PROTOCOL

Production and Purification of Hybrid Ty-VLPs

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Abstract

This article describes how pure Ty-VLPs (virus-like particles) can be prepared from hybrid Ty-VLPs. Many different hybrid Ty-VLPs have been produced and may be easily purified. Since the sedimentation properties of different hybrid Ty-VLPs are similar, a simple purification process can be used for any VLP. This fast, versatile, and easy process allows for the production of a variety of recombinant proteins.

Index Entries: Yeast retrotransposon; hybrid purification; recombinant proteins.

1. Introduction

The self-assembly properties of a protein encoded by the *TYA* gene of the yeast Ty element can be exploited to produce hybrid Ty-VLPs (virus-like particles) *(1,2)*. There has been developed a series of expression vectors that allow the construction of Ty fusion genes containing protein coding sequences of interest (*see* previous article). Many different hybrid Ty-VLPs have now been produced that carry additional proteins that range in size from 3 to 42 kDa. These include regions from human immunodeficiency virus-1 (HIV-1) *env, pol, tat, rev, nef,* and *vif* genes; influenza virus hemagglutinin; human α-interferon, feline leukemia virus *env*; and bovine papillomavirus E1 and E2 (*1–5* and unpublished data).

An important feature of the Ty-VLP system is the ease with which pure VLPs can be prepared as a result of their particulate nature (Fig. 1). The sedimentation properties of different hybrid Ty-VLPs are similar, and this characteristic has been exploited to develop a simple purification process that can be used for any VLP, irrespective of the sequence of the additional protein. The system is therefore extremely versatile, allowing rapid production of a variety of recombinant proteins. Such ease of purification would, for example, make it feasible to survey a genome for regions that encode important antigenic determinants, such as those able to induce a protective immune response against a particular pathogen.

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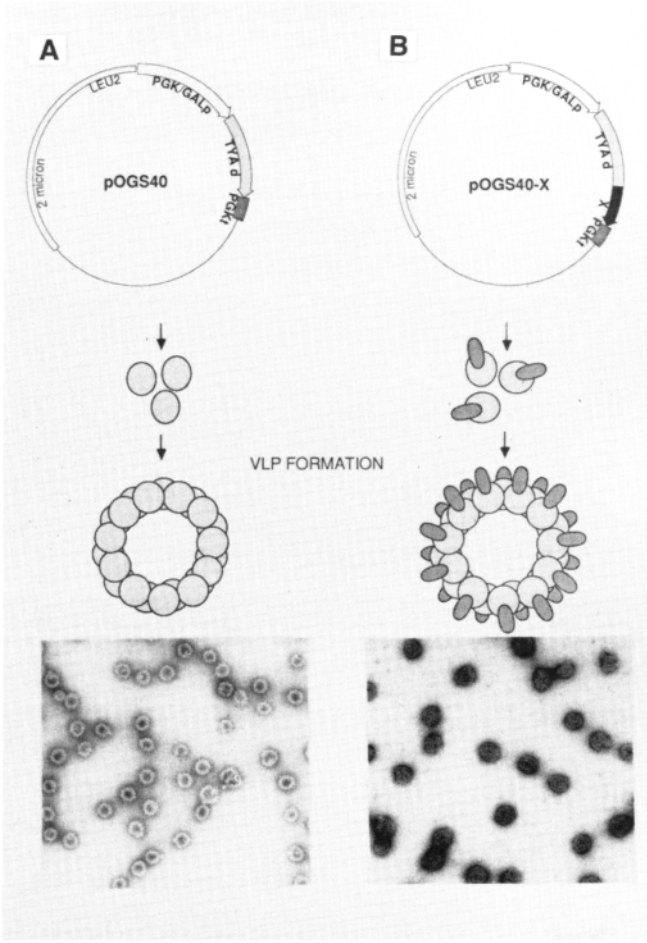

Fig. 1. Ty-VLP formation. Purified control **(A)** and hybrid **(B)** Ty-VLPs are shown below their relevant expression vectors and a schematic diagram of VLP formation. The vectors contain a hybrid PGK/GAL promoter (PGK/GALp; open arrow), the first 381 codons of the *TYA* gene (TYAd; lightly shaded arrow), a PGK terminator region (PGKt; darkly shaded box), selection and replication modules for yeast (open box) and *E. coli* (thin line). The purified VLPs in panel B contain regions of the HIV core p24 and p17 proteins fused to the Ty particle-forming protein.

