology.

Material and Methods

- **Thermocycler-Assisted Protein Digestion**

Bovine Serum Albumin (BSA) and phage lysates proteins from previous isolated phages corresponding to bacteriophages strains (*EC1KELCTY* and *EC3KAMCTY*) will be denatured by thermocycler-assisted protein digestion without the use of chemical denaturants, employing a known protein (BSA, 2mg/ml) as standard. The thermocycler-assisted protein digestion will be performed on pre-sterilized PCR tubes using a PCR-type thermocycler, Trypsin Platinum will be reconstituted in 200 µL of ultrapure water to archive a final concentration of 0.5µg/µL, and aliquoted in 10µl volumes into the PCR tubes for storage until use at -80°C. Ammonium Bicarbonate (NH_4HCO_3) at a concentration of 100Mm, this will be used as a reducing agent. For each digestion, 1µL of protein sample will be mixed with 1µL of DTT (100Mm in NH_4HO_3) and

1uL of proteomic grade trypsin in pre sterilized PCR tube the protein digestion will be carried out using a thermocycler programmed to cycle through temperatures of 49°C, 50°C, 51°C, 52°C, 53°C, 54°C, and 55°C, each for 20 seconds, with a ramp rate of 2.5°C/s, without the use of chemical denaturants (Turapov et al., 2008). A known protein standard, Bovine Serum Albumin (BSA) at a concentration of 20 mg/mL, will be used to validate the thermocycler-assisted protein digestion procedure.

- **SDS-PAGE gel**

A quality check will be performed on BSA to ensure it is suitable for use in proteomics by running a precast SDS-PAGE gel and staining it with Coomassie brilliant blue. Pure BSA should show as a single band around 66 kDa. This methodology ensures precise and efficient protein digestion, facilitating subsequent mass spectrometry analysis.

- **Sample Preparation for MALDI-TOF-MS**

For the MALDI-TOF-MS analysis 2 matrices will be prepared in standard solvent. For the acquisition of the Mass spectra proteolytic mixtures α-Cyano-4-hydroxycinnamic acid (HCCA, 10 mg/mL) and Sinapinic acid (SA, 10 mg/mL) will be used. Following proteolytic digestion, 1 µL of the mixture is applied onto a steel MALDI-TOF MSP 96 target plate (Bruker Daltonics GmbH, Germany). Subsequently, 1 µL of the appropriate matrix (HCCA or SA) is overlaid. The samples are air-dried at room temperature, and mass spectra is acquired using FlexControl software within the range of 2 kDa to 15 kDa.

Expected Results

This research project will aid to develop a methodology for protein sample preparation with the objective of facilitating and finding a cheaper and faster alternative for microorganism identification using MALDI-TOF MS, if Thermocycler-protein digestion method is successful it should be possible to identify Bacteriophage peptide fragments with MALDI-TOF MS. In future research the use of a thermocycler for protein digestion will be paired with SDS-PHAGE gel digestion to separate phage protein bands for MALDI-TOF MS analysis.

Expected Expenses

Supplies	Amount (CAD $))
Trypsin gold	145.00
SurePAGE™, Bis-Tris, 10x8, 4-12%, 12 wells per 10	115.00
MOPS Buffer	40.00
MES Buffer	40.00(Free)
Bovine Serum Albumin PER 10G	176.00
Trifluoroacetic acid	62.26
Total	538.26

TIMELINE of the project

July 2023: Proposal written

September 2024: Method Development and Optimization

- **Week 1-2**: Optimize thermocycler-assisted protein digestion parameters (temperature cycles, ramp rates) using BSA and phage lysates.
- **Week 3-4**: Validate digestion efficiency using SDS-PAGE gel electrophoresis. Ensure BSA shows a single band around 66 kDa.

October 2024: MALDI-TOF-MS Sample Preparation

- **Week 1-2**: Prepare matrices (HCCA and SA) and optimize conditions for MALDI-TOF-MS analysis of proteolytic mixtures.
- **Week 3-4**: Perform MALDI-TOF-MS analysis on BSA and phage lysate samples. Evaluate mass spectra quality and reproducibility.

November 2024: Data Analysis and Finalization

- **Week 1-2**: Analyze MALDI-TOF-MS data for identification of BSA and phage peptide fragments.
- **Week 3**: Prepare project reports, finalize methodology documentation, and draft presentations.
- **Week 4**: Present findings. Discuss future research directions integrating thermocycler and SDS-PAGE gel digestion for enhanced phage protein analysis.

Expected Milestones and Deliverables

- **September**: Optimized thermocycler-assisted digestion protocol established.
- **October**: Successful MALDI-TOF-MS analysis of BSA and initial phage samples.
- **November** : Completion of project reports, methodology documentation, and presentation of results

Literature Cited

Bruker . (2024). *MALDI Biotyper® for Microbial Research*. Www.bruker.com. https://www.bruker.com/en/applications/microbiology-and-diagnostics/microbiological-research/maldi-biotyper-for-microbial-research.html

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Štveráková, D., Šedo, O., Benešík, M., Zdráhal, Z., Doškař, J., & Pantůček, R. (2018). Rapid identification of intact staphylococcal bacteriophages using matrix-assisted laser desorption ionization-time-of-flight mass spectrometry. *Viruses*, *10*(4), 176. https://doi.org/10.3390/v10040176

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Turapov, O. A., Mukamolova, G. V., Bottrill, A. R., & Pangburn, M. K. (2008). Digestion of native proteins for proteomics using a thermocycler. *Analytical Chemistry*, *80*(15), 6093–6099. https://doi.org/10.1021/ac702527b

Links for supplies.

Trypsin gold

Price: CAD 145

https://www.promega.ca/products/mass-spectrometry/trypsin/trypsin-gold-mass-spectrometry-grade/?catNum=V5280#protocols

SurePAGE™, Bis-Tris, 10x8, 4-12%, 12 wells per 10

Price: CAD 115

https://www.genscript.com/molecule/M00653-SurePAGE_Bis_Tris_10x8_4_12_12_wells.html

MOPS buffer

Price: CAD 40

https://www.genscript.com/molecule/M00138-Tris_MOPS_SDS_Running_Buffer_Powder_.html

MES buffer

Price: Free

Bovine Serum Albumin PER 10G

Price: CAD 176

https://www.sigmaaldrich.com/CA/en/product/sigma/a7030

Trifluoroacetic acid, 99%, extra pure, Thermo Scientific Chemicals

Price: CAD 62.26

https://www.fishersci.ca/shop/products/trifluoroacetic-acid-99-extra-pure-thermo-scientific/AC139720250#