Production of Human Norovirus Protruding Domains in E. coli for X-ray Crystallography

Video Article

Production of Human Norovirus Protruding Domains in *E. coli* for X-ray Crystallography

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URL: http://www.jove.com/video/53845
DOI: doi:10.3791/53845

Keywords: Molecular Biology, Issue 110, Cloning, norovirus, P domain, protein expression, protein purification, crystallization

Date Published: 4/19/2016

Citation: Leuthold, M.M., Koromyslova, A.D., Singh, B.K., Hansman, G.S. Production of Human Norovirus Protruding Domains in *E. coli* for X-ray Crystallography. *J. Vis. Exp.* (110), e53845, doi:10.3791/53845 (2016).

Abstract

The norovirus capsid is composed of a single major structural protein, termed VP1. VP1 is subdivided into a shell (S) domain and a protruding (P) domain. The S domain forms a contiguous scaffold around the viral RNA, whereas the P domain forms viral spikes on the S domain and contains determinants for antigenicity and host-cell interactions. The P domain binds carbohydrate structures, *i.e.*, histo-blood group antigens, which are thought to be important for norovirus infections. In this protocol, we describe a method for producing high quality norovirus P domains in high yields. These proteins can then be used for X-ray crystallography and ELISA in order to study antigenicity and host-cell interactions.

The P domain is firstly cloned into an expression vector and then expressed in bacteria. The protein is purified using three steps that involve immobilized metal-ion affinity chromatography and size exclusion chromatography. In principle, it is possible to clone, express, purify, and crystallize proteins in less than four weeks, which makes this protocol a rapid system for analyzing newly emerging norovirus strains.

Video Link

The video component of this article can be found at http://www.jove.com/video/53845/

Introduction

Human noroviruses are the major cause of acute gastroenteritis worldwide[1]. These viruses belong to the *Caliciviridae* family, of which there are at least five genera, including *Norovirus*, *Sapovirus*, *Lagovirus*, *Vesivirus*, and *Nebovirus*. Despite their high impact on the healthcare system and wide distribution, the study of human noroviruses is hampered by the lack of a robust cell culture system. To date, there are no approved vaccines or antiviral strategies available.

The norovirus major capsid protein, termed VP1, can be divided into a shell (S) domain and a protruding (P) domain[2]. The P domain is connected to the S domain by a flexible hinge (H) region. The S domain forms a scaffold around the viral RNA, whereas the P domain forms the outmost part of the viral capsid. The P domain assembles into biologically relevant dimers when expressed in bacteria. The P dimer interacts with carbohydrate structures, termed histo-blood group antigens (HBGAs) that are present as soluble antigens in saliva and found on certain host cells[3]. The P domain-HBGA interaction is thought to be important for infection[4]. Indeed, a recent report revealed the importance of synthetic HBGAs or HBGA-expressing bacteria for human norovirus infection *in vitro*[5].

Current studies regarding the host cell attachment of noroviruses are mainly performed with virus-like particles (VLPs) that can be expressed in insect cells or with recombinant P domains expressed in *Escherichia coli* (*E. coli*). To understand the P domain-HBGA interactions at atomic resolution, P domain-HBGA complex structures can be solved using X-ray crystallography. Here, we describe a protocol for P domain expression and purification that allows production of P domain in high quantity and quality to be used for X-ray crystallography. Moreover, this method can be applied for other calicivirus P domains and non-structural proteins.

The P domain is codon-optimized for *E. coli* expression and cloned into a standard transfer vector. The P domain is then re-cloned into an expression vector that encodes a polyhistidine (His) tag and a mannose-binding protein (MBP) that are followed by a protease cleavage site. The MBP-His-P domain fusion protein is expressed in *E. coli*, followed by three purification steps. The MBP-His-P domain fusion protein is purified using immobilized metal ion affinity chromatography (IMAC). Next, the fusion protein is cleaved with human rhinovirus (HRV)-3C protease and the P domain is separated from the MBP-His by an additional IMAC purification step. Lastly, the P domain is purified using size exclusion chromatography (SEC). The purified P domain can then be used for X-ray crystallography. Screening of protein crystallization conditions is performed with commercially available screening kits using different P domain protein concentrations. Crystal growth is observed and the most promising conditions are optimized.

