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should be addressed to
K.D. (dabrok@iitd.
pan.wroc.pl)

A novel approach for separating bacteriophages from other bacteriophages using affinity chromatography and phage display

Izabela Ceglarek, Agnieszka Piotrowicz, Dorota Lecion, Paulina Miernikiewicz, Barbara Owczarek, Katarzyna Hodyra, Marek Harhala, Andrzej Górski & Krystyna Dąbrowska

Bacteriophage Laboratory, Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, R. Weigla 12, Wrocław, 53-114, Poland.

Practical applications of bacteriophages in medicine and biotechnology induce a great need for technologies of phage purification. None of the popular methods offer solutions for separation of a phage from another similar phage. We used affinity chromatography combined with competitive phage display (i) to purify T4 bacteriophage from bacterial debris and (ii) to separate T4 from other contaminating bacteriophages. In ‘competitive phage display’ bacterial cells produced both wild types of the proteins (expression from the phage genome) and the protein fusions with affinity tags (expression from the expression vectors). Fusion proteins were competitively incorporated into the phage capsid. It allowed effective separation of T4 from a contaminating phage on standard affinity resins.

The traditional approach for bacteriophage purification, i.e. a gradient centrifugation in caesium or saccharose^{1–3} is being gradually replaced by multiple variants of chromatography, mostly because of their easiness to be calibrated for industrial use, safety and lack of time-consuming operations. In the space of only the last decade, we can observe an outbreak of innovative methods for phage purification by chromatography. Phages can be purified by size-exclusion chromatography⁴, chromatofocusing⁵ or in a very successful and promising approach of monolithic anion-exchange chromatography^{6–8}. Both investigations of phage biology and applications of bacteriophages in medicine have induced a great need for technologies that provide highly purified phages as a finished product.

Current methods of phage purification focus on separation of phages from bacterial proteins, DNA, lipopolysaccharide, peptidoglycan, etc. However, when propagated on bacteria, lytic phages may also be contaminated with temperate phages. Temperate phages, since existing within bacteria in a form of prophage, can be released irregularly. Prophages seem to be very common in the bacterial world⁹. They are replicable objects carrying independent sets of genes, composed of many macromolecules and exhibiting individual specificity, immunogenicity and other biological properties. Since phages are anticipated as reliable tools for biotechnology or medicine, prophages associated with many bacterial hosts are a very unwelcome additive in bacteriophage cultures.

As yet, none of the proposed methods of phage purification has offered a solution for separation of a phage from another similar (in size, zeta potential, etc) phage. Bacterial proteins, DNA, lipopolysaccharide or peptidoglycan differ from virions substantially in their physical-chemical characteristics, so effective methods for bacterial debris removal cannot be simply adopted.

Previously¹⁰, we have shown that a combination of phage display and affinity chromatography provides a new method for phage purification. In that method a T4-like bacteriophage surface was furnished with standard affinity tags: glutathione S-transferase (GST) and His-tag, thus allowing phage binding and purification on standard affinity resins. The phage was modified with the affinity tags *in vivo*, during its propagation in bacterial cells which expressed necessary fusion proteins (phage protein fused to affinity tag). Here we present studies of applicability of this concept for easy separation of a target bacteriophage from other very similar ones.

In previous studies¹⁰, a genetically modified phage (T4 Δ hoc) was used as a platform accepting fusions of Highly Immunogenic Outer Capsid Protein (Hoc) with selected affinity tags. Genetic modifications are not favourable,



Table 1 | Fusion types of recombinant proteins used for competitive modification of bacteriophage T4 capsid with affinity tags

