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

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Abstract

This study aimed to develop a methodology for the preparation of bacteriophage structural protein samples for MALDI-TOF MS identification, using three bacteriophage strains (EC1KELCTY, EC1KELHOS and EC3KAMCTY). The bacteriophages were propagated on plate lysates using their respective bacterial hosts. For the protein gel, the phage lysates were treated with 1X SDS sample buffer and analyzed on a 10% SDS-PAGE gel. Individual protein bands were excised for in-gel trypsin digestion to extract peptides for MALDI-TOF MS analysis. Mass spectra were acquired using FlexControl software, with a range of 1 kDa to 9 kDa. Data analysis performed using the MASCOT search engine indicated limitations in the methodology, as the BSA control was not accurately identified. While peptide fragments corresponding to structural proteins of the bacteriophages were recognized, their reliability is uncertain due to potential methodological inconsistencies. Incorporation of desalting columns to the sample preparation procedure prior to mass spectra acquisition with MALDI is required to remove salts that may interfere with mass spectrometry results.

Introduction

Today, some of the most common and effective techniques for microorganism identification are 16S rRNA and 18S rRNA gene sequencing, widely regarded as the gold standard for microbial characterization [1]. However, these methods are expensive, time-consuming, and labor-intensive, making them impractical for routine clinical use. In recent years, Matrix-Assisted Laser Desorption/Ionization–Time-of-Flight Mass Spectrometry (MALDI-TOF MS) has emerged as a highly efficient tool for microbial identification and diagnosis [1]. Unlike gene sequencing, MALDI-TOF MS relies on minimal sample preparation and is significantly faster, cheaper, and more accurate for many applications. The primary cost lies in the initial purchase of the MALDI-TOF instrument, but the low operational and consumable expenses make it a highly scalable technique for high-throughput use.

While MALDI-TOF MS is widely used for identifying bacteria and fungi, it has not yet become a routine method for bacteriophage protein identification. One of the main challenges in applying MALDI-TOF to bacteriophage research is the need to establish optimized sample preparation methodologies. An optimized sample preparation procedure is essential to ensure high-quality and reproducible mass spectra, as suboptimal methods can introduce variability and interfere with data analysis [2].

In proteomics, sample preparation is critical for the success of mass spectrometry-based analyses. In-gel digestion with trypsin, an endopeptidase with high cleavage specificity and stability, is one of the most widely used techniques due to its efficiency and ability to produce clean, high-quality peptide samples. Traditional solution-based digestion methods often use chemical denaturants such as urea or guanidine hydrochloride, which require extensive

purification steps to remove contaminants incompatible with mass spectrometry. These contaminants include salts and detergents that can interfere with the ionization during MALDI-TOF MS analysis, resulting in poor spectral quality [4]. In-gel digestion can mitigate these challenges by removing interfering substances during washing steps resulting in peptides optimal for MS analysis standards such as Bovine Serum Albumin (BSA) are commonly used to validate the in-gel digestion and subsequent analysis with MALDI-TOF MS [5]. BSA is a well-characterized protein that serves as a benchmark due to its stability, known sequence, and predictable peptide fragmentation pattern. In addition to BSA, synthetic peptides or commercially available protein standards can also be used to calibrate the MALDI-TOF instrument and verify the accuracy of mass measurements.

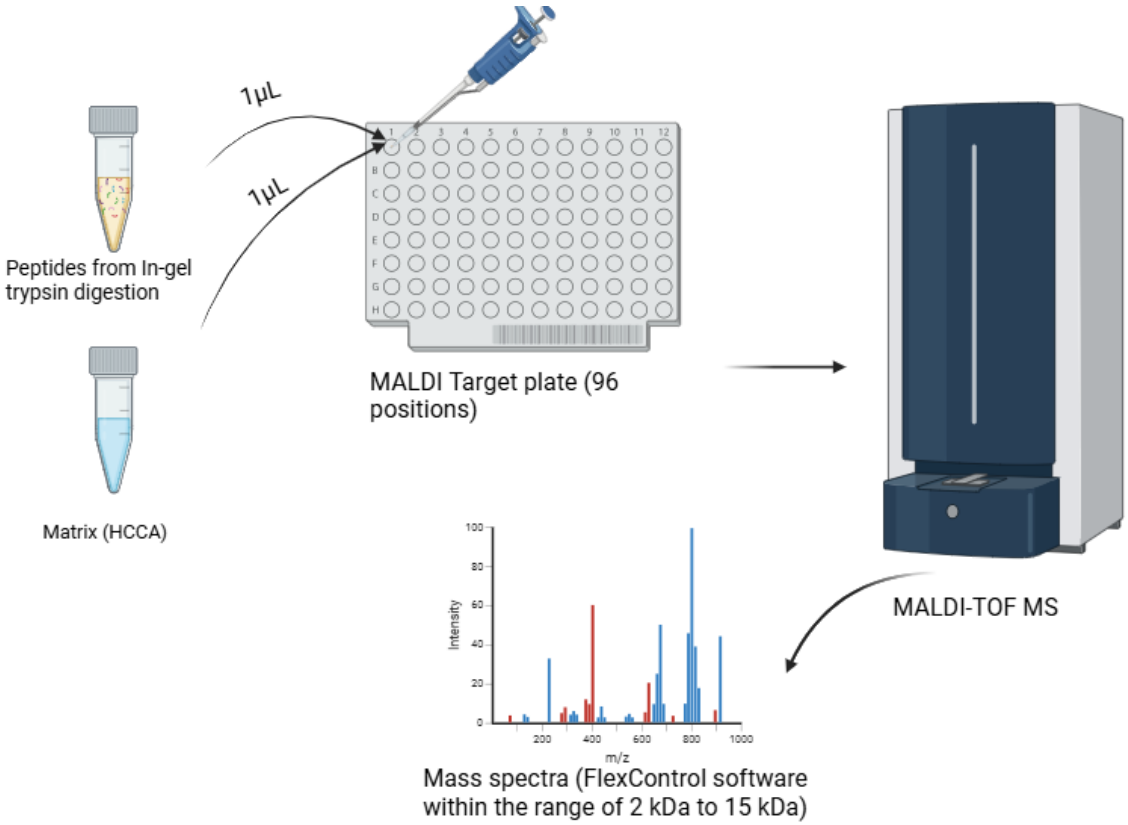

Figure 1. Schematic diagram showing MALDI-TOF MS fingerprinting method and sample preparation. Resulting peptides from in gel trypsin digestion and mixed with equal parts of matrix (HCCA) and pipetted on MALDI target plate, once dry the plate is then placed on MALDI-TOF-MS to obtain mass spectra with FlexControl Software.

This study aimed to identify bacteriophage proteins from different phage strains using SDS-PAGE and in-gel trypsin digestion as a sample preparation for MALDI-TOF-MS analysis (Figure 1). The findings of the study demonstrate the potential of combining SDS-PAGE, in-gel trypsin digestion, and MALDI-TOF MS for bacteriophage protein identification, highlighting its relevance for advancing phage proteomics and optimizing workflows for broader applications.

