RESEARCHREPORT
SWINE HEALTH

Title: Purified PRRS Viral Proteins - **NPB #04-207**

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Abstract: Three porcine reproductive and respiratory syndrome virus proteins, nsp2p, GP5-3', and nucleocapsid, from the prototype North American strain VR2332 have been produced and purified by ATG Laboratories, Inc. The proteins were produced at high levels in bacteria and affinity-purified to near homogeneity. Nsp2p and nucleocapsid are now available to any cooperating researcher on an as needed basis at no charge to the researcher.

Introduction: Porcine reproductive and respiratory syndrome (PRRS) has had devastating economic effects on the swine industry, and essential research required for a molecular understanding of infectivity, as well as treatment and vaccine development, will advance most efficiently if high quality reagents are available to the research community. Therefore, the major goal of this project was to make a large supply of three PRRS virus proteins, nsp2p, GP5-3', and nucleocapsid, available to PRRS researchers without charge. Each recombinant protein has a myc epitope tag as well as a polyhistidine tag. Previously, the laboratory of Michael P. Murtaugh, Ph.D. (University of Minnesota) engineered the plasmids, and developed methods for production of each protein in bacteria and for affinity purification of each protein from bacterial extracts. Production and purification methods were optimized (ATG Laboratories, Inc.) resulting in improved yields of the purified proteins.

Objectives: The objective of this project was to facilitate study of the PRRS virus by creating a stock of affinity-purified PRRS viral proteins (ATG Laboratories, Inc.) and to make them available at no charge to the community of PRRS researchers across the country (University of Minnesota).

Materials and Methods: *Small-scale protein expression screening*-Plasmids, pET 24b myc-*PRRS virus gene*-His (supplied by the Murtaugh lab), were transformed into CodonPlus[DE3]-RP cells (Stratagene, Inc.) or Rosetta2[DE3] (Novagen, Inc) which contain eukaryotic tRNAs not present in *E. coli* on a chloramphenicol resistant plasmid. Transformed cells were screened for protein expression by growing small cultures, each inoculated with a single colony, overnight at 37 ºC in 1.5 ml of 2xYT media containing 10 □g/ml kanamycin and 34 □g/ml chloramphenicol. The overnight culture was used to inoculate 1.5 ml of 2xYT containing the same antibiotics. These cultures were grown at 37 ºC for 30 minutes and then protein expression was induced with IPTG at a final concentration of 1 mM. Cultures

were grown for an additional three hours and then the cells were harvested by centrifugation. Expression of each recombinant protein was assessed in whole cell lysates of each culture by SDS-PAGE analysis on Coomassie blue stained gels. One positive isolate for each plasmid/strain was chosen and used to prepare a glycerol stock that was archived at –80 ºC for future use in larger-scale production cultures.

Larger-scale cultures for protein production-Cultures were grown at 37 ºC with shaking (250 rpm) in baffled 2 liter flasks containing 500 ml of 2xYT culture media and 10 □g/ml kanamycin and 34 ug/ml chloramphenicol. Each flask was inoculated 1:25 or 1:50 (v/v) from an overnight culture (inoculated with cells from a fresh streak of the glycerol stock) produced at 37 ºC. When the culture OD_{600nm} reached 0.5-0.6, protein expression was induced with IPTG at a final concentration of 1 mM, and grown for an additional 3 hours. The cells were harvested by centrifugation, Sorvall GS3 rotor at 7000 rpm for 10 minutes at 4 °C. The cell pellets were stored at -80 °C.

Preparation of washed inclusion bodies-Cell pellets from 2 liters of *E. coli* culture were resuspended in 50 ml of 50 mM Tris(HCl) pH 8.0, 500 mM NaCl, 2 mM EDTA, 10 mM □-ME, and 1 mM PMSF and lysed by sequential addition of the following reagents: 1. Lysozyme at a final concentration of 0.2 mg/ml (w/v). 2. One ml of 10% Triton X-100. 3. Benzonase (Novagen, cat. # 70664-3) at a final concentration of 8 U/ml. 4. Nine ml of 10% Triton X-100. After each addition, the mixture was briefly homogenized (Virtis, Inc., Handishear) and incubated at room temperature for about 10 minutes. The final volume of the lysate was brought to 100 ml with the above buffer. Insoluble material was sedimented at 16,000 rpm for 15 minutes at 4 ºC (four tubes, 25 ml/tube). The supernatants were removed and the pellets were washed with 50 mM Tris(HCl) pH 8.0, 500 mM NaCl, 2 mM EDTA, 10 mM □-ME, and 1 mM PMSF (25 ml/tube) two more times (nsp2p only) and once (nsp2p) or twice (GP5-3' and nucleocapsid) with 0.1% Triton X-100, 10 mM □-ME, and 1 mM PMSF and once with ultrapure water, 10 mM □-ME, and 1 mM PMSF. Each wash consisted of resuspending the pellet in each tube with 25 ml of wash solution, homogenizing the mixture (Virtis, Inc., HandiShear), and sedimenting the insoluble material as above. Each supernatant and was saved on ice until after SDS-PAGE analysis. The protein present in each wash, supernatant and pellet, was examined by SDS-PAGE and the concentration of total protein in the washed inclusion bodies was determined by Bradford assay. Nsp2p and GP5-3' were found in the final pellet, and the nucleocapsid protein was found as a suspension in the final supernatant (ultrapure water wash, last wash). The samples for the Bradford assay were prepared by taking a small aliquot of the final homogenized sample containing the protein of interest and making two 1:10 dilutions in 100 mM NaH_2PO_4, 10 mM Tris(HCl), 6 M guanidine hydrochloride at pH 8.0.

