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NEW APPROACH FOR IDENTIFICATION OF BACTERIOPHAGE VIRION STRUCTURAL PROTEINS

The ability of the phage structural polypeptides to undergo post-translational modification makes the task of correlation of the primary nucleotide sequence data with the actual structural proteins of a virion extremely challenging. This study describes an alternative model approach based on two-stage chromatography for allocation of virion structural components and identification of their major polypeptides.

Bacteriophage T4D, its amber mutant T4D23 (amH11) and its tail preparations were purified, concentrated and separated by ion exchange chromatograpgy based on fibrous DEAE-cellulose. The major tail fraction was then exposed to size-exclusion chromatography which enabled to separate tail components by size. This method proved itself as a highly efficient and gentle enough to save most of the biological material without changing the basic properties of the native phage.

The result also shows that the accumulation of individual phage tails in the course of the amber mutant T4D23 (amH11) propagation on the permissive host Escherichia coli CR63 was resulted by changes in the conditions of reproduction. The ability of bacteriophages to form an excess of tails, capsids and other structures during reproduction on a non-traditional host provides an alternative way for obtaining highly concentrated preparations of virion components for further analysis of their major proteins and determination of the genes responsible for their synthesis.

Key words: bacteriophage T4, amber mutant, virion components, ion-exchange, size-exclusion chromatography, polypeptides.

An understanding of the key processes that take place in biological systems requires not only the availability of genomic data but also a detailed information on the proteins responsible for certain functions [15]. Sequence analysis of phage genomes does not provide a full description of the variety of morphological processes that occur in the natural course of the phage-host interaction. And the ability of the structural polypeptides to undergo a post-translational modification creates one of the basic problems [7, 10]. As a result of this phenomenon, the correlation of the primary nucleotide sequence data with the actual structural proteins of the virion becomes an extremely challenging task.

The solution of the mentioned problem requires highly concentrated phage preparations containing an excess of capsids, tails, fibers or other structural components of the virion. In classical experiments phage amber mutants with a defect in a virion component synthesis are used for this purpose [13]. In some cases a partial degradation of virions under the influence of physical factors is carried out with subsequent enrichment of relevant structures [6]. Unfortunately, both of these methods cannot always be applied to less studied bacterial viruses.

In the course of work with the temperate bacteriophage ZF40 of *Pectobacterium carotovorum* subsp.*carotovorum* it was observed that its propagation in the cells of a non-traditional host results in the accumulation of excessive amounts of certain structural components of the phage [3, 8]. This phenomenon was also noted when working with the model of *Escherichia coli* bacteriophage T4.

In this study we describe an alternative model system based on two-stage chromatography for the allocation of the main structural components of the virion by the example of bacteriophage T4.

Materials and Methods

Bacteria and bacteriophages: The objects of the study were bacteriophage T4D and its amber mutant T4D23 (amH11), deprived of the ability to synthesize the major capsid protein gp23 when reproduced in the nonpermissive strain *E. coli* B^E.

Bacteriophage T4D was obtained from the lysed culture of *E. coli* B^E, the amber mutant T4D23(amH11) – from the permissive host strain *E. coli CR63* [13]. All bacterial cultures were grown on LB [14] medium at 37 °C. Optimal lysis time was determined based on the growth curves of bacteria and phages and equaled 2.5 to 3.0 hours. The final phage concentration in the lysates amounted approximately $5.0x10^{10}$ PFU/ml.

Bacteriophage T4 tail preparation was obtained by infecting an exponentially growing nonpermissive culture *E. coli* B^E with an amber mutant T4D23(amH11). The multiplicity of infection

constituted 10 phages per cell. Intact phage T4D23(amH11) titer in the stock determined by the plaque assay was $4.0x10^7$ PFU/ml. The titer of revertants able to propagate on *E. coli* B^E equaled $4.0x10^6$ PFU/ml. Purification of all preparations from the cell debris was done by means of filtration using sterile Whatman GF/B discs.