Methods

1. Phage Lysate Preparation

Bacteriophage lysates were prepared by inoculating nutrient broth containing a respective overnight bacterial host (E. coli #1 and E. coli #3 strains) (Table 1) with 100 µL of diluted phage suspension to yield a high-titre final product. The mixture was combined with 3 mL of soft agar (0.3%) [6] and poured evenly over the bottom nutrient agar layer. Control plates containing only the *E. coli* overnight culture and 0.3% soft agar was prepared in parallel. All plates were incubated at room temperature overnight. The following day, 3 mL of phosphate-buffered saline (PBS, 1X) was added to each plate, and the plates were incubated again at room temperature overnight. The PBS containing phage particles was recovered the next day, centrifuged at 5,000 × g for 10 minutes, and the resulting supernatant was filter-sterilized and stored in sterile tubes at 4°C for further analysis [6].

Table 1. Summary of bacteriophage strains samples used and respective host.

Bacteriophage strains	**Bacterial Host**
EC1KELCTY	*Escherichia coli (E. coli 1)*
EC1KELHOS	*Escherichia coli (E. coli 1)*
EC3KAMCTY	*Escherichia coli (E. coli 3)*

2. Small Drop Plaque Assay

Eight 10-fold serial dilutions of bacteriophage lysates from three strains (EC1KELCTY, EC1KELHOS, and EC3KAMCTY) were prepared in sterile 1.5 mL Eppendorf tubes using 1X phosphate-buffered saline (PBS). For each dilution, 20 µL of the respective bacteriophage lysate was mixed 180 µL of 1X PBS. A nutrient agar plate was divided into nine equal sections to accommodate the serial dilutions (Figure 2) and a control spot containing only the *E. coli* culture [6]. Each bacteriophage dilution mixed with *E. coli* was carefully spotted onto its respective section on the nutrient agar plate. The plates were incubated at room temperature overnight. The following day, plaques were counted to determine the titer of phage lysates.

3. SDS-PAGE Gel Sample Preparation

Bovine Serum Albumin (BSA)

A BSA stock solution was reconstituted in 1X phosphate-buffered saline (PBS) to a final concentration of 10 mg/ml. The stock (500 µL) was subsequently diluted to the desired concentration by serial dilution (1:1) using 1X PBS and mixed with an equal volume of SDS sample buffer (1X) containing freshly added dithiothreitol (DTT, 100 mmol/L) in PCR tubes [6].

The prepared samples were heated in a thermocycler at 95°C for 5 minutes and then transferred to 1.5 mL Eppendorf tubes. The samples were centrifuged at 15,060 rpm for 5 minutes to remove debris and stored at -20°C until further use [6].

Bacteriophage Lysate Processing

Equal volumes of bacteriophage lysate and SDS sample buffer (1X) with freshly added DTT (100 mmol/L) were mixed in PCR tubes [6]. The mixture was heated in a thermocycler at 100°C for 10 minutes, no centrifugation was performed after reduction of disulfide bonds with DTT. Samples were stored at -20°C until further use.

SDS-PAGE Gel Preparation and Electrophoresis

The resolving gel (10%) for SDS-PAGE was prepared using 30% acrylamide, 1.5 M Tris-HCl buffer (pH 8.8), 10% (w/v) sodium dodecyl sulfate (SDS), 10% (w/v) ammonium persulfate (APS), tetramethylethylenediamine (TEMED), and deionized water. Polymerization was initiated by adding APS and TEMED immediately before pouring the gel into the casting apparatus [7].

After the resolving gel had fully polymerized, a stacking gel (5%) was prepared using 30% acrylamide, 1.0 M Tris-HCl buffer (pH 6.8), 10% (w/v) SDS, 10% (w/v) APS, TEMED [7], and deionized water. The stacking gel was poured over the resolving gel, and a comb was inserted to create wells. Once the stacking gel polymerized, the gel was ready for protein electrophoresis.

Protein electrophoresis was performed at a constant voltage of 200 V for 30 minutes.

SDS-PAGE gel staining with Coomassie Blue Staining G-250

Gel staining was conducted using a solution containing 80 mg of Coomassie Blue G-250 dissolved in 1 liter of deionized water, acidified with HCl to a final concentration of 35 mM [8].

The gel was washed three times with deionized water, followed by incubation in the staining solution overnight at room temperature. After staining, the gel was destined in deionized water until the background became clear. All steps were performed at room temperature.

SDS-PAGE gel band processing:

In- gel digestion procedure was based on Boisvert Lab procedure [9]. Protein bands of interest were excised from the gel using a clean scalpel and further sliced into ~2 × 2 mm cubes. Gel slices were placed into sterile 1.5 mL LoBind Eppendorf tubes and stored at –20°C until further processing.

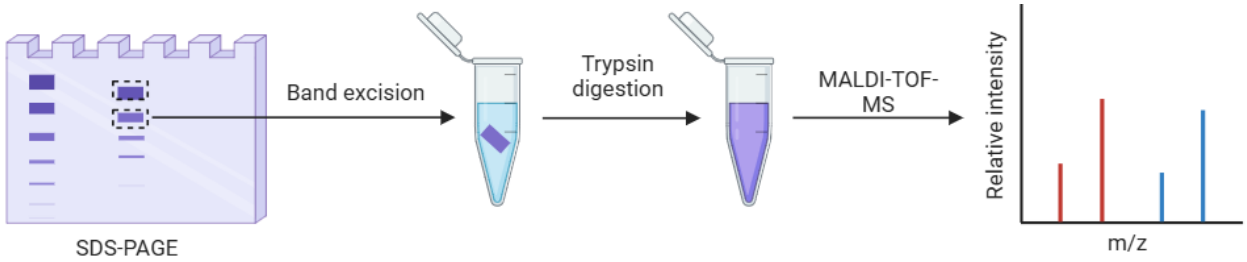

Figure 2. In gel trypsin digestion methodology. Desired gel bands are excised from SDS-PAGE gel and placed in a LoBind eppendorf tube, bands are washed in order to remove the dye from bands and trypsin digestion is perform, once peptides had been extracted solution is mixed with matrix and analyzed with MALDI-TOF-MS to obtain mass spectra.

To prepare the gel pieces for digestion, band slices were washed to remove the dye. Extracted bands were washed with 300 µL of deionized water and incubated at room temperature for 15 minutes. Secondly, 300 µL of acetonitrile (CH_3CN) were added and bands were incubated at room temperature for a further 15 minutes. The supernatant was removed using a P1000 micropipette fitted with a P10 tip to prevent loss of gel pieces.

Bands were washed with 300 µL of 20 mM ammonium bicarbonate (NH_4HCO_3) and left in solution for 15 minutes, supernatant was removed and followed by a wash with 300 µL of a 50:50 (v/v) solution of 20 mM NH_4HCO_3, CH_3CN and left in solution for 15 minutes till bands look shrink and opaque [9]. The supernatant was discarded, and the gel pieces were visually inspected for complete destaining.

