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Electrophoretic techniques for purification, separation and detection of *Kayvirus* with subsequent control by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and microbiological methods

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ABSTRACT

The bacteriophage K1/420 is a member of genus *Kayvirus* that was extensively studied as an alternative treatment to combat bacterial infections caused by antibiotic-resistant *Staphylococcus aureus* strains. Despite the promise of phage therapy, the development of clinical applications of phages is facing regulatory and technical hurdles before it can receive acceptance in the Western World. Suitable simple and accurate diagnostic techniques to control the quality of the phage, which would satisfy the requirements of regulatory authorities are still being discussed. Here, we present the conditions for the simultaneous separation and detection of phage K1/420 and *S. aureus* by CZE and by CIEF were found, and the phage isoelectric point was determined to be 3.6. After removing the cell debris, the phage was successfully purified from the crude phage lysate and pre-concentrated by preparative isoelectric focusing. Its zone was localized by the positions of colored p*I* markers in the cellulose bed. The phage from the harvested zone had a decreased ability to infect its host. However, it was suitable for its separation, detection and identification by capillary electrophoretic methods, MALDI-TOF MS and electron microscopy.

1. Introduction

Antibiotic resistance is a serious problem in the protection of public health. A recent Centers for Disease Control and Prevention (CDC) report [1] states, that from 2 million people infected with antibiotic-resistant bacteria at least 23 000 will die [2]. If no immediate actions are taken, the estimated death toll due to antimicrobial resistance will reach 10 million by the year 2050, surpassing the mortality rate, for example, of cancer [3]. Bacterial viruses, bacteriophages, appear to be a suitable alternative treatment for infections caused by antibiotic-resistant bacteria [4,5]. In addition, with advances in molecular biology and gene engineering, the phage application spectrum has been expanded to various medical and biotechnological fields, such as the detection of pathogenic organisms, delivery vehicles for proteins and DNA vaccines, gene therapy delivery vehicles, and phage display technology [6].

The production of bacteriophages is a complex process which involves the selection of appropriate purification and pre-concentration methods, e.g., centrifugation, membrane filtration (microfiltration, tangential-flow filtration), depth filtration (normal flow filtration), or HPLC using methacrylate monolith columns [7], for their efficient production [8]. The subsequent quality control of bacteriophage preparations includes stability testing, determination of pyrogenicity, sterility and cytotoxicity, confirmation of the absence of temperate bacteriophages and transmission electron microscopy-based confirmation of the expected virion morphology as well as of the specific interaction of the phage with the target bacteria [9,10].

In this study, we would like to propose a possible simplification of the analytical methods for the cost-effective upstream, downstream and final product quality-control monitoring [11] required by drug regulatory authorities. There is a wide range of analytical methods [12], including reversed-phase chromatography (RPLC), size-exclusion chromatography (SEC), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [13], and capillary electrophoresis (CE) [14] to assess the identity, hetero-

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geneity, impurity content, and activity of viral and subviral particles or vaccines [13]. In addition, the total concentration of viruses is then usually determined by viral plaque assay, quantitative polymerase chain reaction (qPCR) or anion exchange-HPLC [15] as an important quality attribute.

Due to the amphoteric properties of viruses [14,16–18], electrophoretic techniques such as capillary isoelectric focusing (CIEF) or capillary zone electrophoresis (CZE) were evaluated as an alternative to the current methods [12,17]. The isoelectric point of viruses is a crucial value [16,19–24] which provides information about their surface charge [19,25,26]. However, it is not relevant for describing their surface properties; e.g. adhesion [27]. In contrast to CIEF, the experimental conditions of CZE can influence the electrophoretic mobility of viruses [12,22,28]. This allows the analysis of intact, i.e. non-denatured, viruses moving freely in aqueous solution, including their identification, quantification, or characterization [15,17,29,30].

The objective of our study is to describe the electrophoretic properties of the polyvalent bacteriophage strain K1/420, belonging to the family *Myoviridae*, genus *Kayvirus* [31,32], which is commercialized for the therapy of the serious bacterial pathogen methicillin-resistant *Staphylococcus aureus* (MRSA). The genomic [31,33], proteomic [34] and structural [35] characterization of phages from the *Kayvirus* genus suggests that the phages are safe for phage therapy. It is therefore important to optimize the separation conditions for both bacteriophage particles and cells of *S. aureus* together. The properties of the virus particles, such as their colloidal nature, large molecular size, aggregation tendency and sensitivity to environmental changes [14,16] can influence the separation conditions. Simultaneously, the contaminants in a sample matrix may include buffers, sucrose and bio-molecules of viral or host origin, in concentrations depending on the type and extent of purification, which are prone to capillary wall interactions [36]. Reproducible results can be obtained by the addition of an ionic or nonionic surfactant to the background electrolyte (BGE) [37,38]. Several studies have been done using CZE for detecting previously purified intact viruses [25,26,29,39,40], purified sub-viral particles [41,42], virus complexes with antibodies prepared in vitro or in urine [43–45]. The use of the CIEF technique in the characterization of virus particles and biological particles in general has been reported by several groups [31,46].