Recombinant product name	Recombinant product description	origin
Hoc-His	C-terminal fusion of His-tag to Hoc	cloned for this work
Hoc-GST	C-terminal fusion of GST tag to Hoc	cloned for this work
Soc-His	C-terminal fusion of His tag to Soc	cloned for this work
Soc-GST	C-terminal fusion of GST tag to Soc	Bacteriophage Laboratory IJET collection (Miernikiewicz et al. 2012 PLoS ONE)
His-Hoc	N-terminal fusion of His tag to Hoc	Bacteriophage Laboratory IJET collection (Miernikiewicz et al. 2012 PLoS ONE)
GST-Hoc	N-terminal fusion of GST tag to Hoc	Bacteriophage Laboratory IJET collection (Miernikiewicz et al. 2012 PLoS ONE)
His-Soc	N-terminal fusion of His tag to Soc	Bacteriophage Laboratory IJET collection (Miernikiewicz et al. 2012 PLoS ONE)
GST-Soc	N-terminal fusion of GST tag to Soc	Bacteriophage Laboratory IJET collection (Miernikiewicz et al. 2012 PLoS ONE)

mostly because of formal limitations to genetically modified organisms (GMO) and often due to technological difficulties. Here we present this method developed so that it does not require genetic modifications of the phage prior to purification procedures: a wild phage strain can be purified and the process has no effect on the phage genome. We propose to define this method as a competitive phage display, since it is based on competition of wild capsid proteins and recombinant capsid proteins fused to affinity tags, during phage capsid formation in a bacterial cell.

Results

Selection for optimal localization of affinity tags on phage capsid.

Effective incorporation of the proteins bearing affinity tags into phage capsid as well as appropriate exposure of the tags on the capsid surface are key factors that determine further binding of the phage to specific resins. These two factors may depend on selected localization of the affinity tags on the capsid: to which proteins the tags are fused and in what position. Therefore we compared as presenting molecules two non-essential external phage proteins, Hoc and Small Outer Capsid Protein (Soc), with both C-terminal and N-terminal localizations of two different affinity tags: GST and His-tag.

All types of the investigated recombinant proteins are listed in Table 1. In order to obtain functional *Escherichia coli* (*E. coli*) clones expressing Hoc-His-tag, Hoc-GST and Soc-His-tag, new expression vectors based on pDEST24 and pDEST42 were created. Other

products were expressed from previously prepared expression vectors based on the same plasmids¹¹. Expression of all necessary fusion proteins (Table 1) was tested in an *E. coli* B834 expression strain before they were used in the procedure of phage capsid modification by phage display. Effective production of all selected proteins was confirmed (Figure 1).

E. coli B834 strains effectively expressing all investigated types of Hoc or Soc fusions with affinity tags were used for propagation of T4 phage. Bacterial cells were infected by the phage after induction for recombinant protein expression; thus the phage display was completed *in vivo*. Since a wild T4 phage was used, bacterial cells were impelled to produce both wild types of the proteins (expression from the phage genome) and the protein fusions with affinity tags (expression from the expression vectors). These two variants of the proteins were expected to compete for their incorporation into the phage head during capsid formation in a bacterial cell.

Effectiveness of this competition was compared by evaluation of phage binding to specific affinity resins and phage purification. Lysates produced by each type of *in vivo* phage display were purified by affinity chromatography. The affinity of modified bacteriophages to standard chromatography resins (glutathione Sepharose and NiNTA-agarose) was examined by analyzing their elution profile from the specific resin and from the negative controls (the same titre of modified phages with a non-specific tag) (Figure 2).

Efficacy of T4 phage binding to the resins was clearly dependant on the type of T4 capsid protein presenting the affinity tags (Hoc