To dehydrate the gel pieces, 150 µL of CH_3CN was added, incubated for 5 minutes, and then removed. This step was repeated until the gel pieces appeared fully white and opaque. Gel pieces were dried in a SpeedVac concentrator for 5 minutes.

Trypsin Digestion

Trypsin digestion was performed by rehydrating gel slices in a digestion buffer containing 12.5 ng/µL modified trypsin (Promega, V5280) in 20 mM NH_4HCO_3. Each gel slice was covered with 50–75 µL of digestion buffer and allowed to rehydrate for 30 minutes at room temperature. The samples were incubated in LoBind Eppendorf tubes at 30°C overnight to ensure complete protein digestion [9].

Peptide Extraction

Peptides were extracted from the digested gel pieces through the following steps:

An equal volume of CH_3CN (50–75 µL) was added to each tube and incubated at 30°C for 30 minutes. The supernatant, containing extracted peptides, was transferred to new LoBind Eppendorf tubes.

Gel pieces were incubated twice with 50–75 µL of freshly prepared 1% formic acid for 20 minutes each. Supernatants were collected after each step and pooled with the extracts.

A final extraction step was performed with 150 µL of CH_3CN, incubated for 10 minutes. The collected extracts were dried completely using a SpeedVac concentrator at 60°C [9]. The dried peptide pellets were resuspended in 0.1% trifluoroacetic acid (TFA) before further analysis.

MASCOT Search Criteria:

The Swiss-Prot database was utilized to identify peptide fragments, with the taxonomy restricted to viruses for bacteriophage mass spectra analysis and Eukarya for BSA.

Table 2. Mascot search parameters for peptide fragments identification.

Parameter	**Value**
Type of Search	Peptide Mass Fingerprint
Enzyme	Trypsin
Mass Values	Monoisotopic
Protein Mass	Unrestricted
Peptide Mass Tolerance	± 0.2 Da
Peptide Charge State	1+
Max Missed Cleavages	1
Database	Swiss-Prot

Results and discussion

Bacteriophage lysate: Small Drop Plaque Assay

The small drop plaque assay was conducted to assess the viability of the bacteriophages and to determine the optimal dilution factor for phage lysate preparation. The assay confirmed that the bacteriophages remained viable, as evidenced by the formation of clear plaques.

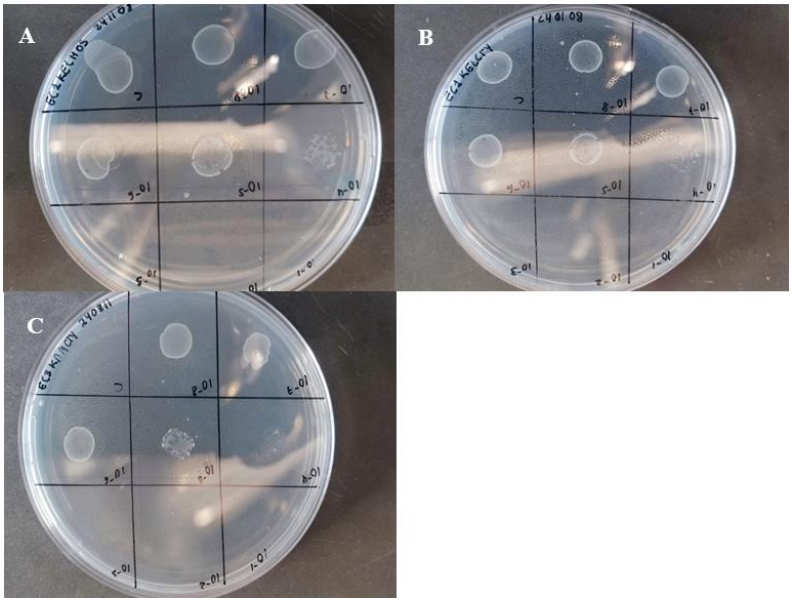

Figure 3. Small drop plaque assay results used to determine dilution necessary to produce phage lysates. A: EC1KELHOS plate optimal dilution 10^{-4}. B: EC1KELCTY plate optimal dilution 10^{-4}. C:EC3KAMCTY optimal dilution was found between 10^{-4} and 10^{-5} due to the almost clear results using a lower dilution.

Bacteriophage lysates preparation

Based on the results of the small drop plaque assay, a consistent dilution factor of 1×10^{-4} was applied for the preparation of bacteriophage lysates for EC1KELHOS and EC1KELCTY. Ultimately, the lysates were recovered from the plates with the lower dilution factor (1×10^{-4}), as these plates displayed a more distinct and uniform plaque pattern (Figure 4).

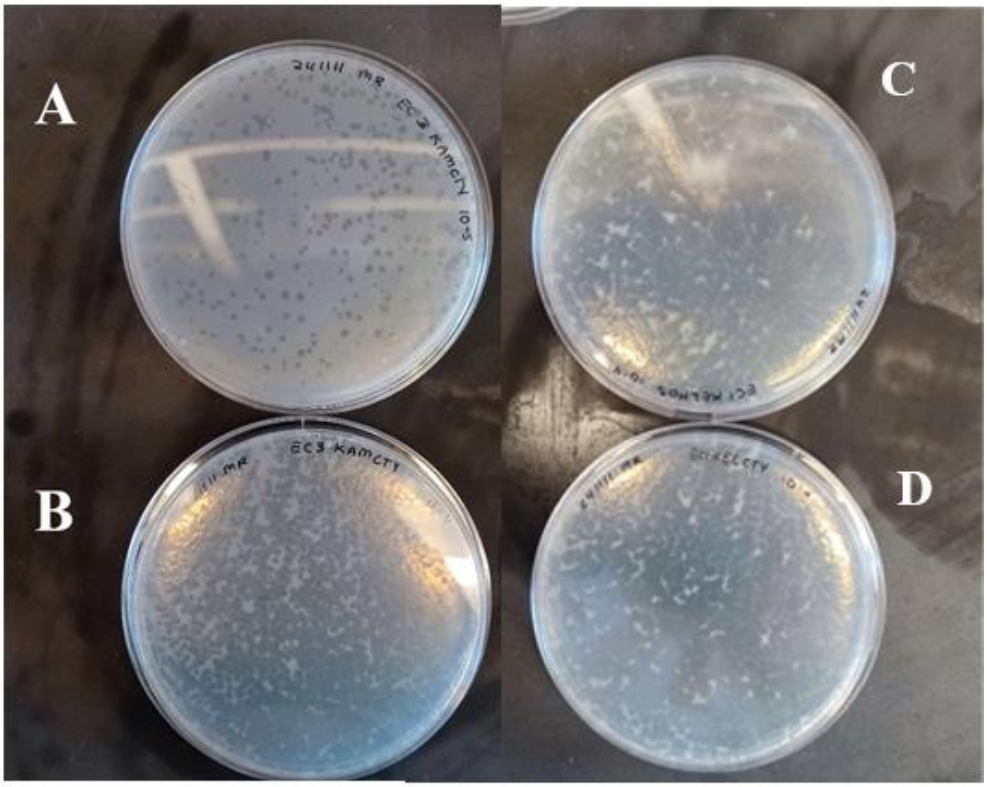

Figure 4. Phage lysate plates with characteristic plaque pattern where some bacteria cells are still visible. A:EC3KAMCTY plate (dilution factor used 10^{-5}). B: EC3KAMCTY plate (dilution factor 10^{-4}).C:EC1KELHOS plate (dilution factor 10^{-4}). D: EC1KELCTY plate (dilution factor 10^{-4}).

