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High-level expression and purification of bacteriophage GA virus-like particles from yeast *Saccharomyces cerevisiae* and *Pichia pastoris*

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Abstract

The recombinant virus-like particles (VLPs) generated by heterologous expression of RNA bacteriophage coat protein genes have been proposed as promising carriers of foreign epitopes and nucleic acids for development of novel vaccines and gene therapy tools. Here, we investigated the possibility to produce bacteriophage GA coat protein-derived VLPs in yeast *Saccharomyces cerevisiae* and *Pichia pastoris*. To optimize growth conditions, three expression systems have been explored: GAL1 and GAL10 promoter-directed expression in *S. cerevisiae* as well as AOX1 promoter-directed expression in *P. pastoris*. Synthesis of GA coat protein and formation of VLPs was observed in all three cases. GA VLPs were purified by a single size-exclusion chromatography step till 80 to 90 % of homogeneity. The final amount of purified VLPs varied between 0.6 to 2.0 mg per 1 g of cells for *S. cerevisiae*, while expression in *P. pastoris* resulted in VLP yield of up to 3 mg from the same amount of cells. The recombinant VLPs obtained may be further used for exposition of foreign epitopes on their surface via chemical coupling and/or packaging of immunostimulatory DNA sequences internally.

Key words: bacteriophage GA, coat protein, expression, virus-like particles, yeast.

Introduction

The RNA bacteriophages (phages) are small viruses with a simple organization. Their T = 3 icosahedral shell is composed of 180 copies of the coat protein (CP) and one copy of the maturation protein that encapsidates approximately 3,500-nucleotide-long genomic RNA. These phages were first isolated from *Escherichia coli* (Loeb, Zinder 1961), but later were also found in *Caulobacter* (Schmidt 1966), *Pseudomonas* (Bradley 1966) and *Acinetobacter* (Coffi 1995). To date, the coliphages have been classified into four groups based on their serological and physicochemical properties. Groups I and II with MS2 and GA phages as the type species are quite similar and are collectively called group A. Phages Qβ and SP, members of groups III and IV, respectively, together form group B (Furuse 1987).

The molecular biology of the RNA phages has been extensively studied (van Duin 1999; Weber 1999). In addition, high-resolution X-ray structures of several RNA phages have been determined (Valegård et al. 1990; Liljas et al. 1994; Golmohammadi et al. 1996; Tars et al. 1997; Tars et al. 2000). These data together with the observation that phage CPs alone in

absence of the viral nucleic acid are able to form non-infectious virus-like particles (VLPs) in *E. coli* (Kozlovska et al. 1993; Pushko et al. 1993) have made icosahedral phage shells attractive as objects for gene and protein engineering manipulations. Thus, recombinant VLPs formed by CPs of group-I RNA phages fr and MS2 have been successfully used for presentation of foreign protein sequences on their surface via genetic fusion (Mastico et al. 1993; Heal et al. 1999; Voronkova et al. 2002). However, steric factors limit the length of peptides that can be added to the CP while still preserving its ability to self-assemble into VLPs. Alternatively, the desired peptides can be chemically coupled to surface-exposed lysine residues (Jegerlehner et al. 2002).

Recently, we and others have demonstrated the potential of yeast cells as a host for producing properly folded phage MS2 and Qβ CP-derived VLPs (Legendre, Fastrez 2005; Freivalds et al. 2006). Here, we continue our investigations to show the formation of group-II RNA phage GA VLPs in two different yeasts to therefore extend a way for further development of a yeast-derived phage VLP technology.

Materials and methods

Strains and plasmid constructions

The GA CP-encoding gene was amplified with desired oligonucleotide primers by polymerase chain reaction (PCR) from *Escherichia coli* expression plasmid pGA-355-24 (I. Cielēns, personal communication). The CP sequence encoded by this plasmid differs from that deposited in GenBank (Acc. No. X03869; Inokuchi et al. 1986) at positions 59 and 79 and is identical to that published by Tars et al. (1997). Construction details are summarized in Table 1. For expression in *S. cerevisiae* strain YPH499, the appropriate PCR fragment was digested with *Bam*HI/*Hind*III and cloned in pESC-URA vector, resulting in a pESC-GA plasmid. For expression in the *S. cerevisiae* strain AH22, the PCR fragment was digested with *Xba*I/*Bgl*II and cloned in pFX-Qβ, resulting in a pFX-GA plasmid. The *P. pastoris* expression plasmid pPIC-GA was generated by cloning of the respective PCR fragment into pPIC3.5K vector using *Bam*HI and *Sna*BI restriction sites. PCR and cloning procedures were carried out using standard molecular biology protocols (Sambrook et al. 1989).