Ion-exchange chromatography (IEC). IEC was performed on a column filled with fibrous DEAE-cellulose produced by SERVA (SERVAGEL, Cellulose Ion Exchanger, analytical grade, type DEAE 23SS, capacity 0.74). Cellulose was prepared according to the standard procedure [1]. Pure lysate (400 ml) was absorbed on the column of 2 cm in diameter with the layer of ion-exchanger of approximately 25 cm. The column was stabilized with 0.01 M sodium-phosphate buffer (pH 7.0) at a flow rate 1.3-2.0 ml/min. Fractionation was performed with preliminary washing of the column with sodium phosphate (NaP) buffer after the lysate application. Based on the previous experience and literature data [4] the following solutions were used for stepwise elution: 0.15 M NaCl, 0.01 M NaP; 0.25 M NaCl, 0.01 M NaP; 0.4 M NaCl, 0.01 M NaP. All solutions had pH 7.2 and included 0.02 % of NaN_3 to prevent bacterial contamination. The elution rate comprised 1.5-2.0 mL/min and the fraction volume equaled 1.5-2.0 ml. Chromatographic procedure was performed at 20 °C. The obtained fractions were tested for the presence of viral particles and their components by means of spectrophotometry at wavelengths of 260 and 280 nm using the Thermo scientific Nanodrop 1000. All fractions were stored at 4 °C.

Concentration and purification of phage preparations. The content of the peak fractions was concentrated by differential centrifugation in rotor SW 28 Spinco L7-70 at 26 000 rpm for 1.5 h at 10 ˚C. The precipitate was resuspended in a minimal volume of the STM buffer (NaCl – 200 mM; Tris–HCl (pH 7,5) – 10 mM; $MgCl_2$ – 10 mM), which constituted 1/50 of the initial volume. The obtained suspension was freed of the remnants of cells and phage conglomerates by precipitation on the microcentrifuge ELMI at 11,000 rpm for 10 min.

Size-exclusion chromatography (SEC). Separation of the content of peak fractions was carried out by SEC on a column with sorbent ToyoPearl HW75 characterized by a throughput for particles from 500 000 to 50 million daltons. The diameter of the column constituted 1 cm, the height – 20 cm.

Electron microscopy. Morphological identification of intact phage particles and phage assembly products was carried out using electron microscopy. Purified samples were spread on copper microscopy grids covered with a layer of nitrocellulose and then contrasted with 2% uranyl acetate. Electron micrographs were taken at magnification 20000X – 40000X with the JEOL JEM 1400.

SDS-PAGE. Virion polypeptides were separated by means of denaturating SDS-PAGE in accordance with the standard Laemmli protocol in one-dimensional T=12% gels [9]. Protein bands were visualized with *Coomassie brilliant blue R-250* and using the Bio-Rad Silver Stain Kit (Bio-Rad Laboratories, CA). The Bio-Rad Broad Range Protein Standard (Catalogue # 161-0317) was applied. Molecular weight analysis was performed using Total Lab 2.01.

Results

Bacteriophage T4 is a classical object of biological studies and the most studied representative of T-even bacteriophages. To a great extent it is due to the large collection of mutants that enables application of a variety of biochemical and biophysical methods for molecular analysis [13]. Considering this the phage T4 was chosen as the foundation for this approach.

Purification, concentration and separation of individual components of a phage virion was performed by two-step chromatography: ion exchange, where the division occurs according to the total surface charge of a particle followed by size-exclusion chromatography to further separate the content of peak fractions by size. The ion exchanger used in this study consists of fibrous DEAE-cellulose and has a capacity for salt solutions of high concentration, which allows obtaining highly concentrated preparations of viable phages and their components [1].

Fig. 1 demonstrates the separation profile of 400 ml of the phage T4D *E. coli* suspension on a column with DEAE-cellulose. It is characterized by two distinguished nucleoprotein peaks. The first was obtained in the course of elution with 0.25 M NaCl solution. It corresponds to the release of native phage particles the concentration of which in the peak fractions constituted $1.0x10^{12}$ PFU/ml. The second less expressed peak was obtained by elution with 0.4 M solution of NaCl. Along with a small amount of phage particles, the concentration of which in this case was only $7.0x10^{10}$ PFU/ml, these fractions contained some tails and base plates as illustrated by electron microscopy

(Fig. 2 A, B). Thus, the experimental data has proven the efficiency of ion-exchange chromatography as of a method for concentration of native phage particles. This also points at high level of stability of bacteriophage T4, obtained on the traditional indicator strain.