Effect of Storage Time on Phage Lysates for Protein Detection Using SDS-PAGE.

Lysates from three bacteriophage strains (EC1KELHOS, EC1KELCTY, and EC3KAMCTY) were stored for different durations (5 months, 1 month, and 1 week) (Figure 5) and analyzed to assess the impact of storage time on protein integrity. Results indicated that freshly prepared phage lysates exhibited higher protein density and reduced degradation compared to older samples. Lysates stored for 5 months showed less than half the protein density of the 1-week samples, emphasizing the importance of lysate freshness for protein stability.

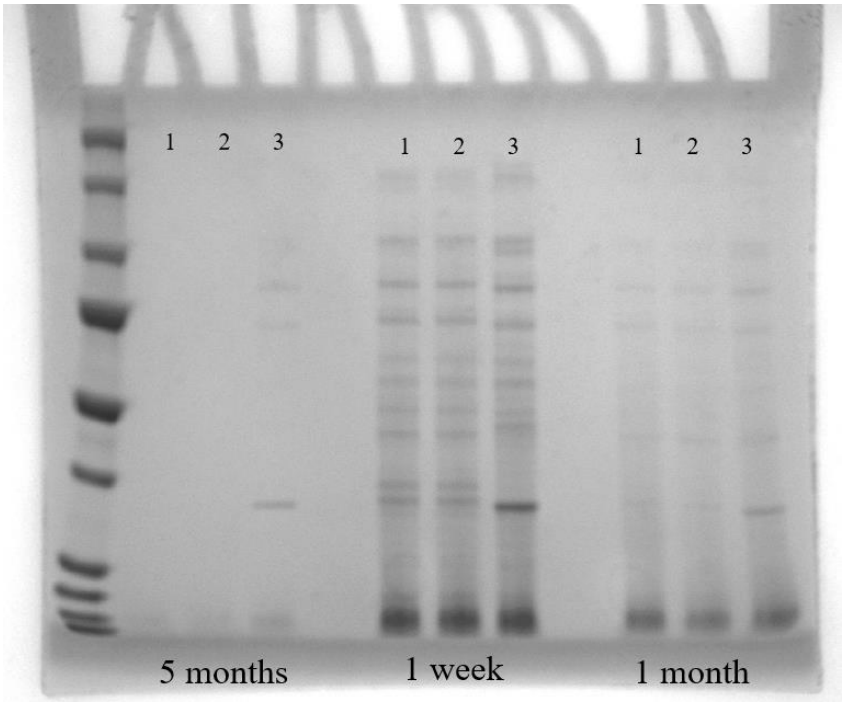

Figure 5. SDS-PAGE pre-cast gradient gel (4%-12%) first well containing BioRad precision Plus Protein Standards, (1) EC1KELHOS, (2) EC1KELCTY, (3) EC3KAMCTY.

MALDI-TOF analysis of peptides from in gel trypsin digestion.

The 10% SDS-PAGE gel demonstrates the separation of proteins from bacteriophage lysates and BSA standards to evaluate protein composition and estimate protein concentrations in the lysates (Figure 6).

Lane 1 contains molecular weight markers (Bio-Rad Precision Plus Protein Standards), serving as a reference for estimating protein sizes. Lanes 2, 3, and 4 show BSA standards at concentrations of 50 µg/mL, 25 µg/mL, and 12.5 µg/mL, respectively. These BSA standards were included to estimate the protein concentration in the bacteriophage samples by comparing the intensity of the lysate protein bands with the BSA standards.

Lanes 6, 8, and 10 display the protein profiles of bacteriophage lysates: EC1KELHOS (magenta bands), EC1KELCTY (blue bands), and EC3KAMCTY (green bands). The distinct banding patterns observed in each lysate highlight differences in protein composition among the phage strains. Proteins of interest, indicated by highlighted bands, were excised for further analysis through in-gel trypsin digestion.

The intensity of the protein bands in the bacteriophage lysates, when compared to the BSA standards, provides an approximation of protein concentration, with differences potentially attributable to lysate preparation, storage conditions, or intrinsic properties of the phage strains. This approach underscores the utility of BSA as a standard for quantitative comparison in SDS-PAGE analysis.