Yeast transformation and expression conditions

S. cerevisiae strains AH22 and YPH499 were transformed with pFX-GA and pESC-GA, respectively, using standard lithium acetate/polyethylene glycol procedure as decribed by Gietz et al. (1992). The YPH499/pESC-GA transformants were selected on uracil-free agarized synthetic dextrose (SD) minimal medium according to manufacturer's protocol. For expression, individual transformants were cultivated in liquid synthetic galactose (SG) medium for up to 72 h, until OD_{590} reached 6 - 7.

Transformed AH22/pFX-GA clones were selected on agarized rich YEP medium containing 2 % glucose (YEPD medium) supplemented with 10 mM formaldehyde. Individual transformants were then incubated in liquid YEPD medium supplemented with 5 mM formaldehyde for 20 to 24 h until optical density OD_{590} reached 6 - 8. For induction, the cells were collected by low-speed centrifugation and resuspended in YEP medium with 3 % galactose (YEPG medium), and cultivation was continued for another 20 - 24 h, with final OD_{590} 10 - 14.

Electroporation of *P. pastoris* with the *Ecl*136II-linearized pPIC-GA plasmid and selection of clones containing multiple integrations of expression cassette into yeast chromosome were performed as described by Freivalds et al. (2006). GA CP gene expression in *P. pastoris* was achieved according to recommendations of the manufacturer. Briefly, selected clones were incubated in BMGY medium for 20 to 24 h until OD_{590} reached 4 - 6; then the cells were collected by low-speed centrifugation and resuspended in BMMY induction medium and cultivated for 72 h. All cultivations were performed in 500 mL flasks with 100 mL of expression media at 30 °C on a rotary shaker either at 200 rpm (*S. cerevisiae*) or at 250 rpm (*P. pastoris*). The cells were collected by low-speed centrifugation, washed with distilled water and stored at –20 °C until use.

Purification of GA VLPs

For purification of GA VLPs, 1 g of yeast cells was resuspended in 4 mL of lysis buffer (20 mM Tris-HCl, 5 mM EDTA, 0.65 M NaCl, 1 mM PMSF, pH 7.8). To disrupt the cells, suspension was applied to the French press (three strokes, 20 000 psi). The unsoluble cell debris was separated by centrifugation (1 h, 15 500 g) and discarded. Soluble supernatant proteins were concentrated by addition of solid ammonium sulfate to 60 % of saturation and incubation overnight at 4 °C. After centrifugation 20 min at 8000 g, the proteins were solubilized into 1 mL of lysis buffer without PMSF and loaded onto a Sepharose CL4B gelfiltration column (V = 90 mL, h = 110 cm), with the buffer flow rate approximately 1.0 mL h^{-1}, and 1.5 mL fractions were collected. All of the purification steps were performed at 4 °C.

Protein content in cell and protein samples was analyzed in denaturating polyacrylamide gels (PAAG), with 4 % stacking and 15 % separating gel, according to standard protocols. To visualize protein bands, the gels were stained with Coomassie Brilliant Blue (CBB). Ouchterlony's double radial immunodiffusion with cell lysates was performed using rabbit polyclonal anti-GA antibodies. VLP electrophoresis in 1 % native agarose gels was performed in TAE buffer (pH 8.4) for about 1 h at a constant 90 mA current. VLPs were concentrated

Table 1. Description of vectors and strains used for expression of phage GA CP gene in yeast *S. cerevisiae* and *P. pastoris*. Cloning sites in forward (Fw) and reverse (Rv) primers are underlined. Start and termination codons of the GA CP gene are shown in bold