Hybrid VLPs can also be used in many other laboratory applications. The most obvious use is the production of defined polyclonal and monoclonal antisera as research reagents by selecting particular regions of a protein as the added antigen. These antibodies can then be used as research tools themselves, perhaps to purify native protein, as immunodetection reagents, or to map functional domains of a protein. Conversely, hybrid VLPs can be

used as a rapid primary screen to map monoclonal antibodies raised against non-VLP antigens. For example, a series of VLPs containing overlapping fragments of the HIV envelope protein gp120 have been used to map a monoclonal antibody to a 30 amino acid sequence *(1)*. The cost of fine mapping with peptides can therefore be decreased significantly by reducing the length of sequence to be covered by overlapping peptides.

The VLP system can also be manipulated to produce nonparticulate proteins or protein domains. By engineering a protease cleavage site at the C-terminus of the particle-forming protein p1, hybrid VLPs can be produced with the general structure of p1:cleavage site:added antigen. Hybrid VLPs have been constructed that contain various antigens downstream of the recognition sequence for the blood coagulation factor Xa. Purification of the particles followed by factor Xa cleavage results in a mixture of three proteins from which the protein of interest can be purified (Fig. 2). This technology has been used successfully to purify several HIV antigens, providing sufficient material for structural and functional analyses *(5,6)*. Such systems could result in a better understanding of disease pathology and the development of novel screening systems for antiviral compounds directed at virus-specific targets.

2. Materials

1. Synthetic complete-glucose (SC-glc) medium: 0.67% (w/v) Yeast nitrogen base without amino acids, 1% (w/v) glucose. Add appropriate amino acids after autoclaving. The amino acids required will be dependent on the auxotrophy of the yeast strain used. The yeast strain used must be auxotrophic for leucine biosynthesis, and leucine is omitted from the media to ensure selection for the VLP plasmid following transformation. Amino acids can be prepared as a 50X stock solution and filter-sterilized.
2. SC-glc plates: 0.67% (w/v) Yeast nitrogen base without amino acids, 1% glucose (w/v), 2% Bacto-agar. Add appropriate amino acids after autoclaving.
3. SC-glc/gal medium: 0.67% (w/v) Yeast nitrogen base without amino acids, 0.3% (w/v) glucose, 1% (w/v) galactose. Add amino acids after autoclaving.
4. TEN buffer: 10 m*M* Tris-HCl, pH 7.4, 2 m*M* EDTA, 140 m*M* NaCl. This buffer is usually made up as a 10X stock solution.
5. Protease inhibitors: Separate solutions of 25 mg/mL chymostatin, antipain, leupeptin, and pepstatin A are made in dimethyl sulfoxide and stored in 25-µL aliquots. A 25-mg/mL solution of aprotinin is made in water and stored in the same manner. Then 25 µL of each of the above-mentioned solutions is added to 1 L of TEN buffer plus 1 mL of fresh 5 m*M* phenylmethylsulfonyl fluoride dissolved in ethanol. The protease inhibitors can be obtained from Sigma.
6. Acid-washed glass beads: Glass beads, 40-mesh, can be obtained from BDH Ltd. The beads are washed in concentrated sulfuric acid, rinsed 10 times in tap water, 10 times in distilled water, dried, and baked at 150°C for 2 h.