With the methods described here, it is possible to go from gene to protein to structure within less than four weeks. Therefore, our method of P domain expression, purification, and crystallization is suitable to study norovirus-host interaction at the molecular level and provide important data to assist in up-to-date vaccine design and drug screening.

Protocol

1. P Domain Cloning

1. Determine the P domain coding region by sequence alignment of norovirus strains *(e.g.,* GII.10 strain, GenBank: AF504671, pdb-ID: 3ONU)[6]. Moreover, remove the flexible region at the C-terminal end of the P domain (**Figure 2A**). Codon-optimize the DNA for *E. coli* expression and include BamHI (N-terminal) and NotI (C-terminal) restriction sites in order to sub-clone the P domain coding region into the pMalc2x expression vector[6,7].
   Note: The P domain coding region is optimized and synthesized by a commercial service. The P domain coding region (insert) is approximately 1 kb in length and delivered in a standard transfer vector.
2. Digest 2 µg of the transfer vector with each 1 µl BamHI (20,000 U/ml) and NotI (10,000 U/ml) restriction enzymes for 1 hr at 37 °C with manufacturer supplied buffers.
3. Separate the digested insert on a 1% agarose gel for 20 min at 135 V and purify the insert DNA from the gel using a commercial kit.
4. Prepare the pMalc2x expression vector by digesting 2 µg of this vector with each 1 µl BamHI (20,000 U/ml) and NotI (10,000 U/ml) restriction enzymes for 1 hr at 37 °C. Purify the vector from an agarose gel as described above (1.3). Note: Both samples (1.2 and 1.4) can be stored at -20 °C.
5. Ligate the purified insert into the digested pMalc2x vector at the BamHI and NotI restriction sites with 1 µl T4-DNA ligase (400,000 U/ml) for 15 min at room temperature (RT) (**Figure 2B and 2C**). Use at least 20 ng of the pMalc2x vector and a vector:insert ratio 1:3 (molecular weight). The ligation mix is usually ~ 20 µl.
6. Transform 2 µl of the ligation mix into 50 µl chemically competent *E. coli* DH5α bacterial cells using a standard transformation protocol (10 min on ice, heat shock 45 sec at 42 °C) and grow in 600 µl S.O.C. medium for 1 hr at 37 °C. Centrifuge the transformed cells for 3 min at 1,000 x g, discard the supernatant, and resuspend the pellet in 30 µl of S.O.C. medium.
   1. Plate the transformation mix on LB-Agar plates, containing 100 µg/ml ampicillin for selection, and grow overnight at 37 °C. Select at least five colonies.
7. For each of the five colonies, inoculate 2-3 ml culture of LB-medium supplemented with 50 µg/ml ampicillin (LB-amp) and grow by shaking overnight at 160 rpm at 37 °C.
8. Extract the plasmids from the overnight culture using a commercial kit. Verify the presence of the P domain insert by sequencing with a pMalc2x forward primer (5'-TCAGACTGTCGATGAAGC-3') and reverse primer (5'-GATGTGCTGCAAGGCGAT-3').