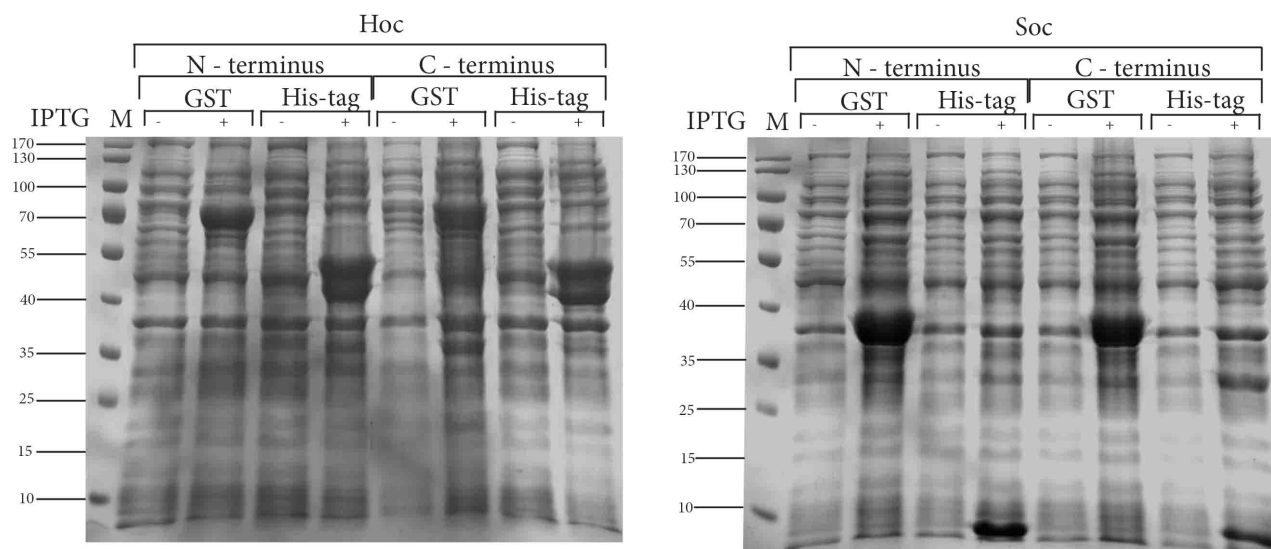


Figure 1 | Expression of recombinant GST- or His-tagged proteins Hoc and Soc. M – molecular weight marker.