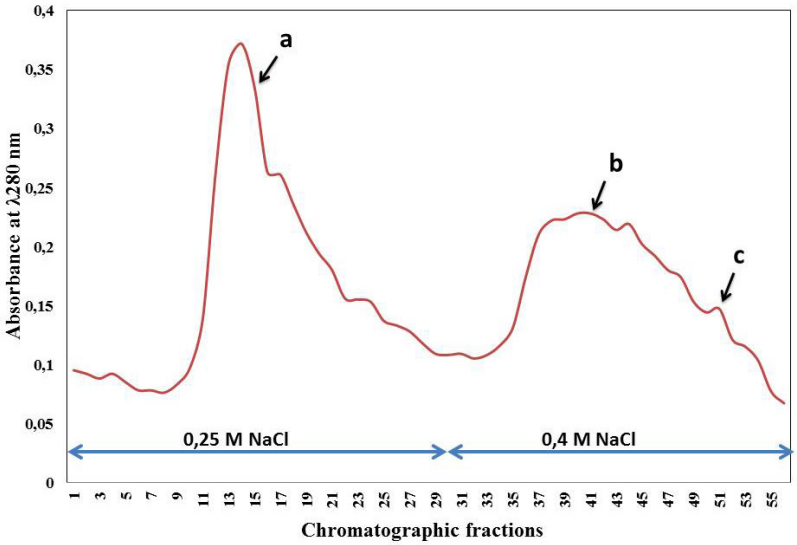

Fig. 1. Ion-exchange chromatography profile of bacteriophage T4D *E. coli* B^{E} preparation: a – intact phage particles, b – phage tails, c – base plates.

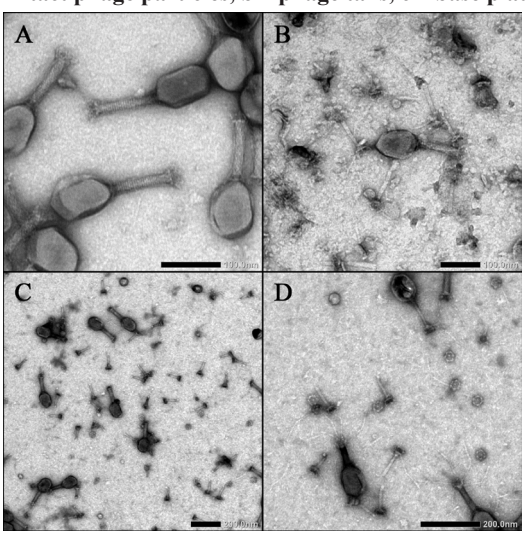

Fig. 2. Electron micrograph of the bacteriophage T4 and its amber mutant T4 am23(H11) peak fractions content. A – intact bacteriophage T4D virions (bar 100 nm), B – bacteriophage T4D structural components obtained at elution with 0,4 M NaCl (bar 100 nm), C – phage tails of the amber mutant T4 am23(H11) (bar 200 nm), D – base plates of the phage mutant T4 am23(H11) (bar 200 nm).

The chromatogram of the amber mutant T4D23 (amH11) phage particles obtained on the permissive host *E. coli* CR63 does not show any significant difference from the T4D phage profile. It also offers two nucleoprotein peaks at 0.25 M and 0.4 M NaCl. The concentration of native phages in the main peak equaled 6.0×10^{11} PFU/ml, while its accompanying second peak was characterized by an increase in the number of structural components of the phage tail. In particular, as shown in Fig. 2 (C, D), fractions that are located at the beginning of the peak contain an excess of tails deprived of a sheath while fractions in the end include base plates. This fact indicates that the possibility of obtaining preparations with a high content of certain structural components of the virion by phage propagation on a non-traditional host can be used as a foundation for a universal methodological approach.

The table below summarizes the data on the efficiency of concentration of phages by ion-exchange chromatography using fibrous DEAE-cellulose. The concentrated phage suspension (18-21 ml) was obtained from the initial volume of 400 ml lysate. The loss of viable phage reached only 10 to 35% while the concentration of native virions capable of infecting the bacterial host increased 12-20 times.

Table

Efficiency of phage particles concentration using ion-exchange chromatography on fibrous DEAE-cellulose

Bacteriophage	Initial phage concentration in lysate, PFU/ml	Lysate volume applied on the column, ml	Phage concentration after IEC, PFU/ml	Final volume after IEC, ml	Concentration efficiency, %	Concentration efficiency
T4D	$5x10^{10}$	400	$1,0x10^{12}$	18,0	90	20-fold
T4D23 (amH11)	$5x10^{10}$	400	$6,0x10^{11}$	21,0	65	12-fold

The chromatogram of purified tails obtained during reproduction of the amber mutant T4D23 (amH11) in the non-permissive host – *E. coli* B^E contained only one major peak at elution with 0.4 M NaCl (Fig.3). This peak forms a plateau and is characterized exclusively by tails of the T4 phage, most of which are deprived of the sheath (Fig.4). In addition to the tails a significant amount of base plates was found in the last fractions.