2. P Domain Expression

1. Transform 1 µl (150 ng/µl - 400 ng/µl) of the pMalc2x vector coding for the MBP-His-P domain fusion protein into 50 µl of competent *E. coli* BL21 cells using a standard transformation protocol (10 min on ice, heat shock 45 sec at 42 °C) and grow in 600 µl S.O.C. medium for 1 hr at 37 °C. Subculture into 120 ml of LB-amp overnight at 160 rpm and 37 °C.
2. Inoculate nine liters (*e.g.,* 6 x 5 L flasks with 1.5 L medium each) of LB-amp with the subculture (1:100). Grow the cells shaking at 160 rpm and 37 °C until the OD_{600} reaches 0.4 - 0.6. Subsequently, lower the temperature to 22 °C for ~ 1 hr and then induce the protein expression with 0.66 mM of isopropyl-β-D-thiogalactopyranoside (IPTG)[8]. Grow the cells overnight at 22 °C (~ 18 hr).
   Note: The temperature can be varied, but we recommend to use 22 °C or lower.
3. Harvest the cells by centrifugation (10,543 x g, 15 min, 4 °C). Discard the supernatant and freeze the cell pellet at -20 °C.

3. 1st Purification Step and Protease Cleavage

1. Prepare buffers that are used during the protein purification steps from stock solutions to guarantee reproducibility and stability of the experiments. Prepare four different buffers for the immobilized metal ion affinity chromatography (IMAC), each containing a different concentration of imidazole (10 mM, 20 mM, 50 mM, and 250 mM). For the SEC, prepare a gel-filtration buffer (GFB) with a higher salt concentration, but without imidazole. Use deionized water and filter all buffers before use with a pore size of 0.45 µm.
   Note: For a detailed buffer preparation scheme, refer to **Table 1**.
2. Thaw the cell pellet from the nine liter culture and dissolve in 150 ml PBS at 4 °C. Sonicate the cell suspension three times for 2 min (power 130 W, amplitude 20%, pulse frequency 50%) to disrupt the cells. Keep the cell suspension on ice during sonication.
3. Centrifuge the sonicated cell suspension (43,667 x g, 30 min, 4 °C) to separate cell debris from the supernatant containing expressed protein. Collect the supernatant and discard the pellet.
4. Wash and equilibrate 10 ml (=1 column volume [CV]) slurry of Nickel (Ni)-NTA agarose beads with 10 mM imidazole buffer in a chromatography column. Add the equilibrated Ni beads to the supernatant from step 3.3 containing expressed MBP-His-P domain fusion protein and incubate for 30 min at 4 °C with slow rotation.
5. After incubation, apply the entire Ni-bead-protein mixture to a chromatography column. Wash the column slowly with each 5 CVs of 10 mM, 20 mM, and 50 mM imidazole buffers, starting with 10 mM, then 20 mM and last 50 mM (**Figure 3A**).
6. Elute the MBP-His-P domain fusion protein using 250 mM imidazole buffer (**Figure 3A**). During elution, check the OD_{280nm} to verify the elution of the fusion protein (the rise in OD_{280nm}). Continue elution until the OD_{280nm} drops to ~ 0.1. Wash the beads with excessive amounts of 250 mM imidazole buffer (at least 10 CVs), followed by at least 10 CVs of 10 mM imidazole buffer. Save the beads for the second purification step (section 4).
7. Verify the presence of the MBP-His-P domain fusion protein with SDS-PAGE using a 12% SDS-polyacrylamide gel (10 x 8 cm)[9] (**Figure 3A**). Perform gel electrophoresis at 45 A and 200 V for 45 min.

8. Concentrate (*e.g.,* using a commercial concentrator) the eluted MBP-His-P domain fusion protein to a final concentration of ~ 3 mg/ml. Cleave the MBP-His-P domain fusion with HRV-3C protease during dialysis against 2 L of 10 mM imidazole buffer (~ 1:100) overnight at 4 °C (**Figure 3A**). Depending on the final volume of concentrated protein, perform dialysis in a dialysis cassette or dialysis tubing.
   Note: The amount of HRV-3C protease for protein cleavage is calculated according to the specific protease activity (2 U/µl, 1 U is sufficient to cleave 100 µg of protein) and amount of eluted fusion protein that varies on the expression level and can be estimated from the SDS-PAGE result (3.7).