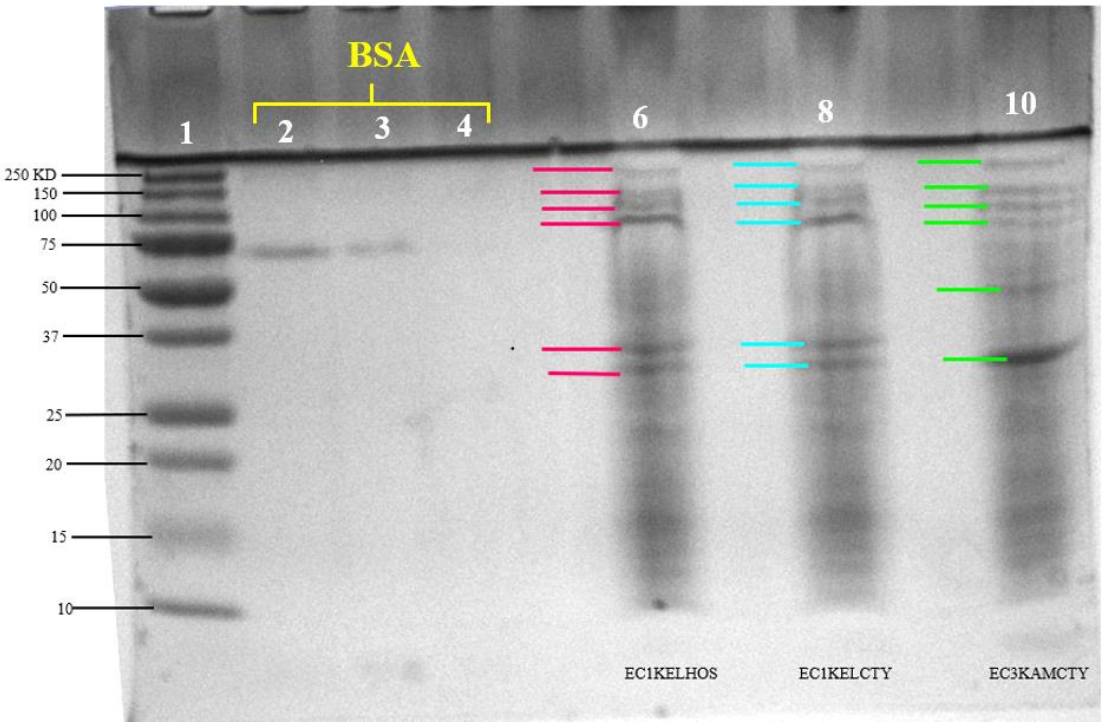

Figure 6. 10% SDS-PAGE gel run with Tri's buffer (pH 8.3) at 200V for 30 minutes. Lane 1 contains Bio-Rad Precision Plus Protein Standards (molecular weight markers labeled from 10 kDa to 250 kDa). Lanes 2, 3, and 4 contain bovine serum albumin (BSA) at concentrations of 50 µg/mL, 25 µg/mL, and 12.5 µg/mL, respectively. Lanes 6, 8, and 10 contain bacteriophage lysates: EC1KELHOS (magenta bands), EC1KELCTY (blue bands), and EC3KAMCTY (green bands). Highlighted bands correspond to proteins of interest excised for in-gel trypsin digestion. All bands extracted were digested together according to the strain these were isolated from.

The bands obtained in the gel presented some background degradation that could be due to the time that the phage lysates were stored.

For the gel samples preparation, different digestion temperatures were used for BSA (95°C) and bacteriophage samples (100°C), following established protocols in literature. This difference raises brings an important consideration regarding the structural complexity of the proteins in each sample and their response to thermal denaturation. For bacteriophages, which contain highly stable structural proteins, increasing the temperature to 100°C helps to overcome intermolecular interactions and disulfide bond that may otherwise impede digestion.

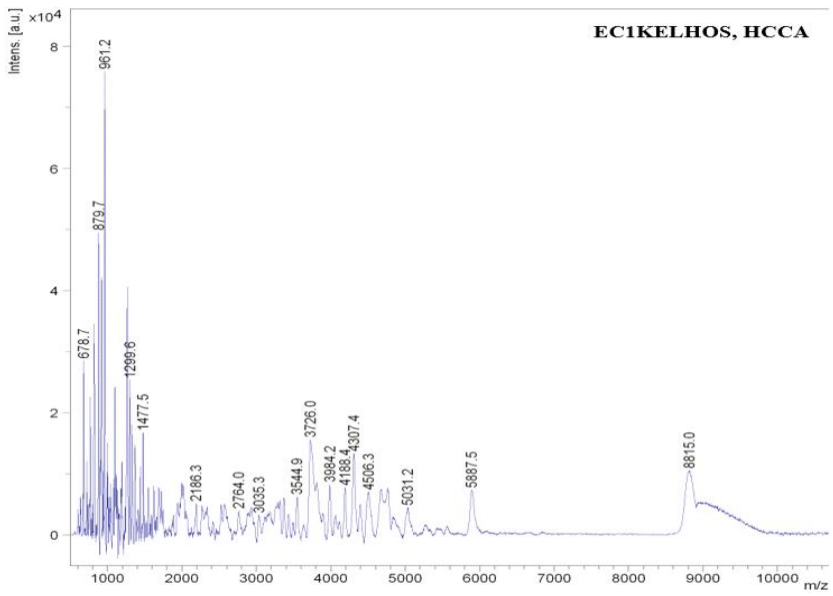

Figure 7. MALDI mass spectra of EC1KELHOS bacteriophage strain proteolytic digest with trypsin obtained using HCCA matrix. Bands excised from a phage lysate (3×108 PFU/mL) protein gel (10% SDS PAGE) (Figure 6) gel were used as the analytical input for the in-gel trypsin protein digestion.

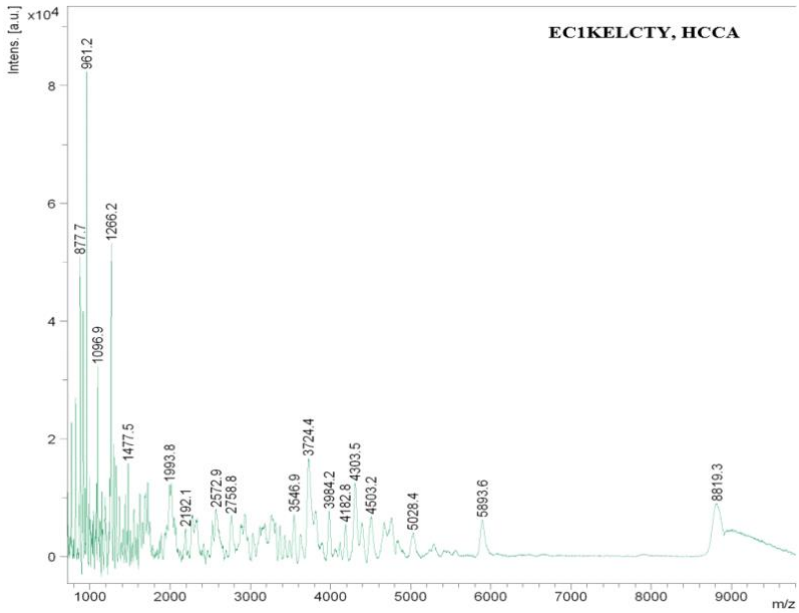

Figure 8. MALDI mass spectra of EC1KELCTY bacteriophage strain proteolytic digest with trypsin obtained using HCCA matrix. Bands excised from a phage lysate (3×108 PFU/mL) protein gel (10% SDS PAGE) (Figure 6) gel were used as the analytical input for the in-gel trypsin protein digestion.

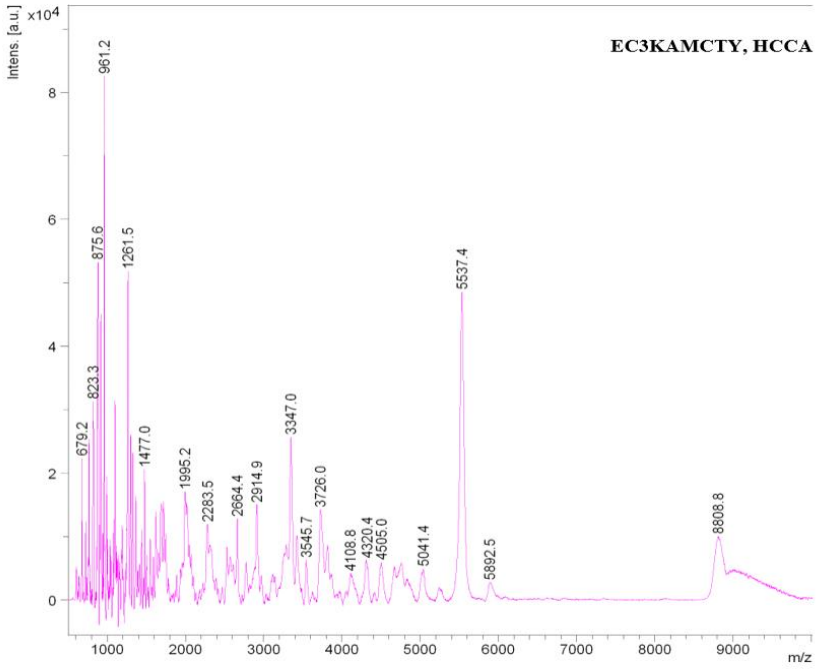

Figure 9. MALDI mass spectra of EC1KAMCTY bacteriophage strain proteolytic digest with trypsin obtained using HCCA matrix. Bands excised from a phage lysate (3×108 PFU/mL) protein gel (10% SDS PAGE) (Figure 6) gel were used as the analytical input for the in-gel trypsin protein digestion.