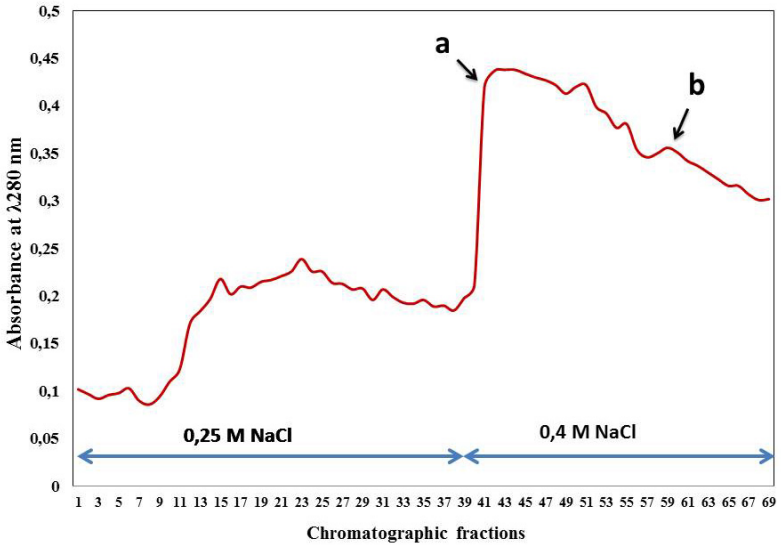

Fig. 3. Ion-exchange chromatography profile of bacteriophage T4 amber mutant am23(H11). Peak fractions containing phage tails (a) and base plates (b).

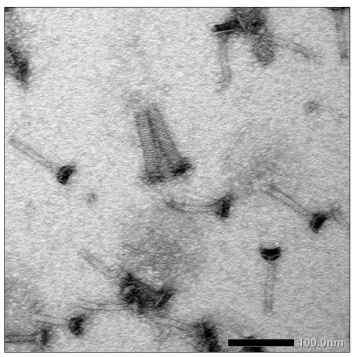

Fig. 4. Electron micrograph of the amber mutant T4 am23(H11) tails (bar 100 nm).

Fractions comprising separate nucleoprotein peaks for all three phage preparations were collected and concentrated by differential ultracentrifugation. The final concentration of particles of the native phages T4D and T4D23 (amH11) in preparations obtained by elution with 0.25 M NaCl

solution was $5.6x10^{12}$ and $2.2x10^{12}$ PFU/ml, respectively, which is 4-5 times higher than the initial concentration of the viral particles in chromatographic fractions. Thus, a gradual application of chromatography and ultracentrifugation methods allows obtaining well-purified preparations of extremely high content of biological material.

The subsequent analysis of polypeptide profiles of the obtained fractions confirmed the data of electron microscopy: in fractions containing an excess of tails lacking a sheath the polypeptide corresponding to the major sheath protein of 69 kDa was found in minor amounts, while the major core protein was identified (Fig. 5).

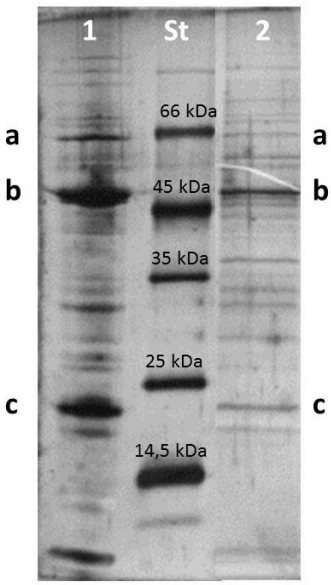

Fig. 5. Polypeptide profiles of the native phage T4 (1) and the phage tails (2). a – the major tail sheath protein, b – the major capsid protein, c – the major tail core protein.

Size-exclusion chromatography was applied to the concentrated preparation of phage tails obtained by propagation of the amber mutant on the non-permissive host *E. coli*B^E (sample volume 0.5 ml). The stationary phase presents the ToyoPearl HW75 sorbent able to separate large particles with molecular weight up to 50 million daltons including intact phage virions. Three peaks were found in the resulting chromatographic profiles (Fig. 6). The first and the major one corresponds to the output of phage tails. The third peak contained base plates, which was illustrated by electron microscopy (data not provided). Thus, SEC enabled to obtain separate fractions containing major building blocks of the phage tail.