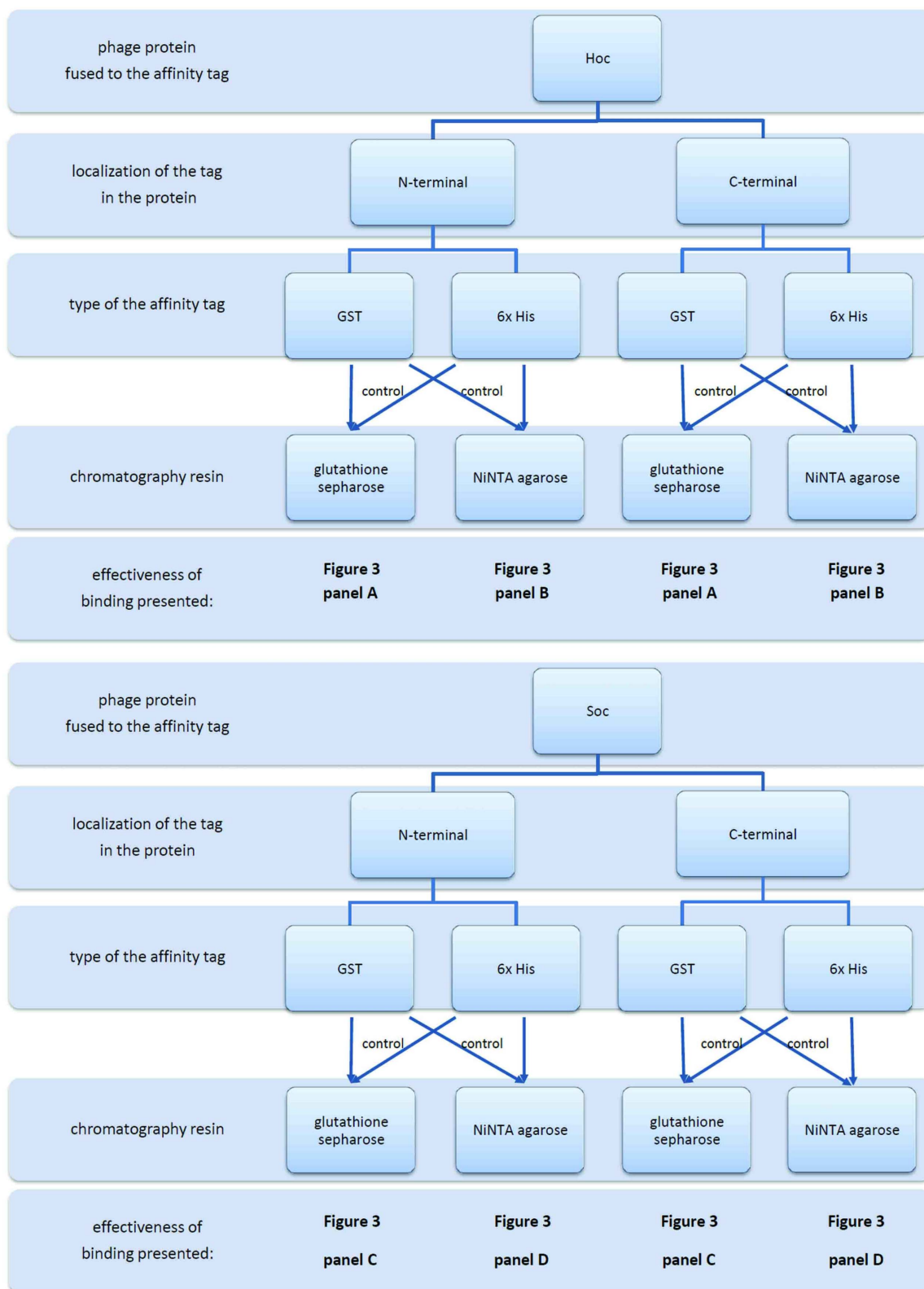


Figure 2 | Modifications of bacteriophage T4 capsid with affinity tags before affinity chromatography. Schema of recombinant protein types. Information on relevant panels in Figure 3 presenting results of purification of all types is given in the bottom of each chart.

protein or Soc protein), type of the affinity tags presented (GST approx. 27 kDa or His-tag approx. 1 kDa), and to localization of the affinity tags in the protein (N- or C-terminal). Figure 3 presents the results of phage purification as elution profiles: phage titre in three subsequent elution fractions, with wash fraction presented. The elution profiles revealed the best suitability of Hoc N-terminal

fusions for this method. Both GST and His-tag in this position substantially increased phage affinity to the specific resins. C-terminal fusions of Hoc were inefficient (Figure 3 A and B). In contrast to Hoc, no clear differences between N- and C-terminal fusions of the affinity tags to Soc protein were observed (Figure 3 C and D). Further, the big affinity tag (GST) was not effectively incorporated into the phage

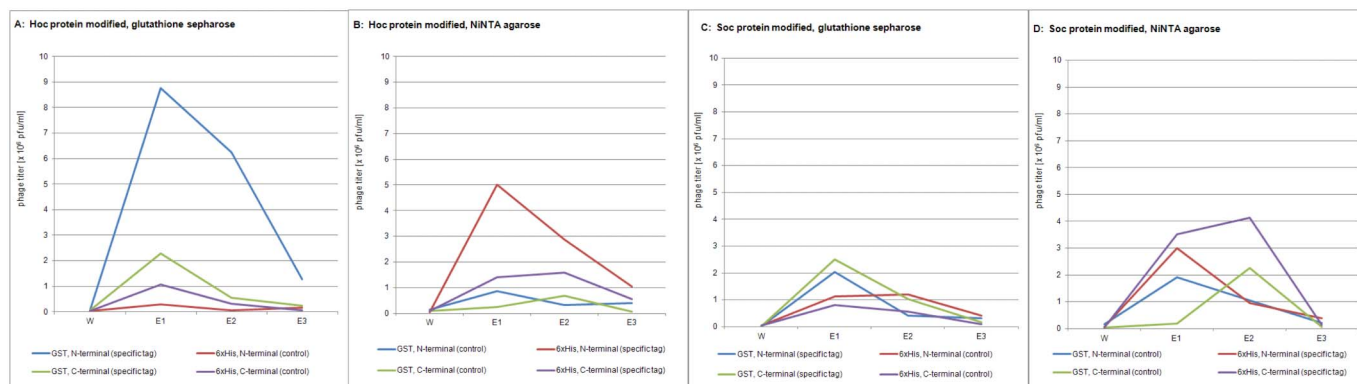


Figure 3 | Efficacy of T4 phage binding to standard affinity chromatography resins. Presented according to: type of T4 capsid protein presenting affinity tags (Hoc protein or Soc protein), type of the affinity tags presented (GST or His-tag), and to localization of the affinity tags in the protein (N- or C-terminal). Phages were modified competitively, i.e. without genetic manipulation of the phage genome. Phage titres [pfu/ml] in wash fraction and in three subsequent elution fractions are presented (each fraction was eluted by 500 mM of imidazole or 20 mM of glutathione). W – wash fraction, E1 to E3 – successive elution fractions. (A): Hoc protein of T4 was modified with the affinity tags and then the phage was isolated by GST-affinity chromatography. (B): Hoc protein of T4 was modified with the affinity tags and then the phage was isolated by Ni²⁺-affinity chromatography. (C): Soc protein of T4 was modified with the affinity tags and then the phage was isolated by GST-affinity chromatography. (D): Hoc protein of T4 was modified with the affinity tags and then the phage was isolated by Ni²⁺-affinity chromatography.