Strain/genotype	Vector	Primers (5' _ 3')	Construct
S. cerevisiae YPH499	pESC-URA (Stratagene)	Fw: CAGGATCC**ATG**GCAACTTTACGCAGTTTCGT	pESC-GA
MATa ura3-52 lys2-801_amber		Rv: TGAAGCTT**TA**CGCGTAGAAGCCACTCTG	
ade2-101_ochre trp1-_63 his3-_200 leu2-_1			
S. cerevisiae AH22	pFX-Qβ (Samuel et al. 2002;	Fw: TTTCTAGAACAA**ATG**GCAACTTTACGCAGTTTCG	pFX-GA
MATa leu2 his4	Freivalds et al. 2006)	Rv: TTAGATCT**TA**CGCGTAGAAGCCACTCTG	
P. pastoris GS115	pPIC3.5K (Invitrogen)	Fw: TTGGATCCACC**ATG**GCAACTTTACGCAG	pPIC-GA
his4		Rv: TTTACGTA**TTA**CGCGTAGAAGCCACTC	

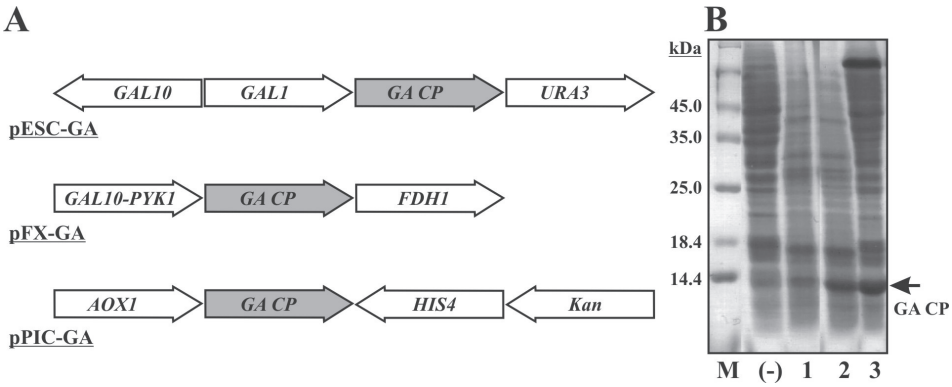

Fig. 1. Expression of the GA CP gene in yeast. A, schematic presentation of the vectors used. The relative direction of genes and promoters is indicated by arrows. *URA3*, *HIS4*, and *FDH1* encode for genes used as primary markers for selection of yeast transformants, while the Kan gene allows secondary screening of *P. pastoris* for high-copy integrants. B, CBB-stained PAAG demonstrating the total synthesis level of GA CP. M, protein molecular weight marker, (-), non-transformed *P. pastoris* cells as a negative control. Lanes 1, 2 and 3 represent cell lysates from strains YPH499, AH22, and GS115, respectively. Accumulation of GA CP is indicated by an arrow.

by dialysis against storage buffer (50 % glycerol, 10 mM Tris-HCl, 2.5 mM EDTA, 325 mM NaCl, pH 7.8) for at least 24 h. Protein concentration measurements were made according to Bradford (1976).

For electron microscopy, samples were adsorbed on carbon-formvar coated grids and stained with 2 % phosphotungstic acid (pH 6.8); the grids were examined with a JEM 100C electron microscope (JEOL Ltd., Tokyo, Japan) at an accelerating voltage 80 kV.

Results

Design of constructs and expression

In order to establish optimal conditions for generation of GA VLPs in yeast, we aimed to compare three well-described expression systems by cloning of phage GA CP gene in three different vectors. The resulting expression units are schematically presented in Fig. 1A. In the first approach, the pESC-URA vector was selected as a template for cloning and *GAL1* promoter-directed expression. This vector was previously used for generation of phage MS2 VLPs (Legendre, Fastrez 2005). In a second approach, we focused on the pFX-derived plasmid, which represents an already established VLP producing system exploiting hybrid *GAL10-PYK1* promoter. In addition, this vector encodes for the *FDH1* gene of *Candida maltosa*, conferring resistance to formaldehyde (Sasnauskas et al. 1992), which is very convenient for quick selection of transformants on rich media. In a third approach, the strong *AOX1* promoter-directed expression provided by the *P. pastoris* expression vector pPIC3.5K was undertaken.