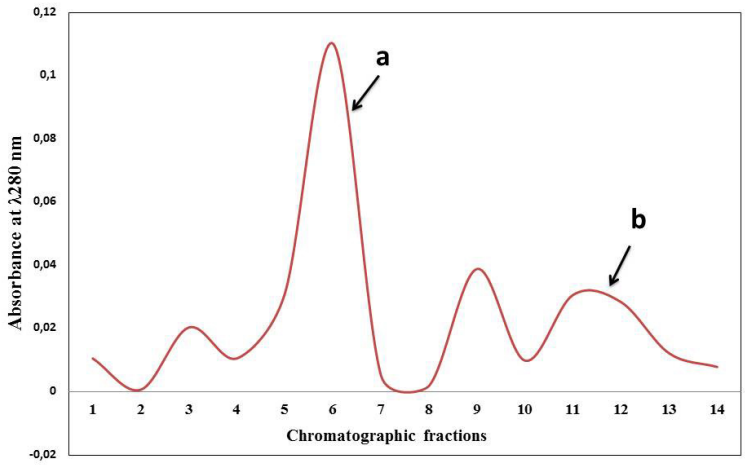

Fig. 6. Size-exclusion chromatography profile of bacteriophage T4 tails preparation. a – phage tails, b – base plates.

Discussion

Correlation of the genetic structure of a polypeptide with its real function is one of the most challenging tasks of molecular biology. It is well known that in the process of maturation T4 phage capsid passes several stages of procapsid which requires proteolysis of certain structural polypeptides [10, 12, 16]. These stages of head formation are controlled not only by the phage genes, but may also be a subject to the bacterial host influence. Therefore, obtaining highly concentrated preparations of individual capsids, tails, base plates, fibers and etc. is the key task for effective determining whether a particular virion polypeptide belongs to a certain structural component.

As a rule phage structural components are obtained using mutants that have a defect in one of the major proteins of a capsid or a tail, respectively [13, 11]. As mentioned above, the mutant bacteriophage T4D 23(amH11) cannot synthesize the major capsid protein – gp23. As a result phage heads are not formed in the course of propagation on the traditional indicator culture *E. coli*B^E [13]. This allows one to obtain preparations containing exclusively tails. Base plates are obtained by using T4 phage that carries a mutation in the gene 19 encoding the major core protein. Individual fibers are accumulated by mutations in genes responsible for their connection to the base plate [11]. The disadvantage of this method is in the fact that for less studied phages the search for such targeted mutants is extremely complicated and sometimes impossible to conduct.

Another approach for accumulating the components of a phage virion is a deliberate degradation of native particles. The influence of osmotic shock is used in most cases for this purpose, when as a result of gradient ultracentrifugation individual structural components are separated from the intact virions forming a separate band at the top of the gradient with the lowest density [6]. The disadvantage of this method is the possibility of complete disintegration of phage particles which are unstable by their nature, resulting in a complete loss of biological material. Moreover, the amount of the resulting fractions containing an excess of tails or capsids is limited.

It is known that in the course of phage propagation on a permissive host a small pool of individual structural components is formed. Changing the host and, therefore, changing the conditions of reproduction affects the course of the phage virion formation. It leads to an increase in the amount of morphogenesis and morphopoiesis errors, resulting in the accumulation of individual capsids, tails etc.

Previous studies of virulent variants of the bacteriophage ZF40 *P. carotovorum* subsp. *carotovorum (Pcc)* have shown that changes in its morphogenetic development occur when it is propagated on an alternative host [3, 8]. For instance, a virulent variant ZF40-421AK obtained in the strain *Pcc*5297 produces an excess of individual tails [8] and RT80 variant on the *Pcc*J2 culture creates a large number of capsid structures [3]. In this case, a defective phage development may be caused by the influence of both the restriction-modification systems and the host chaperonin complexes involved in the folding of the main structural polypeptides of the capsid [7]. This characteristic of the ZF40 phage can be used to identify the key proteins of its unique tail, which in contrast to most of Myoviridae phages has criss-cross striations, whereas the sequence data do not provide any clear information on the major sheath protein [5].

The results of the phage T4 study presented in this paper show that the accumulation of individual tails in the course of the amber mutant T4D23 (amH11) propagation on the permissive host *E. coli* CR63 has led to changes in the conditions of reproduction. Therefore, the ability of bacteriophages to form an excess of tail structures during reproduction on a non-traditional host provides an alternative way for obtaining highly concentrated preparations of virion components for further analysis of their protein profiles and determining genes responsible for the synthesis of those polypeptides.