capsid as a fusion with Soc protein, resulting in a very low phage yield in elution fractions (Figure 3 C).

Endotoxin activity by Limulus Amebocyte Lysate assay in crude lysates was 10⁵–10⁶ pfu/ml. After the purification procedures, it was controlled in the three preparations of best phage yield; in GST-Hoc modified phage preparation endotoxin activity was 6.5 EU/ml, in His-Hoc modified phage preparation it was 4.8 EU/ml, and in His-Soc modified phage preparation it was 7.8 EU/ml. Thus, the purification level was very similar to that obtained previously in the procedure established for the genetically modified phage¹⁰.

This part of the work delivered information on the best localization of affinity tags on T4 phage capsid for purification purposes. These observations also show general limitations for phage display on T4 phage capsid, which depend on the size of displayed elements, and on their localization on decorative capsid proteins.

Separation of two types of congenial bacteriophage virions. As a model of T4 phage lysate contaminated with other bacteriophages a mixture of T4 and other phages (1 : 1) was used. Three bacteriophage strains were tested as contaminants, all belonging to Myoviridae, and thus similar to T4: ϕ 9, whose capsid is smaller than T4 (120 nm \times 86 nm); 76, whose capsid is larger than T4; and Tu1b, which has the same dimensions and very high similarity to T4 as a T4-like phage. Before purification, T4 bacteriophage was competitively modified with GST-Hoc, identically to the protocol established in the previous section. Lysates were mixed 1:1 (T4: ϕ 9, or T4:76, or

T4:Tu1b) and incubated with glutathione Sepharose, then the resin was intensively washed, and phages were sequentially eluted with a glutathione buffer. Concentration of both phages was determined in elution fractions 1, 2, and 3 by phage titration; T4 phage was titrated on *E. coli* B strain that was resistant to ϕ 9, 76 and Tu1b phages; contaminant phages were titrated on *E. coli* strains resistant to T4.

Contaminating phages of all types were in significantly lower concentration than T4 phage (Figure 4). The titre of phage ϕ 9 in the first elution fraction was only 0.2% that of T4, the titre of phage 76 was 1.7% that of T4, and the titre of Tu1b was 11% that of T4. This result shows applicability of competitive phage display combined with affinity chromatography for separation of target phages from other contaminating ones.

Discussion

In this work we propose a new method for bacteriophage purification, which combines competitive phage display and affinity chromatography (Figure 5). In ‘competitive phage display’ bacterial cells infected with a phage produce both wild types of the proteins (expression from the phage genome) and the protein fusions with affinity tags (expression from the expression vectors). Wild type and fusion proteins are competitively incorporated into the phage capsid. Thus, phage display of foreign peptides or proteins can be done without any modification to a phage genome, also without deletions of genes coding acceptor proteins. Genetic modifications are not favourable in practical applications, mostly because of formal