All three constructs pESC-GA, pFX-GA, and pPIC-GA were transformed in their corresponding yeast host (YPH499, AH22, and GS115, respectively). While both *S. cerevisiae* vectors exist in the cells as episomes, pPIC3.5K does not contain a yeast replication origin and needs to be integrated into the host genome via homologous recombination. Due to

Table 2. Summary of generation of phage GA VLPs in yeast. Replication in at least two independent experiments

Strain/plasmid	Amount of cells after cultivation (g L^{-1})	Immunologic anti-GA titers	GA VLPs (mg from 1 g of cells)
YPH499/pESC-GA	15 - 20	1:4 - 1:8	0.6 - 0.8
AH22/pFX-GA	40 - 50	1:16	1.5 - 2
GS115/pPIC-GA	40 - 50	1:32	2.5 - 3

the presence of *Kan* gene in the expression unit, we have selected clones with multiple expression units integrated in the yeast chromosome, which accordingly exhibited increased resistance to Geneticin in *P. pastoris*.

The selected clones were cultivated in appropriate conditions ensuring maximal expression level in each particular case. Optical densities of yeast cells notably varied between strains due to the content of cultivation media, resulting in different amounts of cells obtained at the end of cultivation (Table 2). Total synthesis of GA CP was monitored by CBB-stained PAAG (Fig. 1B) showing well-detectable accumulation of ~13.6 kDa protein in strains AH22 and GS115, while in strain YPH499 the production was significantly lower. Nevertheless, presence of the specific product in the latter case was verified by Western blot with GA-specific antibodies (data not shown). Therefore synthesis of GA CP was confirmed in all three cases and we proceeded to analyze solubility and self-assembly of the target protein.

Purification and characterization of GA VLPs

As the first step of purification, the cells were disrupted by French press and the soluble protein fraction was analyzed by Ouchterlony double radial immunodiffusion with anti-GA antibodies. The obtained titres (Table 2) correlated well with the absolute GA CP amounts (Fig. 1B), suggesting that the majority of the target protein was in the soluble protein fraction and also providing indirect evidence of the presence of VLPs in cell lysates, since unassembled CP usually accumulates as unsoluble aggregates in cells (our unpublished observations).

For further purification, a concentrated mixture of soluble proteins was subjected to size-exclusion chromatography on Sepharose CL4B beads. As expected, the majority of the target protein was eluted between 36 to 42 ml, which corresponds to the calculated volume where VLPs may appear. The respective part of the elution profile is presented in Fig. 2A. Importantly, the larger part of contaminants was removed during the chromatography, indicating the effectiveness of the particular method.

To verify the presence of VLPs, the same fractions were also subjected to native agarose gel electrophoresis (Fig. 2B). The gel was stained with ethidium bromide demonstrating a strong nucleic acid signal that was correlated with the amount of GA CP (Fig. 2A). Taken together, these data strongly suggest formation of VLP nucleoprotein complexes migrating towards anode in native agarose gel. In part, this might be explained by presence of a large amount of negatively charged nucleic acid non-specifically packed inside the VLPs.

Based on the information presented above, the peak VLP-containing fractions were pooled and concentrated. Overall amounts of the obtained VLPs are presented in Table 2. These data correlated well with both the total synthesis level of GA CP (Fig. 1B) and

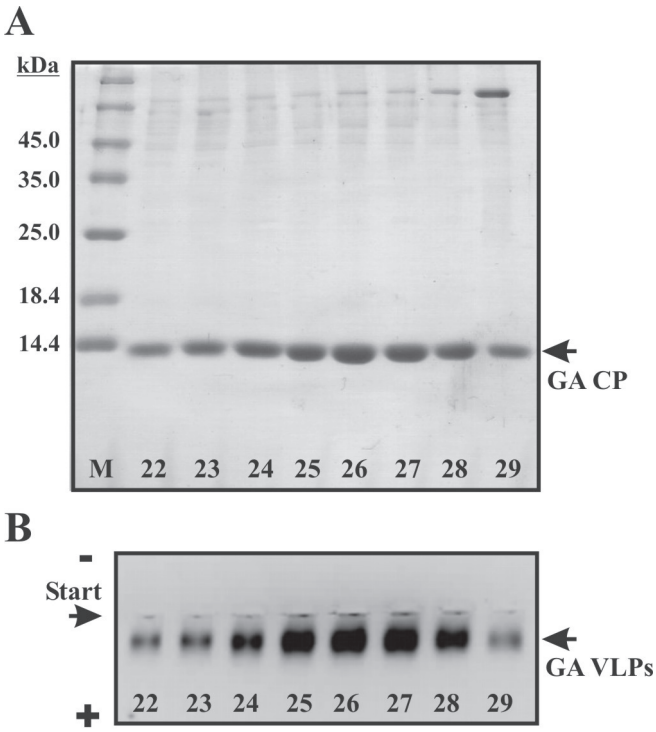

Fig. 2. Purification and detection of recombinant GA VLPs. A, CBB-stained PAAG that demonstrates protein content in *P. pastoris*-derived peak column fractions. The numbers below correspond to appropriate fractions. Both S. cerevisiae strains gave similar profiles but with accordingly lower protein amount (data not shown). M, protein molecular weight marker. The same samples were analyzed in ethidium bromide stained native agarose gel (B). Target proteins are marked by arrows. Anode and cathode are designated as "+" and "-", respectively.