4. 2nd Purification Step

1. Equilibrate the Ni-beads from step 3.6 in 10 mM imidazole buffer.
2. Incubate the dialyzed protein from step 3.7 (containing cleaved P domain, MBP protein, and HRV-protease) with the equilibrated Ni-beads (4.1) for 30 min at 4 °C with slow rotation.
3. Apply the Ni-bead mixture to a column and collect the flow-through (cleaved P domain) (**Figure 3B**). Measure the concentration of protein as it comes off the column until the OD_{280nm} reaches ~ 0.1.
   Note: The MBP-His should remain bound to the Ni-beads (**Figure 3B**).
4. Check the presence of cleaved P domain using SDS-PAGE with a 12% gel as described above (**Figure 3B**). Concentrate the eluted P domain to ~ 3 mg/ml and dialyze overnight at 4 °C against GFB for subsequent SEC purification.

5. 3rd Purification Step

1. Wash pumps and pipes of the HPLC purification system and pre-equilibrate the SEC-column (see Materials List) with GFB.
2. Inject the P domain to the column at a flow-rate of 1 ml/min using a superloop (up to 12 ml) or loop (up to 3 ml), depending on the volume of the concentrated sample. After the injection is finished, increase the flow rate to 2.5 ml/min.
3. As the OD increases and the P domain comes off the column, collect fractions of 1.5 ml. Check fractions using SDS-PAGE with a 12% gel (**Figure 4A and 4B**). Pool only the purest fractions and concentrate to ~ 3 mg/ml and ~ 8 mg/ml.
   Note: After ~110 ml (void volume) most impurities are eluted from a SEC column with 320 ml bed volume. The P domain is usually eluted as a dimer. Elution time/volume of the P domain dimer is dependent on the prep grade (pg) of the SEC column.

6. Crystallization of the P Domain

1. Use the P domain at ~ 3 mg/ml and 8 mg/ml for initial crystallization screening. Prepare at least 100 µl of P domain per concentration for initial screens with 384 commercially available screening conditions. Perform screening at 18 °C in a 96-well plate format, where the reservoir contains 100 µl of mother solution and a drop is composed of 0.2 µl mother solution and 0.2 µl protein.
2. Repeat and optimize successful crystallization conditions. Therefore, use 15-well plates that contain 3 rows. Set up the first row with 100% mother solution, the second row with 90% mother solution and 10% water, and the third row with 80% mother solution and 20% water. Use a drop size of 2 µl (1 µl protein + 1 µl mother solution) and 500 µl of mother solution as a reservoir volume.
3. Use optimized crystal conditions for co-crystallizing the P domain with ligands. Prepare plates as described in 6.2. Instead of 2 µl drop size, set up drops containing 1 µl mother solution, 1 µl protein, and 1 µl of ligand at a concentration of 1 mg/ml.
4. Collect data sets of single crystals using synchrotron radiation. Perform molecular replacement using published P domain structures with a high sequence similarity as initial search model[6,10-13].
   Note: Presence of a ligand shows up as un-modeled blob of electron density.

Representative Results

The schematic of the described protocol is depicted in **Figure 1**. The protocol covers 6 major parts that include cloning of the target gene, expression, a three-step purification, and crystallization. **Figure 2** illustrates the design of the expression construct (EC) and characteristics of the pMalc2x expression vector. The sequence of the multiple cloning site (MCS) of the pMalc2x vector shows restriction and protease cleavage sites. **Figure 3** shows representative SDS-PAGE results of MBP-His-P domain fusion protein and cleaved P domain with the corresponding schematics of the first two steps of purification. The third purification step is depicted in **Figure 4** and involves a purification scheme, the elution chromatogram of purified P domain and a representative SDS-PAGE result of collected fractions. The purest fractions (according to SDS-PAGE) are pooled, concentrated, and used for X-ray crystallography.