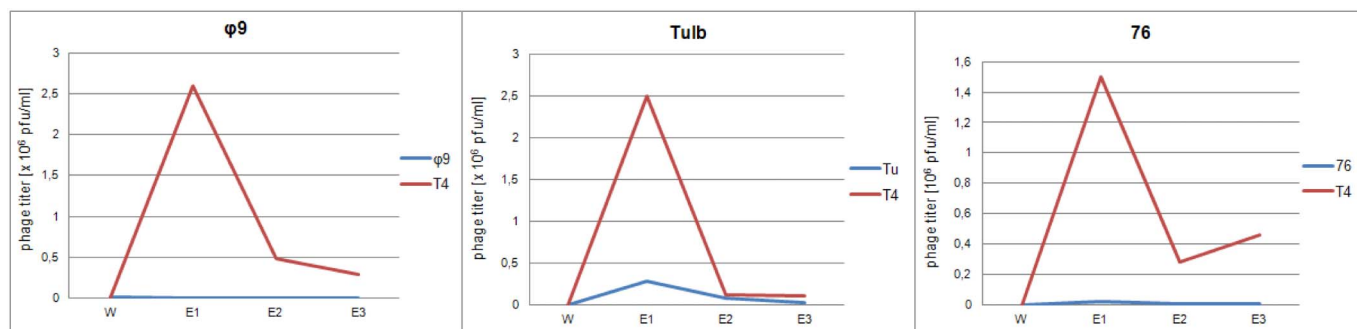


Figure 4 | Separation of T4 bacteriophage modified with GST-Hoc by competitive phage display from contaminating phages: ϕ 9, Tu1b, and 76. Bacteriophages were mixed 1 : 1, incubated with glutathione Sepharose, washed and eluted. Phage titre in subsequent fractions is presented: W – wash fraction, E1 to E3 – elution fractions.

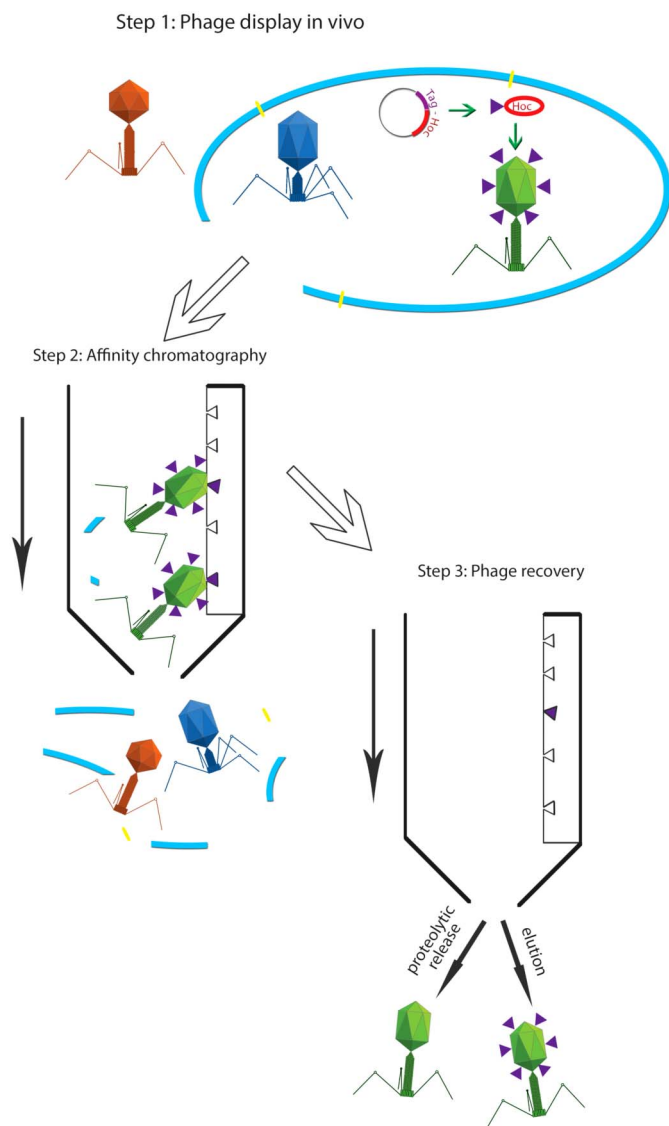


Figure 5 | The new method for bacteriophage purification: competitive phage display combined with affinity chromatography.

limitations to GMO; our method allows purification of unmodified phages. The prerequisite of the method is the genomic sequence of a phage which must be known to design expression vectors for bacterial production of tag-protein fusions.

We used T4 bacteriophage to show applicability of the method. Optimum localization of affinity tags presented on the phage capsid was determined by comparison of N- and C-termini in two decorative proteins of the T4 phage capsid: Hoc and Soc; N-terminal position in Hoc results in the highest affinity of the modified phage and the best purification results. This observation also shows general limitations for phage display on the T4 phage capsid, which depend on the size of displayed elements, and on their localization in decorative capsid proteins.