immunological anti-GA titers obtained in supernatant (Table 2). Visually, the overall purity of the VLP samples in PAAG can be estimated as 80 to 90 %, which is rather high after only a single purification step (Fig. 3A). Not surprisingly, the highest purity was associated with the highest expression level, obtained for *P. pastoris*. Finally, the samples were subjected to electron microscopy, which confirmed the formation of icosahedral phage GA-like particles in all three cases (Fig. 3B).

Taken together, an efficient GA VLP generation system was established in both yeast *S. cerevisiae* and *P. pastoris*. The highest yield of VLPs was found in the case of *P. pastoris*. Such recombinant wild-type GA VLPs may be further used for exposition of foreign peptides on their surface via chemical coupling and/or packaging of immunostimulatory DNA sequences internally.

Discussion

Highly immunogenic VLPs generated by heterologous expression of viral structural genes have become a powerful tool for vaccine development. In addition to being effective

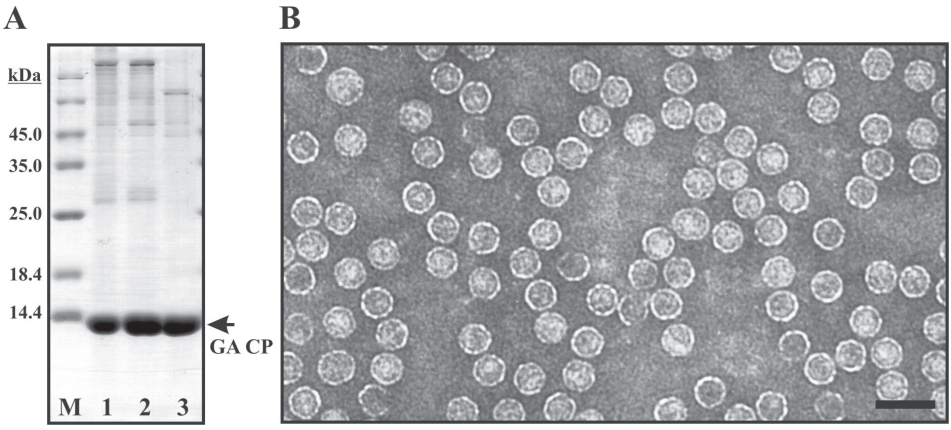

Fig. 3. Characterization of purified GA VLPs. A, CBB-stained PAAG of the final product after size-exclusion chromatography. M, protein molecular weight marker. Lanes 1, 2 and 3 demonstrate the purity of VLPs obtained from strains YPH499, AH22, and GS115, respectively. B, direct evidence of VLP formation by electron microscopy. Only VLPs purified from yeast *P. pastoris* are presented. Scale bar: 50 nm.

vaccines against the corresponding virus from which they were derived, VLPs can also be used to present foreign epitopes to the immune system. This approach might be combined with the packaging of selected genes and drugs inside VLPs (for recent review articles see Georgens et al. 2005; Xu et al. 2006; Jennings, Bachmann 2008).

Icosahedral capsids of the simple RNA phages have attracted attention of scientists as promising carriers of foreign epitopes and nucleic acids. A special interest has been devoted to group III phage Qβ VLPs. Bacterially expressed Qβ VLPs have been explored for chemical coupling of desired peptides to surface-exposed lysine residues. To increase their immunogenicity, such chimeric VLPs can be further engineered by loading them with short synthetic DNA sequences (Storni et al. 2004; Schwarz et al. 2005). Several Qβ phage-derived therapeutic vaccine candidates have already entered phase I to III clinical trials (Kündig et al. 2006; Maurer, Bachmann 2007; Tissot et al. 2008).

