Methods

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 [1].

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 [1]. 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.

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 [1]. 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 [1].

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 [1]. 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 [2].

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 [2], 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 [3]. 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 [4]. 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.

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 [4]. 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 [4].

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 [4]. 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

References

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