Biological samples are usually available in small volumes, for which the use of CE is highly suitable [17,19]. Pre-separation and pre-concentration are important steps, due to the low concentration of particles in the matrix [36,47]. Isoelectric focusing (IEF) also provides an opportunity to increase the sample concentration during the focusing in a single step [48–53]. Recently, the potential of cellulose-based preparative IEF [54] was demonstrated for the pre-separation and pre-concentration of microbial cells [47].

On the basis of this knowledge, the large sample volume of this phage was pre-concentrated by preparative IEF. The pH gradient profile and the local pH are visually traced by selecting colored low-molecular p*I* markers [52,53,55]. The results from the preparative IEF were examined and compared to each other by CIEF, CZE, MALDI-TOF MS and by bacteriophage plaque assay and electron microscopy.

2. Materials and methods

2.1. Chemicals

High-resolution ampholyte, pH 2–4, ampholyte pH 3–4.5, 2-morpholino-ethanesulphonic acid monohydrate, 3-morpholino-propanesulphonic acid, and *N*-[tris-(hydroxymethyl)-methyl]-3-amino-2-hydroxy-propanesulphonic acid were purchased from Fluka Chemie GmbH (Buchs, Switzerland). The solution of synthetic carrier ampholytes (Biolyte, pH 3–10) was obtained from Bio-Rad Labs (Hercules, CA). N-(2-acetamido)-2-aminoethanesulphonic acid and 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulphonic acid were obtained from Merck (Darmstadt, Germany). L-aspartic acid was obtained from LOBA Chemie (Vienna, Austria). Polyethylene glycol, Mw 10 000 (PEG 10 000), acetone, acetonitrile (ACN), (3-glycidyloxypropyl)trimethoxysilane (GOTMS), trifluoroacetic acid (TFA), ethanol (EtOH), α–cellulose (powder, cat No C8002, average fibre length 200 μm), ethylene glycol, glycerol, Triton X-100, 2-(4-Sulfophenylazo)-1,8-dihydroxy-3,6-naphthalenedisulfonic acid trisodium salt (SPADNS), and individual amphoteric compounds (used as simple buffers in a separation medium) were purchased from Sigma-Aldrich (Schnelldorf, Germany). 3,5-Dimethoxy-4-hydroxycinnamic acid (SA) and protein calibration mixture ProMix2 were purchased from LaserBio Labs (Sophia-Antipolis Cedex, France). All chemicals were of analytical grade. The specifications [56,57] of the simple buffers used as spacers have been described elsewhere [54,58]. Colored p*I* markers, p*I* 2.0 (green), 2.6 (red), 3.9 (orange), 5.3 (lavender), 5.7 (orange), 6.2 (red), 7.2 (yellow), 8.0 (orange), 9.0 (yellow), and 10.1 (violet) and for CIEF p*I* 2.0, 2.7 and 3.7 were developed and synthesized at the Institute of Analytical Chemistry of the CAS, v. v. i. [48,52,53,55,59,60].

2.2. Bacteriophage and bacterial strains

Bacteriophage K1/420, a broad host-range mutant of phi812 [61], was isolated from the medication Stafal® (lot no. 40105214, Bohemia Pharmaceuticals s.r.o., Prague, Czech Republic). *S. aureus* CCM 8428 was used for phage propagation and titration and in phage-bacteria mixture experiments.

2.3. Bacteriophage propagation and purification

K1/420 was purified according to Nováček et al. [35] with minor modifications. The phage was propagated on its propagation strain, grown to OD_{600} = 0.5 at 37 °C in meat-peptone broth prepared from 13 g of nutrient broth (Oxoid, UK, CM0001), 3 g of yeast extract (Oxoid, LP0021) and 5 g of peptone (Oxoid, LP0037) dissolved in distilled water to 1000 mL (pH 7.4). The crude phage lysate, obtained after complete lysis of the bacteria, was centrifuged at 4500 × g for 30 min at 4 °C and filtered through polyethersulfone syringe filters with pore size 0.45 μm (Techno Plastic Products, Switzerland) to remove bacterial debris. Phages were pelleted by centrifugation at 54,000 × g for 2.5 h at 4 °C in a JA-30.50 Ti rotor (Beckman Coulter, IN). The resulting pellet was re-suspended in 350 μl of the phage buffer (5×10^{-2} mol L^{-1} Tris pH 8.0, 10^{-2} mol L^{-1} $CaCl_2$, 10^{-2} mol L^{-1} NaCl) overnight at 4 °C, and soluble proteins were removed by extraction with an equal volume of chloroform. The resulting aqueous fraction (approximately 1.2 mL) was overlaid onto a preformed cesium chloride (Sigma) density gradient (1 mL of each 1.45 g mL^{-1}, 1.50 g m L^{-1}, 1.70 g m L^{-1} of CsCl in phage buffer) and centrifuged at 194,000 × g for 4 h at 12 °C using a SW 55 Ti rotor (Beckman). Phage particles forming a visible zone were collected by puncturing the tube with an 0.8 mm gauge needle and syringe. CsCl was removed from the phage-containing fraction by dialysis against an excess of phage buffer at 4 °C overnight using Visking dialysis tubing type 8/32″, 0.05 mm thick (Carl Roth, Germany). The titer of the purified phage was approximately 10^{11} PFU mL^{-1}.

2.4. Enumeration of phage particles using drop plaque assay on double agar overlay

The enumeration of phage particles was performed in 3 independent experiments. Tenfold serial dilutions of K1/420 in meat

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