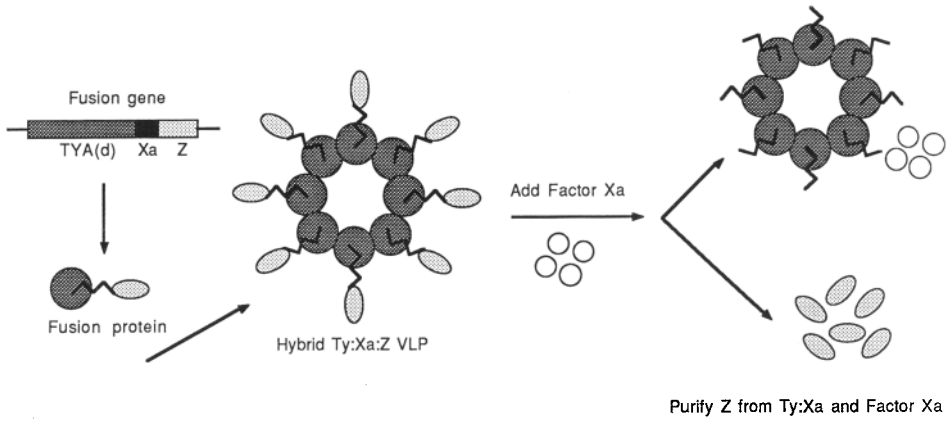

Fig. 2. Purification of proteins following factor Xa cleavage of hybrid Ty-VLPs. The recognition sequence for factor Xa is inserted between the coding sequence of *TYA*(d) and the protein of interest (Z). The resulting fusion protein assembles into VLPs. These are purified and cleaved with factor Xa (open cirles). The enzyme and residual particulate material are then purified from protein Z.

7. 60% (w/v) Sucrose dissolved in TEN buffer.
8. 5–20% Linear sucrose gradients: Prepare a 12.5% stock sucrose solution in TEN buffer and autoclave. Add 30 mL to Beckman SW28 tubes (or equivalent) and freeze at –20°C. The day before the gradients are needed, remove the required number and thaw slowly at 4°C for 16 h. Add 2 mL of 60% sucrose to the bottom of the tube using a Pasteur pipet before loading the gradients.
9. Sephacryl S1000 superfine can be obtained from LKB. The column is equilibrated in TEN buffer before use.
10. Ultrafiltration units with a 30,000-Dalton cutoff can be obtained from Millipore (Immersible CX-30 units, catalog number PTTK11K25).
11. 5X DB: 20% (v/v) Glycerol, 10% (v/v), β-mercaptoethanol, 10% (w/v) SDS, 0.125*M* Tris-HCl, pH 6.8, 0.1% (w/v) bromophenol blue.
12. SDS-polyacrylamide gels:
    a. Separating gel: 10% stock acrylamide solution (acrylamide:*bis*-acrylamide ratio of 30:0.8), 0.375*M* Tris-HCl (pH 8.8), 0.05% (wv) ammonium persulfate, 0.1% (w/v) SDS, 0.0005% (v/v) TEMED.
    b. Stacking gel: 5% stock acrylamide solution, 0.125*M* Tris-HCl, pH 6.8, 0.1% (v/v) ammonium persulfate, 0.1% (w/v) SDS, 0.001% (v/v) TEMED.
13. Coomassie blue stain: 0.25% (w/v) Coomassie blue in 40% (v/v) methanol and 10% (v/v) acetic acid.
14. Destaining solution: 20% (v/v) Methanol, 10% (v/v) acetic acid.

3. Methods

In this protocol, 16-L cultures containing transformed yeast are grown, and Ty-VLPs are purified from them. Procedures are described for the production of Ty-VLPs using constitutive expression from the yeast phosphoglycerate kinase (PGK) promoter and using galactose-induced expression from a hybrid PGK-GAL promoter (*see* previous article).

3.1. Growth of Cultures—Constitutive Expression

The procedures for transforming yeast with recombinant plasmids and preparing glycerol stocks of transformants are described in the previous article. Large cultures of transformed yeast should be grown from glycerol stocks.

1. Inoculate 100 mL of SC-glc medium (plus amino acids) with a 1-mL glycerol stock of the appropriate yeast transformant. Incubate, with vigorous shaking, at 30°C until a cell density of at least 2×10^7 cells/mL is achieved. This will take approx 2 d.
2. Put 1 L of SC-glc medium (plus amino acids) into each of two 2-L flasks, and inoculate each with 50 mL of preculture. Incubate overnight as before until the cell density is 5×10^7 cells/mL.
3. Split the 2 L of culture among 16 individual liters of SC-glc medium (plus amino acids). Grow overnight as before. Harvest the cells when the cell density is 4×10^7 cells/mL by centrifugation at 3500*g* for 20 min. Resuspend the pellets in 10 mL of water/L of culture. Transfer to 50-mL Falcon tubes (pool the cells from 2 L of culture into one tube) and centrifuge at 3500*g* for 5 min in a bench-top centrifuge. Repeat the washing procedure twice more. Finally, wash each pellet with 20 mL of TEN buffer plus protease inhibitors. Remove the supernatant and freeze the cell pellets at –20°C until required.

For a smaller-scale preparation, the cells can be harvested and washed at Step 2.