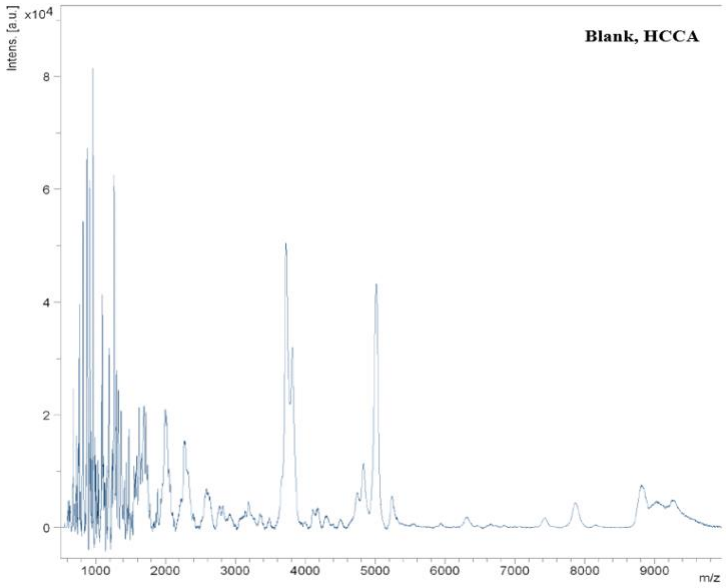

Figure 10. MALDI mass spectra of blank obtained using HCCA matrix. The spectrum displays characteristic matrix peaks predominantly in the lower m/z region (<1000 m/z) and minimal signal intensity in the higher m/z range, indicating the absence of analytes in the blank. Any peaks observed outside the expected matrix region may be attributed to background noise or potential contamination.

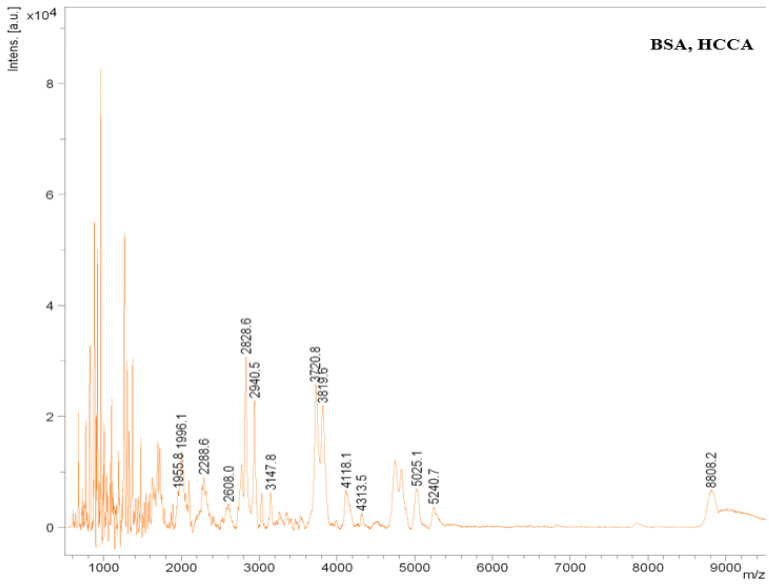

Figure 11. MALDI mass spectra of BSA proteolytic digest with trypsin obtained using HCCA matrix. Bands excited (Figure 6) from BSA (50 ug/ml) sample ran with SDS sample buffer (1x) in 10% SDS-PAGE gel were used as the analytical input for the in-gel trypsin protein digestion method validation.

The identification of proteins from bacteriophage strains EC1KELHOS, EC1KELCTY, and EC3KAMCTY. Mass spectra for EC1KELHOS and EC1KELCTY (figure 7 and 8) present similar m/z pattern as expected due to the similarity between these two strains, EC3KAMCTY (figure 9) presented a slightly different spectra than the other strains, with significant peak around 5537m/z.

A MASCOT search was performed using the mass spectrometry. For the EC1KELHOS strain (figure 7), the highest protein score obtained was 32 (P > 55) for the D3_FOWPN core protein (Fowlpox virus strain NVSL), with 25% sequence coverage. Similarly, for the EC1KELCTY strain (figure 8), the highest protein score was 36 (P > 55) for CAPSD_BPPM2 major capsid protein P2 (Pseudoalteromonas phage PM2), with 28% sequence coverage. The EC3KAMCTY strain (figure 9) yielded a maximum protein score of 42 (P > 55) for MATRX_BDV1 matrix protein (Borna disease virus 1), with 42% sequence coverage.

Despite these results, none of the analyzed samples achieved statistically significant protein scores based on the Mascot scoring system. The Mascot score is calculated as $-10 \times \log(P)$, where P represents the probability that the observed match is a random event. Scores below the threshold ($P > 55$) indicate low confidence in the protein identifications, suggesting that the matches are likely random and statistically insignificant.

Furthermore, bovine serum albumin (BSA), used as a control, was not successfully identified. The protein score for P02769 (bovine serum albumin precursor) was 3 ($P > 36$), with only 2% sequence coverage.

The low protein scores observed across all samples may be attributed to poor data quality, potentially arising from suboptimal experimental conditions such as sample preparation, digestion efficiency, or instrument calibration. Improving experimental conditions could enhance data quality and yield higher protein scores, thereby increasing confidence in the protein identifications. Optimizations may include refining the protein digestion protocol, increasing sample purity, or ensuring adequate instrument sensitivity and accuracy. These adjustments are critical for reliable protein identification and downstream applications.

One critical step often required prior to MALDI-TOF analysis during in-gel trypsin digestion is the desalting of peptide samples using ZipTips. These tips, containing a polymer matrix, effectively remove salts and other contaminants that interfere with MALDI ionization and compromise spectral accuracy. This step was omitted due to the unavailability of ZipTips during the experiment, which likely contributed to lower signal intensity and reduced peptide matches. Incorporating this step in future experiments is essential to improve peptide recovery, enhance ionization efficiency, and ensure accurate mass spectrometric analysis.

Previous successful identification of bacteriophage structural peptides has been achieved using in-gel trypsin digestion, as demonstrated in studies such as Clément's (2021) research on bacteriophage Vp16T [10]. Their findings reinforce the efficacy of this approach in phage proteomics, highlighting its potential for characterizing viral proteins.