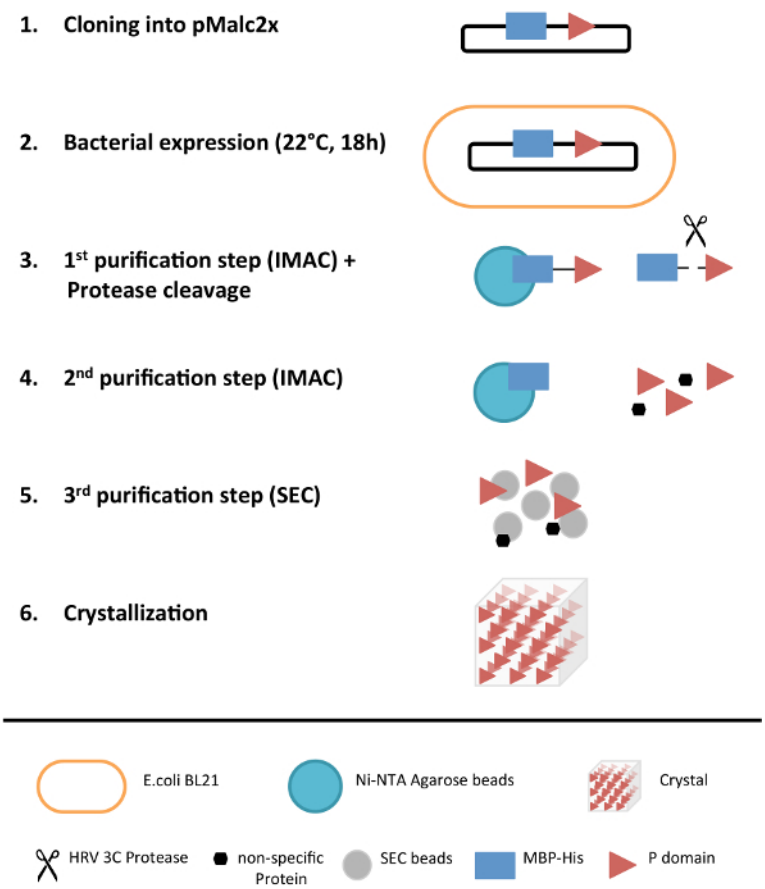

Figure 1. Schematic of Norovirus P Domain Expression and Purification. The protocol for norovirus P domain expression and purification contains six major parts, covering cloning and expression (**1 and 2**), purification (**3 to 5**), and crystallization (**6.**). Red triangles represent the P domain (gene and protein), whereas blue rectangles represent the MBP-His. Ni-NTA agarose beads that are used during IMAC (**3 and 4**) are illustrated as big cyan spheres. The SEC beads are depicted as grey spheres.