3.2. Growth of Cultures—Inducible Expression

1. Inoculate 100 mL of SC-glc medium (plus amino acids) with a 1-mL glycerol stock of the appropriate yeast transformant. Incubate, with vigorous shaking, at 30°C until the cell density is $2–4 \times 10^7$ cells/mL. This will take approx 2 d.
2. Put 1 L of SC-glc medium (plus amino acids) into each of two 2-L flasks, and inoculate each with 50 mL of the preculture. Grow for approx 20 h, until the cell density is $4–6 \times 10^7$ cells/mL.
3. Split the 2 L of culture among 16 individual liters of SC-glc/gel medium (plus amino acids). Grow for 24 h.
4. Harvest the cells when a density of $4–8 \times 10^7$ cells/mL is reached. Wash the cells with water and TEN buffer as described above. Freeze the cell pellets at –20°C until required.

For a smaller-scale preparation, inoculate 50 mL of the preculture into 1 L of SC-glc medium (plus amino acids) at Step 2 and 150 mL from this culture into each of 2 × 1 L of SC-glc/gel medium (plus amino acids) at Step 3.

3.3. Purification of Ty-VLPs (see Note 1)

The following procedure is used for the purification of hybrid Ty-VLPs from 16 L of culture. The procedure is summarized in Fig. 3. Yields are generally in the range of 20–100 mg/16 L, and the VLPs constitute >90% of the final preparation. For smaller-scale preparations, the procedure can be scaled down proportionally, and a smaller column can be used for the final purification step. All procedures are performed at 4°C using prechilled buffers.

1. Thaw cells (in eight 50-mL Falcon tubes). Add 4 mL of TEN buffer containing protease inhibitors to each tube and resuspend the cells. Add 5 mL of acid-washed glass beads to each tube.
2. Vortex the tubes for 10, 30-s periods interspersed with 30-s periods of cooling in ice. Centrifuge the suspension at 2000*g* for 5 min. Remove and retain the supernatant on ice (*see* Note 2).
3. Add 4 mL of fresh TEN buffer (containing protease inhibitors) to the cell pellet and repeat the vortexing and centrifugation as above. Remove and retain the supernatant.
4. Add 3 mL of fresh TEN buffer (containing protease inhibitors) to the cell pellet and again repeat the vortexing and centrifugation. Pool the supernatants and centrifuge at 13000*g* (Sorvall HB4 rotor or equivalent) for 20 min.
5. Centrifuge the cleared supernatant at 100,000*g* (Beckman SW40 rotor, 30,000 rpm for 1 h; SW28 for 1.5 h at 28,000 rpm; or equivalent) onto a 2-mL 60% sucrose cushion (in TEN buffer). The use of a sucrose cushion prevents pelleting of the VLPs, which can result in a decrease in yield. Collect the cushions with a Pasteur pipet and dialyze overnight against 1 L of TEN buffer plus protease inhibitors (*see* Notes 3,4).
6. Centrifuge the dialysate at 13,000*g* for 20 min and retain the supernatant. Add 2 mL of 60% sucrose (in TEN buffer) to the bottom of each of six 5–20% sucrose gradients (Beckman SW28 tubes or equivalent) with a Pasteur pipet. Load each gradient with up to 2 mL of the supernatant and centrifuge at 53,000*g* (25,000 rpm Beckman SW28 rotor, or equivalent) for 6 h.
7. Following centrifugation, remove the sucrose cushions containing the VLPs using a Pasteur pipet. Remove the sucrose from this crude particle preparation by dialyzing against TEN buffer for 36 h.
8. Centrifuge the dialysate at 13,000*g* for 20 min, filter the supernatant through a 0.45-μm filter, and concentrate the sample to 16 mL using a Millipore ultrafiltration device. Load the sample onto a Sephacryl S1000 superfine 5 × 100 cm column, and develop at a rate of 16 mL/h with TEN buffer (*see* Notes 5,6).

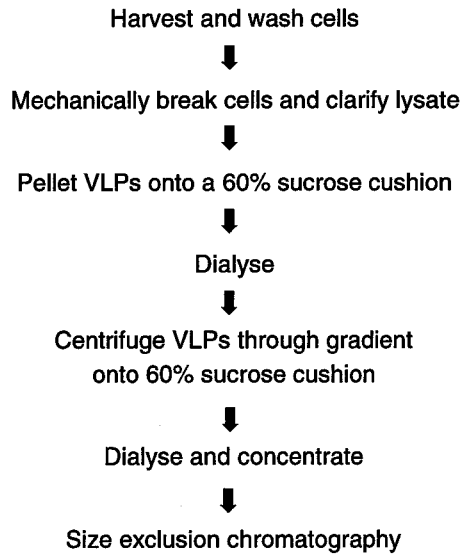

Fig. 3. Flowchart for the purification of hybrid Ty-VLPs.