We tested a model of target phage contamination with other similar phages using T4 phage mixed with other Myoviridae strains (ϕ 9 phage, 76 phage or Tu1b phage). Separation of the target bacteriophage (modified T4) from non-modified contaminating bacteriophages resulted in up to 500 times higher concentration of the T4 phage (from 1:1 before purification to 500:1 after purification). Thus, competitive phage display combined with affinity chromatography provides a novel tool for bacteriophage purification, which enables separation of bacteriophage particles from other contaminating bacteriophages, even highly similar ones.

Phage display of foreign peptides or proteins can be done without any modifications in a phage genome, and also without deletions of genes coding acceptor proteins. We propose this method as ‘competitive phage display’, in which bacterial cells produce both wild types of the proteins (expression from the phage genome) and the protein fusions with affinity tags (expression from the expression vectors). Wild type and fusion proteins are competitively incorporated into the phage capsid.

In T4 phage, the N-terminus of Hoc protein is the localization that offers the best exposure of foreign elements. Competitive phage display enables effective exposure of affinity tags on the phage capsid, thus providing a tool for bacteriophage purification, including separation of bacteriophage particles from other contaminating bacteriophages.

Methods

Bacteriophages and bacteria. T4 phage was purchased from the American Type Culture Collection (ATCC, USA). ϕ 9 phage, 76 phage and Tu1b phage come from the Institute of Immunology and Experimental Therapy collection of bacteriophages (<http://www.iitd.pan.wroc.pl/en/PCM/index.html>).

E. coli expression strain B834 (Novagen) carrying expression plasmids with the gene *hoc* or *soc* in the N-terminal or C-terminal fusion with affinity tags.

Expression vectors. All expression vectors applied in this work were based on GATEWAY recombination technology (Invitrogen). The following types of vectors were used: pDEST17 (N-terminal His-tag), pDEST15 (N-terminal GST-tag), pDEST24 (C-terminal His-tag), pDEST42 (C-terminal GST-tag). All these vectors carry ampicillin resistance, so this antibiotic was a selection agent in all appropriate procedures. Previously made expression constructions¹¹ were applied: pDEST15 with the gene *hoc*, pDEST15 with the gene *soc*, pDEST17 with the gene *hoc*, pDEST17 with the gene *soc*, pDEST24 with the gene *soc*.

GATEWAY recombination technology was used to prepare vectors: pDEST24 with the gene *hoc*, pDEST42 with the gene *hoc*, pDEST42 with the gene *soc*; cloning was done in compliance with the manufacturer’s instructions. Entry clones employed pDONR221 vector (Invitrogen). Products of polymerase chain reaction (PCR) were used to cloning. Primers for PCR consisted of additional sequences: recombination regions and a sequence for the rare protease AcTev (Invitrogen), which were fused to the genes.

Primers:

soc.forward

GGCAAAGTTTGTACAAAAAAGCAGGCTGAAGGAGATATACATATGGC-TAGTACTCGCGGTATG

soc.reverse

GAACCACTTTGTACAAGAAAGCTGGGTCGCCCTGAAAATACAGG-TTTTCGCTGCTGCTACCAGTTACTTTCCACAAAATC

hoc.forward (for both final constructs: based on pDEST24 and on pDEST42)

GGGGCAAAGTTTGTACAAAAAAGCAGGCTGAAGGAGATATACATA-TGACTTTTACAGTTGATATAACTCC

hoc.reverse (for both final constructs: based on pDEST24 and on pDEST42)

GAACCACTTTGTACAAGAAAGCTGGGTCGCCCTGAAAATACAGG-TTTTCGCTGCTGCTGGATAGGTATAGATGATAC

Control DNA sequencing was performed at the Institute of Biochemistry and Biophysics, Polish Academy of Sciences, DNA Sequencing and Oligonucleotide Synthesis Laboratory, Warsaw, Poland. Isolated plasmid DNA (PlasmidMini A&A Biotechnology) was applied in the reaction of sequencing (3730 DNA Analyzer, Applied Biosystems, Hitachi, DNA Sequencing Kit Big Dye™ Terminator Cycle Sequencing version 1.1): 94°C for 10 s, 52°C for 20 s, 60°C for 4 min, 25 cycles; 100 ng DNA, 1 μ l of 5 μ M primer, 3 μ l buffer, 1 μ l enzyme premix, H₂O adjusted to 10 μ l (<http://oligo.ibb.waw.pl>).