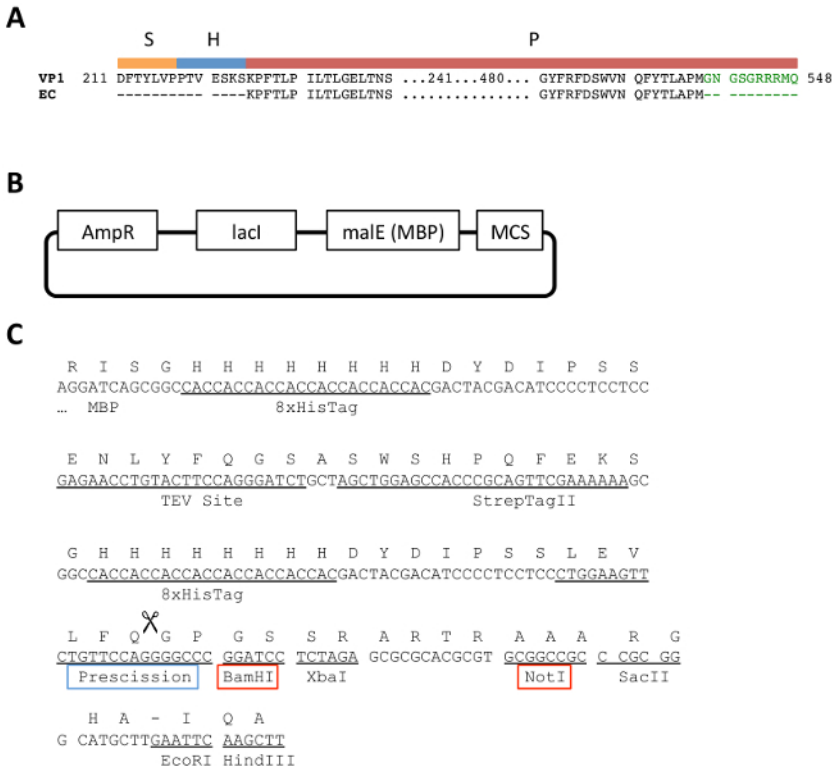

Figure 2. Design and Cloning of the Expression Construct (EC). The P domain EC, the expression vector map, and the multiple cloning site (MCS) are shown. **A**) Alignment of the norovirus capsid protein (VP1) and the P domain EC illustrating the design of the EC with the C-terminal deletion (green). **B**) Schematic representation of the pMalc2x expression vector used for P domain expression in *E. coli* with ampicillin-resistance cassette (AmpR), lac-operon (lacI), mannose-binding protein (malE, MBP) and a MCS. **C**) Sequence of the MCS of the pMalc2x expression vector. Highlighted are the restriction enzyme cleavage sites (red boxes) and the recognition sequence LEVLFQGP for the HRV 3C protease (precision, blue box).