9. Fractions containing hybrid Ty-VLPs are identified by running aliquots of the fractions on 10% SDS—polyacrylamide gels. Add 10 µL of 5 × DB to 40 µL of column fraction and boil the mixture for 5 min in a water bath. The quantity of sample loaded will depend on the protein gel apparatus used. If a minigel (e.g., the Bio-Rad Mini-Protean) is used, then 10 µL is sufficient. Run the gel according to the manufacturer's instructions for the apparatus, stain with 0.25% Coomassie Blue solution, and destain.
10. Pool the fractions containing the VLPs, concentrate to approx 1 mg/mL, if necessary, and store in aliquots at –70°C.

4. Notes

1. Confirmation of the presence of fusion protein prior to starting the VLP purification: The presence of fusion protein can be detected by either Coomassie Blue staining, a total protein extract run on a 10% SDS-polyacrylamide gel or by Western blotting using either an anti-Ty antibody, or an antibody directed against the non-Ty component. As a control, a total protein extract of non-transformed yeast should be included. Total yeast protein extracts can be prepared as follows: Remove 5×10^8 cells at the end of the growth period and centrifuge at 2000*g* for 5 min in a 15-mL Falcon tube. Resuspend the cells in 1 mL of TEN buffer. Add 1 g of acid-washed and baked glass beads. Disrupt cells by vortexing the sample three times for 1 min each, interspersed with 1-min cooling periods on ice. Remove beads by centrifuging at 2000*g* for 5 min. Determine the protein concentration of the supernatant using a Bradford dye-binding assay (available from Bio-Rad). Load 30–50 µg of total protein

for detection by Coomassie Blue staining or 3–5 μg for detection by Western blotting.

2. Breakage of yeast cells: In order to maximize yields of VLPs, it is essential to obtain efficient breakage of the yeast cells. When vortexing the cells with glass beads, it is important to hold the Falcon tube in such a manner that the liquid and beads are forced as far as possible up the sides of the tube. This can be achieved by keeping the tube vertical, holding the tube at the top, and pressing into the whirlimixer. Breakage can be evaluated by phase-contrast microscopic examination. Damaged cells will appear phase-dark,whereas unbroken cells will remain phase-bright. An alternative is to use a mechanical homogenizer, such as a Bead Beater (available from Biospec Products, Bartlesville, OK).
3. Harvesting VLPs from sucrose cushions: It is important to avoid mixing the supernatant (containing nonparticulate contaminants) with the sucrose cushion. This can be ensured by drawing off the supernatant with a Pasteur pipet before removing the sucrose cushion.
4. Dialysis: Extended dialysis of the crude VLP preparation often results in the formation of a precipitate. This does not contain the VLPS and is removed by centrifugation (13,000*g* for 20 min).
5. Filtration of VLPs: Hybrid VLPs have a diameter of 50–80 nm, depending on the size of the non-Ty component. They should therefore pass through 0.45- or 0.22-μm filters. However, prior to Sephacryl S1000 column chromatography, some contaminants are still present and filtration through a 0.45-μm filter may be difficult. It is therefore often advisable to prefilter using a 3-μm filter.
6. Alternative to size exclusion chromatography: If column chromatography facilities are not available, it is possible to replace this step with 15–45% sucrose gradients. However, it should be noted that resolution is improved by using the size exclusion column, rather than density gradient centrifugation. Layer 8-mL aliquots of 45, 35, 25, and 15% sucrose (in TEN buffer) into Beckman SW28 tubes (or equivalent) and leave to equilibrate overnight at 4°C. Load a maximum of 3 mL of the centrifuged, filtered, and concentrated dialysate onto each gradient and centrifuge at 53,000*g* (25,000 rpm in Beckman SW28, or equivalent) for 3 h at 4°C. Separate the gradients into 2-mL fractions. Identify the fractions that contain hybrid Ty-VLPs by running aliquots on 10% SDS-polyacrylamide gels as described above.

References

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