Similarly, other studies employing liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) have identified structural peptides from bacteriophages infecting Streptococcus species, providing insights into host-phage interactions [10]. The success of our in-gel digestion protocol in generating identifiable peptides, despite some limitations in sample preparation, aligns with these previous findings and supports its further refinement for enhanced spectral quality and protein identification

Additionally, for the in-gel trypsin digestion method the use of a thermomixer is suggested for better peptide extraction during incubation. Instead, the samples were place in a incubator and vortex every 5 minutes to ensure an efficient peptide extraction, further refinements to the sample preparation process, including the optimization of digestion conditions (e.g., trypsin concentration, incubation time, and temperature), are warranted to improve peptide yield and coverage.

Conclusion

This study attempted to develop and validate a methodology for identifying bacteriophage structural proteins through SDS-PAGE and MALDI-TOF MS analysis. SDS-PAGE results revealed that freshly prepared lysates retained higher protein density and exhibited reduced degradation compared to lysates stored for extended periods. This underscores the critical importance of lysate freshness for preserving protein integrity and ensuring accurate downstream analyses.

The subsequent MALDI-TOF analysis of peptides obtained from in-gel trypsin digestion provided preliminary identification of structural proteins, including capsid proteins from bacteriophage strains EC1KELCTY, EC1KELHOS, and EC3KAMCTY, with sequence coverage ranging from 19% to 28%.

The spectra also showed similarities between EC1KELCTY and EC1KELHOS, suggesting similar structure of these strains, this could be a reason both are able to interact with the same bacterial host. However, issues with sample preparation and the omission of key steps, such as peptide desalting with ZipTips, likely contributed to reduced spectral quality and the poor identification of the BSA control (2% sequence match). Secondly, when selecting bands for in-gel digestion, these were digested together according to its strain. However, samples turned out to be more complex than expected. For analysis optimization, bands could be digested and analyzed separately to improve the quality of spectra and facilitate peptide identification.

The study highlights the need for improvements in the sample preparation process, including the incorporation of peptide desalting steps, optimization of digestion conditions, and enhanced incubation methods (e.g., using a thermomixer). These refinements will improve

peptide recovery, ionization efficiency, and spectral accuracy, thereby facilitating the validation of the proposed methodology for routine application in phage proteomics.

Overall, the results provide valuable insights into the effect of storage time on bacteriophage protein stability and offer a foundation for further optimizing MALDI-TOF MS protocols for structural protein analysis. Future work should focus on addressing the identified limitations to enhance the robustness and reliability of the method, ultimately enabling its application in characterizing bacteriophage proteins with greater precision.

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Appendix

Table 3. EC1KELCTY protein identification results from MALDI-TOF-MS spectra using MASCOT data base. Organized from most relevant to less relevant results.

Protein ID	Mass (Da)	Score	Expect	Matches	Description*
CAPSD_BPPM2	30180	36	4.6	2	Major capsid protein P2 OS=Pseudoalteromonas phage PM2 OX=2905728 GN=II PE=1 SV=2
VE4_HPV39	10191	24	71	1	Protein E4 OS=Human papillomavirus 39 OX=10588 GN=E4 PE=3 SV=2
CAPSD_CTV36	24894	21	1.40E+02	1	Capsid protein OS=Citrus tristeza virus (isolate T36) OX=31712 PE=1 SV=1
VE6_HPV1	16306	21	1.50E+02	1	Protein E6 OS=Human papillomavirus type 1 OX=10583 GN=E6 PE=3 SV=1
PKG9_BPPRD	25770	20	1.90E+02	1	DNA packaging ATPase P9 OS=Enterobacteria phage PRD1 OX=10658 GN=IX PE=1 SV=3
E1A_ADECC	25346	20	1.90E+02	1	Early E1A protein OS=Canine adenovirus serotype 1 (strain CLL) OX=69150 PE=3 SV=1
YO15_ADEG1	11898	19	2.40E+02	1	Uncharacterized protein ORF15 OS=Fowl adenovirus A serotype 1 (strain CELO / Phelps) OX=10553 GN=15 PE=4 SV=1
CAP8_ADE05	24671	19	2.40E+02	1	Pre-hexon-linking protein VIII OS=Human adenovirus C serotype 5 OX=28285 GN=L4 PE=1 SV=1
E1A_ADECR	19323	18	2.50E+02	1	Early E1A protein OS=Canine adenovirus serotype 1 (strain RI261) OX=69151 PE=3 SV=1
HBSAG_HBVF6	43552	18	2.50E+02	1	Large envelope protein OS=Hepatitis B virus genotype F2 subtype adw4q (isolate Senegal/9203) OX=489503 GN=S PE=1 SV=2
DNBI_EHV1	40284	18	2.60E+02	1	Major DNA-binding protein (Fragment) OS=Equine herpesvirus 1 (strain HVS25A) OX=10327 PE=3 SV=1

PACK_BPCPT	12252	18	2.80E+02	1	Putative packaging signal terminase OS=Vibrio phage CP-T1 OX=10689 PE=4 SV=1
ME53_NPVOP	52224	18	2.80E+02	1	Early 53 kDa protein OS=Orgyia pseudotsugata multicapsid polyhedrosis virus OX=262177 GN=ME53 PE=4 SV=1
A1_BPT5	61425	18	3.00E+02	1	Protein A1 OS=Escherichia phage T5 OX=2695836 GN=A1 PE=1 SV=1

**Description: (OS: Organism species), (OX: Organism Taxonomic Identifier), (GN: Gene Name), (PE: Protein Existence) and (SV: Sequence Version).*

Table 4. EC1KELHOS protein identification results from MALDI-TOF-MS spectra using MASCOT data base. Organized from most relevant

to less relevant results.

Protein ID	Mass (Da)	Score	Expect	Matches	Description*
D3_FOWPN	31941	34	6.5	2	27 kDa core protein OS=Fowlpox virus (strain NVSL) OX=928301 GN=FPV069 PE=2 SV=1
D3_FOWPV	31941	34	6.5	2	27 kDa core protein OS=Fowlpox virus OX=10261 GN=FPV069 PE=2 SV=1
HBSAG_HBVB5	43722	31	14	2	Large envelope protein OS=Hepatitis B virus genotype B2 (isolate Vietnam/16091/1992) OX=489462 GN=S PE=1 SV=1
HBSAG_HBVB6	43589	31	14	2	Large envelope protein OS=Hepatitis B virus genotype B2 subtype adw (isolate China/patient4/1996) OX=489463 GN=S PE=1 SV=1
VE8_BPV2	8529	24	74	1	Uncharacterized protein E8 OS=Bos taurus papillomavirus 2 OX=2758382 PE=4 SV=2
X_HBVB3	16677	22	1.20E+02	1	Protein X OS=Hepatitis B virus genotype B2 (isolate Vietnam/9873/1997) OX=489461 GN=X PE=3 SV=1