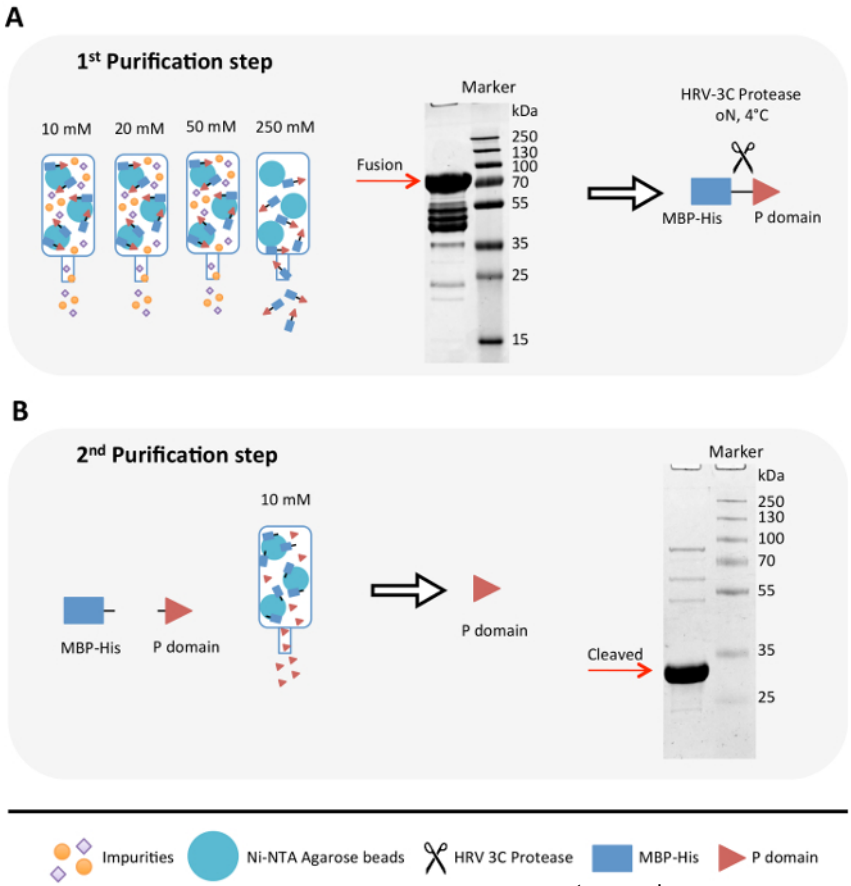

Figure 3. Schematic and Representative Results for the 1st and 2nd Purification Step. Purification overview and representative SDS-PAGE results are shown. **A**) Purification of the MBP-His-P domain fusion protein using Ni-NTA agarose beads (big cyan spheres). The 12% SDS-PAGE gel shows the MBP-His-P domain fusion protein. **B**) Separation of MBP-His (blue rectangle) from cleaved P domain (red triangle). Elution of cleaved P domain is analyzed by SDS-PAGE on a 12% gel.

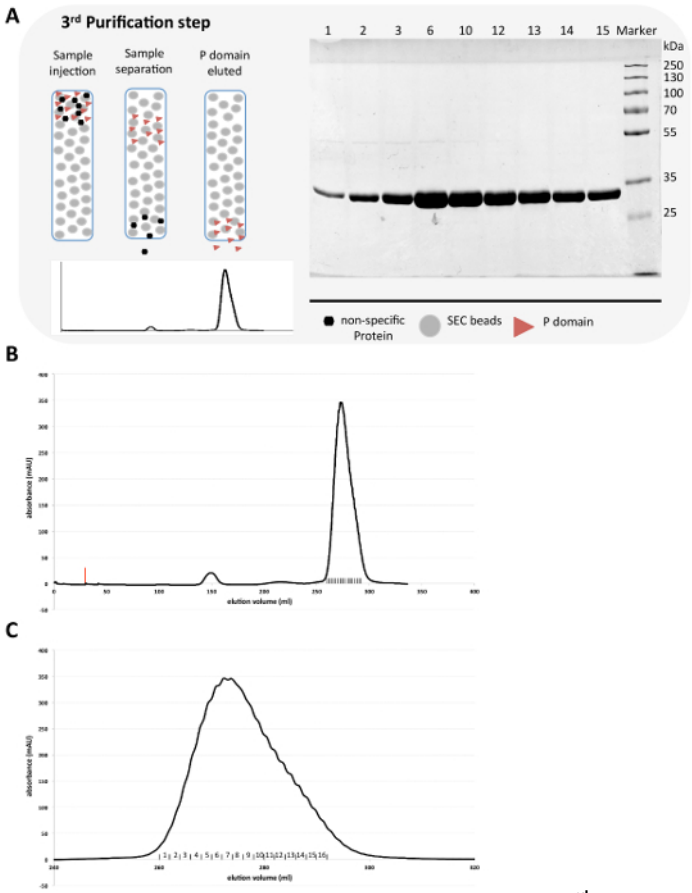

Figure 4. Schematic and Representative Results for the 3rd Purification Step. Chromatogram of P domain elution using SEC and corresponding SDS-PAGE result. Black numbers indicate the fractions that are collected. **A**) Schematic of the separation on the SEC column (SEC beads are depicted as grey spheres). The 12% SDS-PAGE shows the protein present in the fractions that are collected during SEC elution and which are subsequently pooled and used for X-ray crystallography. **B**) The SEC chromatogram shows the measured absorbance (black line) over elution volume. The red line indicates when the P domain was injected into the SEC-column. **C**) Zoom in to second peak of **B**. Please click here to view a larger version of this figure.

Buffer name	1 M Tris pH 7.6	5 M NaCl	3.5 M Imidazole, pH 8
250 mM Imidazole buffer	20 ml	40 ml	71 ml
50 mM Imidazole buffer	20 ml	40 ml	14 ml
20 mM Imidazole buffer	20 ml	40 ml	5.7 ml
10 mM Imidazole buffer	20 ml	40 ml	2.8 ml
GFB (Gel filtration buffer)	25 ml	60 ml	--

Table 1. Pipetting Scheme for Common Buffers used During Purification. Stock solutions of Tris-HCl, sodium chloride (NaCl), and imidazole are prepared as indicated in the header of the table. The amount of stock solution needed to prepare 1 L of the desired buffer in water is represented.