However, for vaccine development, *E. coli*-derived VLPs need to be purified from contamination of bacterial endotoxins, which is costly and time-consuming. Alternatively, VLPs could be produced in "endotoxin-free" organisms, such as yeast, which has been regarded as generally safe for human use. Up to now, a large number of structural genes from mammalian viruses have been expressed in yeast resulting in formation of VLPs (Valenzuela et al. 1982; Kniskern et al. 1986; Sasnauskas et al. 1999; Samuel et al. 2002; Slibinskas et al. 2004; Juozapaitis et al. 2007). The yeast expression system has been used successfully to produce licensed prophylactic vaccines against human hepatitis B virus (McAleer et al. 1984) and human papillomavirus (Bryan 2007).

An important drawback of repetitive vaccination with chimeric VLPs might be their limited effectiveness due to the presence of neutralizing antibodies against capsid protein induced after the first application (Da Silva et al. 2001). Therefore, technologies to generate a broad spectrum of carrier VLPs need to be developed. It should be also noted that humans normally do not possess pre-existing antibodies to RNA phages and the immune response will therefore not be impaired. Taking into account these considerations, we extended our

investigations to optimize generation of phage GA-derived VLPs in yeast. The phage GA CP gene was therefore cloned and expressed in three different vectors.

Despite the rather small production of GA CP observed under GAL1 promoter in pESC-URA, this vector contains another GAL10 promoter located in the opposite orientation. This might be advantageous for co-expression of two genes for protein-protein or protein-nucleic acid interaction studies in *S. cerevisiae*. Significantly higher expression of target protein was observed in case of pFX-GA. This is in line with previous data about pFX-directed high-level expression and VLP formation of polyomavirus VP1 and mumps virus nucleoprotein in *S. cerevisiae* (Sasnauskas et al. 1999; Samuel et al. 2002). Finally, methylotrophic yeast, *P. pastoris*, was superior in production of GA CP and yield of recombinant VLPs, therefore confirming its selection as a host microorganism for high-level expression of recombinant genes for both basic laboratory research and industrial manufacture (for a review see Macauley-Patrick et al. 2005).

Recently, we demonstrated the assembly of phage Qb VLPs in *S. cerevisiae* and *P. pastoris* using pFX- and pPIC3.5K-derived expression vectors, respectively (Freivalds et al. 2006). The results obtained were quite similar to those described in this article in that selection of *P. pastoris* clones with multiple expression units integrated in the yeast chromosome resulted in increased expression and outcome of recombinant VLPs while those with single insertion demonstrated rather low synthesis of the target protein. However, not always more integration events leads to higher production, as shown for synthesis of the measles virus nucleoprotein (Slibinskas et al. 2004). Thus, wide screening and selection of individual *P. pastoris* clones is needed for obtaining maximum production in each particular case.

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Efektīva bakteriofāga GA vīrusveidīgo daļiņu iegūšana no raugiem *Saccharomyces cerevisiae* un *Pichia pastoris*

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Kopsavilkums

RNS bakteriofāgu apvalka proteīnu veidotās rekombinantās vīrusveidīgās daļiņas (virus-like particles; VLPs) uzskata par daudzsološu peptīdu un nukleīnskābju transportformu jauna tipa vakcīnu un gēnu terapijas līdzekļu konstruēšanai. Šajā darbā mēs pētījām iespēju iegūt bakteriofāga GA VLPs raugos *Saccharomyces cerevisiae* un *Pichia pastoris*. Kultivēšanas apstākļu optimizācijai salīdzināja GAL1 un GAL10 promoteru kontrolētu ekspresiju *S. cerevisiae*, kā arī AOX1 promotera kontrolētu ekspresiju *P. pastoris*. Visos trīs gadījumos konstatēja GA apvalka proteīna sintēzi un VLP veidošanos. Pēc viena hromatogrāfijas cikla gēlfiltrācijas kolonnā frakcionētās GA VLPs sasniedza 80 līdz 90 % tīrību. Kopumā attīrīto VLP iznākums bija 0.6 līdz 2.0 mg no 1 g *S. cerevisiae* šūnu, bet *P. pastoris* gadījumā tas sasniedza pat 3 mg no identiska šūnu daudzuma. Iegūtās rekombinantās VLPs potenciāli varētu izmantot ķīmiski piesaistītu peptīdu eksponēšanai uz virsmas, kā arī imunostimulatoru DNS sekvenču pakošanai daļiņu iekšienē.