X_HBVB5	16498	22	1.20E+02	1	Protein X OS=Hepatitis B virus genotype B2 (isolate Vietnam/16091/1992) OX=489462 GN=X PE=3 SV=1
CAPSD_BPMS2	13851	20	1.60E+02	1	Capsid protein OS=Escherichia phage MS2 OX=12022 PE=1 SV=2
M2_I68A5	11212	20	1.90E+02	1	Matrix protein 2 OS=Influenza A virus (strain A/Korea/426/1968 H2N2) OX=488241 GN=M PE=3 SV=1
Y06E_BPT4	20668	20	2.00E+02	1	Uncharacterized 20.7 kDa protein in vs-regB intergenic region OS=Enterobacteria phage T4 OX=10665 GN=y06E PE=4 SV=1
VME1_IBVDE	25540	19	2.20E+02	1	Membrane protein OS=Avian infectious bronchitis virus (strain DE072) OX=233265 GN=M PE=3 SV=2
ENDON_BPLP2	11181	19	2.20E+02	1	Probable HNH endonuclease OS=Lactococcus phage p2 OX=254252 PE=3 SV=1
ENDON_BPLSK	11181	19	2.20E+02	1	Probable HNH endonuclease OS=Lactococcus phage SK1 OX=2905675 PE=3 SV=1
APYC1_BPBSP	29033	19	2.30E+02	1	Anti-Pycsar protein Apyc1 OS=Bacillus phage BSP38 OX=2283013 GN=BSP38_126 PE=1 SV=1

**Description: (OS: Organism species), (OX: Organism Taxonomic Identifier), (GN: Gene Name), (PE: Protein Existence) and (SV: Sequence Version).*

Table 5. EC3KAMCTY protein identification results from MALDI-TOF-MS spectra using MASCOT data base. Organized from most relevant to less relevant.

Protein ID	Mass (Da)	Score	Expect	Matches	Description*
MATRX_BDV1	16248	42	1.2	3	Matrix protein OS=Borna disease virus 1 OX=1714621 GN=M PE=1 SV=1
MATRX_BDVV	16234	42	1.2	3	Matrix protein OS=Borna disease virus (strain V) OX=928296 GN=M PE=1 SV=1
TRX1_SHV21	37361	22	1.20E+02	2	Triplex capsid protein 1 OS=Saimiriine herpesvirus 2 (strain 11) OX=10383 GN=TRX1 PE=3 SV=1
ORF2_TTVI1	8038	20	1.60E+02	1	Uncharacterized ORF2 protein OS=Torque teno sus virus 1 (isolate Sd-TTV31) OX=766190 GN=ORF1 PE=4 SV=1
VNB_INBUS	10994	20	1.60E+02	1	Glycoprotein NB OS=Influenza B virus (strain B/USSR/100/1983) OX=230286 GN=NB PE=3 SV=1

**Description: (OS: Organism species), (OX: Organism Taxonomic Identifier), (GN: Gene Name), (PE: Protein Existence) and (SV: Sequence Version).*

Table 6. BSA protein identification results from MALDI-TOF-MS spectra using MASCOT data base. Organized from most relevant to less relevant.

Protein ID	Mass (Da)	Score	Expect	Matches	Description*
Q7Z3Y9	51878	12	16	2	Keratin, type I cytoskeletal 26 OS=Homo sapiens OX=9606 GN=KRT26 PE=2 SV=1

P01030	192676	3	1.20E+02	2	Complement C4-A precursor OS=Bos taurus OX=9913 GN=C4A PE=1 SV=1
ENSBTAP00000007350	192871	3	1.20E+02	2	Complement C4-A precursor OS=Bos taurus OX=9913 PE=1 SV=1
XP_001474382	11956	9	32	1	RIKEN cDNA 1110025L11 OS=Mus musculus OX=10090 GN=1110025L11Rik PE=2 SV=1
P07477	26541	8	38	1	Trypsin-1 precursor OS=Homo sapiens OX=9606 GN=PRSS1 PE=1 SV=1
P08779	51236	5	77	1	Keratin, type I cytoskeletal 16 OS=Homo sapiens OX=9606 GN=KRT16 PE=2 SV=1
Q6NXH9	58875	5	81	1	Keratin 73 OS=Mus musculus OX=10090 GN=Krt73 PE=3 SV=1
Q3MHH8	57371	5	82	1	Amylase, alpha 2B OS=Bos taurus OX=9913 GN=AMY2B PE=3 SV=1
HIT000292931	49418	4	90	1	Keratin, type II cytoskeletal 8 OS=Homo sapiens OX=9606 GN=KRT8 PE=2 SV=1
Q1RMK2	65016	4	92	1	IGHM protein OS=Bos taurus OX=9913 GN=IGHM PE=3 SV=1
Q3SZH5	45428	4	95	1	Similar to Angiotensinogen OS=Bos taurus OX=9913 GN=ANGPT PE=3 SV=1
P05787	53671	4	97	1	Keratin, type II cytoskeletal 8 OS=Homo sapiens OX=9606 GN=KRT8 PE=2 SV=1
ENSBTAP00000016046	77404	4	97	1	Fibulin-1 C isoform 1 OS=Bos taurus OX=9913 GN=FBLN1 PE=2 SV=1
P19001	44515	4	98	1	Keratin, type I cytoskeletal 19 OS=Mus musculus OX=10090 GN=Krt19 PE=2 SV=1
Q3SZV7	52262	4	1.00E+02	1	Similar to hemopexin OS=Bos taurus OX=9913 GN=HEMOPE PE=3 SV=1

Q8VED5	57517	3	1.10E+02	1	Keratin 79 OS=Mus musculus OX=10090 GN=Krt79 PE=3 SV=1
P02672	66971	3	1.10E+02	1	Fibrinogen alpha chain precursor OS=Bos taurus OX=9913 GN=FGA PE=1 SV=1
P02769	69248	3	1.20E+02	1	Bovine serum albumin precursor OS=Bos taurus OX=9913 GN=ALB PE=1 SV=1
P50446	59299	3	1.20E+02	1	Keratin, type II cytoskeletal 6A OS=Mus musculus OX=10090 GN=Krt6a PE=2 SV=1
P35908	65825	3	1.30E+02	1	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens OX=9606 GN=KRT2 PE=1 SV=1
Q32PI4	68887	3	1.30E+02	1	Complement factor I OS=Bos taurus OX=9913 GN=CFI PE=2 SV=1
Q2KJC7	86804	2	1.40E+02	1	Periostin, osteoblast specific factor OS=Bos taurus OX=9913 GN=POSTN PE=2 SV=1
ENSBTAP00000031900	121378	2	1.40E+02	1	121 kDa protein OS=Bos taurus OX=9913 GN=120kDa PE=1 SV=1
Q3KUS7	85358	2	1.40E+02	1	Complement factor B OS=Bos taurus OX=9913 GN=CFB PE=2 SV=1

**Description: (OS: Organism species), (OX: Organism Taxonomic Identifier), (GN: Gene Name), (PE: Protein Existence) and (SV: Sequence Version).*

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