In this case the use of two-stage chromatographic separation of phage structures is a subsidiary step. It allows concentrating and obtaining pure preparations of intact phage particles, capsids, tails, base plates and fibers separately without losing most of the biological material and without changing the basic properties of the native phage. For comparison: purification and concentration of the phage ZF40, obtained on the *Pcc*5297 strain, by gradient centrifugation in CsCl, results in the loss of 98% of viable virions [2].

Thus, in this paper we propose a new methodological approach for obtaining, effective separation and concentration of individual structural components of a virion by the propagation of phages on alternative hosts followed by two-stage chromatographic separation of lysates. This method may be used as a model. The preparations of such components can be used in molecular genetic studies

for identification of structural proteins. Subsequent mass-spectrometry analysis of the polypeptides enables to determine genes responsible for their synthesis and to detect the phenomenon of their post-translational processing. Equally promising is the use of this method in biotechnology, where phage capsids and tails can be used as vectors for targeted delivery of various therapeutical substances [17].

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НОВИЙ ПІДХІД ДЛЯ ІДЕНТИФІКАЦІЇ СТРУКТУРНИХ БІЛКІВ ФАГОВОГО ВІРІОНА

Р е з ю м е

Здатність фагових структурних поліпептидів до пост-трансляційної модифікації надзвичайно ускладнює співвіднесення даних сиквенсу генома з реальними структурними білками віріона. Дана стаття описує альтернативний модельний метод для отримання окремих структурних компонентів віріона та ідентифікації їх основних поліпептидів за допомогою двохстадійної хроматографії.

Препарати часток бактеріофага T4D, амбер-мутанта T4D23(amH11) та його хвостових відростків T4 були очищені, сконцентровані та розділені шляхом іонообмінної хроматографії на колонці з DEAE-целюлозою. Пікова фракція, що містила окремі хвостові відростки, була в подальшому піддана гель-фільтрації, що дозволило розділити структурні компоненти за розміром. Даний метод довів свою ефективність, не призводячи при цьому до втрати більшості біологічного матеріалу і не змінюючи основних характеристик нативного фага.

Отримані результати також показали: накопичення окремих хвостових відростків при репродукції амбер-мутанта T4D23(amH11) на пермісивному хазяїні *E. coli* CR63, є результатом зміни умов розмноження. Таким чином, здатність бактеріофагів утворювати надлишок структурних частин віріона в ході розмноження на нетрадиційних хазяїнах створює альтернативний шлях для отримання висококонцентрованих препаратів цих компонентів з метою подальшого аналізу їх основних білків і визначення генів, що відповідають за їх синтез.

К л ю ч о в і с л о в а: бактеріофаг T4, амбер-мутант, компоненти віріона, іонообмінна і гель-фільтраційна хроматографія, поліпептиди.

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НОВЫЙ ПОДХОД ДЛЯ ИДЕНТИФИКАЦИИ СТРУКТУРНЫХ БЕЛКОВ ФАГОВОГО ВИРИОНА

Р е з ю м е

Способность фаговых структурных полипептидов к пост-трансляционной модификации чрезвычайно затрудняет соотнесение данных сиквенса генома с реальными структурными белками вириона. Данная статья описывает альтернативный модельный метод для получения отдельных структурных компонентов вириона и идентификации их основных полипептидов с помощью двухстадийной хроматографии.

Препараты частиц бактериофага T4D, амбер-мутанта T4D23(amH11) и его хвостовых отростков были очищены, сконцентрированы и разделены путем ионообменной хроматографии на колонке с волокнистой DEAE-целлюлозой. Пиковая фракция, содержащая отдельные хвостовые отростки, была в дальнейшем подвергнута гель-фильтрации, что позволило разделить структурные компоненты по размеру. Данный метод доказал свою эффективность, не приводя при этом к потере большинства биологического материала и не изменяя основных характеристик нативного фага.

Полученные результаты также показали: накопление отдельных хвостовых отростков при репродукции амбер-мутанта T4D23(amH11) на пермиссивном хозяине *E. coli* CR63 является результатом изменения условий размножения. Таким образом, способность фагов образовывать избыток структурных частей в ходе размножения на нетрадиционных хозяевах создает альтернативный путь для получения высококонцентрированных препаратов этих компонентов с целью анализа их основных белков и определения генов, отвечающих за их синтез.

К л ю ч е в ы е с л о в а: бактериофаг T4, амбер-мутант, компоненты вириона, ионообменная и гельфильтрационная хроматография, полипетиды.

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