Discussion

Here, we describe a protocol for the expression and purification of norovirus P domains in high quality and quantity. Noroviruses are not well studied and structural data are continuously needed. To our knowledge, P domain production using other protocols (*e.g.*, GST-tagged P domains) has been problematic, so far, and sufficient structural data on norovirus-host interaction have been missing. With the method described here, we have recently contributed significantly to the understanding of the molecular details of norovirus carbohydrate binding. The present protocol can be adapted to a variety of proteins. However, successful implementation of this protocol depends on several factors within each part of the purification method.

Design of the expression construct is the first step of importance. We perform codon-optimization of the P domain expression construct to improve the expression yield in *E. coli* and remove relevant restriction sites, present within the coding region. Moreover, we remove a flexible

region at the C-terminus that could be disadvantageous for protein folding during expression and protein packing during crystallization. Expression as MBP fusion protein is a mean to keep the protein in solution during expression.

Regarding soluble protein expression there are additional parameters to be considered. The *E. coli* BL21 strain is optimized for high yield protein expression and therefore used in this protocol. Expression is performed overnight at 22 °C and a reduced amount of IPTG is used for induction. This is favorable for the kinetics of protein expression and as a result, less protein will aggregate into inclusion bodies due to misfolding. Therefore, it is important to cool down the culture to 22 °C before induction of protein expression with IPTG. If the protein yield is not satisfactory, it is possible to further decrease the temperature and adjust the amount of IPTG.

Certain care should be taken regarding the purification columns. In principle, Ni-beads can be reused several times. However, binding capacity will be reduced over time. If the Ni-solution loses its standard blue color, the beads can be stripped and recharged using the instructions in the manufacturer's handbook. Furthermore, it is important to maintain the SEC column in proper condition and clean it regularly to allow high performance. Depending on the protein size that has to be purified a different prep grade (pg) of the SEC column should be chosen. The P domain dimer is ~ 65 kDa in size and can be well separated from impurities of ~ 100 kDa using a 75 pg column, whereas larger proteins can be better separated using a 200 pg column.

As a combination of optimized sequence design, expression, and purification procedure it is possible to gain very pure and high quality P domain using our method. Owing to the high quality of the purified P domain, additional studies are suitable, including immunization for antibody production, NMR experiments, and ELISA-based studies. Moreover, the purified P domain can be used for complex formation with Fab antibodies and Nanobodies[14,15]. To our knowledge, this is the first protocol that allows P domain crystallization in a high throughput manner and, using this protocol, we have determined over 20 complex structures of various norovirus and lagovirus P domains in complex with HBGAs[6,16].

According to our experience, the protocol might be limited to proteins up to 65 kDa. However, capsid proteins of different caliciviruses[17] and non-structural proteins, such as viral protein genome-linked (VPg), protease, and RNA dependent RNA polymerase (RdRp) were successfully expressed and purified using this method (unpublished). When applying this method to capsid proteins of other viruses, it might be necessary to vary and optimize several parameters *(e.g.*, the imidazole concentration of the elution buffer) to gain sufficient amount of protein. In addition, different storage buffers other than GFB (*e.g.*, PBS or TBS) can be tested for optimal protein stability.

The majority of the analyzed constructs yielded in cubic/plate-like crystals, which diffracted to high-resolutions. Therefore, the present protocol provides a tool to obtain pure protein that crystallizes well. As long as there is now robust cell culture model available, this methodology constitutes a significant step to contribute to the understanding of norovirus-host cell interaction.

Disclosures

The authors have nothing to disclose.

Acknowledgements

The funding for this study was provided by the CHS foundation. We acknowledge the protein crystallization platform within the excellence cluster CellNetworks of the University of Heidelberg for crystal screening and the European Synchrotron Radiation Facility for provision of synchrotron radiation facilities.

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