The expression of the recombinant proteins was controlled in the bacterial lysate (lysis by freeze-thawing) by polyacrylamide electrophoresis (SDS-PAGE) of total bacterial protein profile and by test affinity chromatography with binding, washing (buffer for GST-tag: 0 mM Na₂HPO₄, 300 mM NaCl, pH 7.5, or buffer for His-tag: 50 mM imidazole, 50 mM Na₂HPO₄, 300 mM NaCl, pH 7.5) and elution (glutathione buffer for GST-tag: 20 mM glutathione, 100 mM Tris, 200 mM NaCl, 0.1% Tween 20, pH 8.0, or imidazole buffer for His-tag: 500 mM imidazole, 50 mM Na₂HPO₄, 300 mM NaCl, 0.1% Tween 20, pH 7.5) followed with SDS-PAGE. Strains were used for further procedures only if effective expression of the modified proteins was observed.

In vivo phage display. Bacterial cells transformed with a selected expression vector were grown at 37°C in Luria broth (LB) with ampicillin as a selection antibiotic until optical density (OD₆₀₀) 0.7 was reached. Next they were transferred to fresh media containing 0.2 mM isopropyl β -D-1-thiogalactopyranoside (IPTG). Addition of approx. 10⁶ pfu/ml T4 (1:100 of total culture volume) took place 15 min after induction of protein expression, and then phage infection started. Infected culture were incubated at 37°C for 8 hours. After bacterial cell lysis, lysates were filtered and used for affinity chromatography. Phage T4 modified with a non-specific affinity



tag - control preparations, and lysates of T4 phage modified with a specific affinity tag – were always diluted (with LB culture medium) up to the same phage concentration: 5×10^8 pfu/ml.

Purification procedure. For phage purification, 1 ml of filtered lysates was mixed with 1 ml of LB medium and incubated with 5 ml of glutathione Sepharose (GE Healthcare) or Ni-NTA agarose (QiaGen) overnight at 4°C. In the model of separation of two bacteriophage strains, phages were mixed 1 : 1 (1 ml of T4 5×10^8 pfu/ml with 1 ml of ϕ 9 phage, or 76 phage, or Tu1b phage 5×10^8 pfu/ml supplemented with 1 ml of LB) and incubated with 5 ml of glutathione Sepharose (GE Healthcare).

The unbound fraction was released, and next the resin was washed with: 5 litres of sodium phosphate buffer (50 mM Na_2HPO_4 , 300 mM NaCl, pH 7.5) for glutathione Sepharose or in the case of His-tag modification the phosphate buffer (50 mM Na_2HPO_4 , 300 mM NaCl, pH 7.5) was enriched with imidazole 500 mM. Elution of specifically bound phage particles from glutathione Sepharose was carried out with glutathione buffer (20 mM glutathione, 100 mM Tris, 200 mM NaCl, 0.1% Tween 20, pH 8.0) or for Ni-NTA agarose was carried out competitively with buffer with 500 mM imidazole (500 mM imidazole, 50 mM Na_2HPO_4 , 300 mM NaCl, 0.1% Tween 20, pH 7.5). Three successive elutions were done, 15 ml each.

The two-layer method of Adams¹² was used to titration of phage preparations and Limulus ameocyte lysate assay (Lonza) was used to determine LPS level. Each experiment was repeated three times; mean values are presented.

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Author contributions

Experiments conducted by IC, AP, DL, PM, BO, KH, MH, KD. Idea of the studies conceived by KD. Data analysis and manuscript preparation by KD, PM. Consultation AG. All authors reviewed the manuscript.

Additional information

Competing financial interests: K.D., P.M., A.P., B.O., A.G. are inventors of a patent (patent application no P 392774) owned by the Institute of Immunology and Experimental Therapy related to phage purification. I.C., D.L., K.H., M.H. declare no potential conflict of interest.

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