Protein purification-Each recombinant protein was purified under denaturing conditions. Inclusion body protein (typically 150-250 mg) was solubilized in 100 ml of 100 mM NaH_2PO_4, 10 mM Tris(HCl) pH 8.0, 6 M guanidine hydrochloride, 10 mM □-ME, and 1 mM PMSF and rotated gently at room temperature for 30 minutes and then incubated (not rotating) overnight at 4 ºC. In the morning insoluble material was sedimented by centrifugation at 16,000 rpm for 15 minutes at 4 ºC. The supernatants were transferred to four 50 ml tubes containing a 5 ml bed of Ni-NTA-agarose (QIAGEN, Inc), 25 ml/tube. The tubes were rotated at room temperature for 60 minutes, and then loaded into 3.0 cm ID x 13 cm (nsp2p and nucleocapsid) or 1.5 cm ID x 13 cm (GP5-3') column. The nsp2p flow-throughs were collected and then re-loaded onto the column once. The column was washed three times with 20-35 ml of 100 mM NaH_2PO_4, 10 mM Tris(HCl), 6 M guanidine hydrochloride, at pH 6.3. The His-tagged PRRS protein was eluted with 100 mM NaH_2PO_4, 10 mM Tris(HCl), 6 M guanidine hydrochloride at pH 4.4. The protein present in each fraction was examined by SDS-PAGE and the fractions containing the highest concentration of the recombinant protein at the highest purity were pooled and stored at –80 ºC. The concentration of total protein in the protein pools was determined by Bradford assay.

Protein refolding-Refolding of the Ni-NTA-purified protein was performed using a variation of the methods described in Buchner *et al.* [1] and Clark [2] in 60 mg batches of Ni-NTA-purified protein. First, the concentration of Tris(HCl) was increased to a final concentration of 100 mM (in 20 ml), EDTA was added to a final concentration of 2 mM (in 20 ml), and the pH was adjusted to 8.0 with 10 N NaOH. Then DTT was added so that the final concentration was 300 mM (in 20 ml). Then 100 mM NaH_2PO_4, 10 mM Tris(HCl), 6 M guanidine hydrochloride at pH 8 was added so that the final volume was 20 ml (final protein concentration of 3 mg/ml). The reduced protein solution was rotated at room temperature for 2 hours and then added in 1 ml aliquots over ~10 minutes to 2 L of 100 mM Tris at pH 8.0, 0.5 M L-arginine, 8 mM oxidized glutathione, 2 mM EDTA, 3 mM DTT, and 0.1 mM PMSF and stirred slowly overnight at 4 ºC. In the morning, the refolding solution was filtered through a bottle top 0.2 um filter (Nalgene, Inc., SFCA #291-4520). The filtrate was loaded to a cross-flow hollow fiber cartridge (AmershamBiosciences, 5 or 10 kDa MWCO, UFP-5-C-4MA or UFP-10-C-4MA, respectively) and concentrated to the minimum volume of about 50 ml. Then refolding buffer was exchanged for storage buffer, 20 mM Tris(HCl) pH 8.0 (nsp2p and GP5-3') or 20 mM Tris(HCl) pH 8, 150 mM NaCl (nucleocapsid) using the same cartridge. The retentate was clarified by centrifugation and then the supernatant was filtered through a 0.2 □m filter, aliquoted, and stored at –80 ºC. Protein concentration was determined by Bradford assay and protein purity was assessed by SDS-PAGE.

Protein concentration: The concentration of total protein was determined by Bradford assay (Pierce Chemical Co., Coomassie Protein Assay Reagent, catalog # 23200 or Amresco, Inc., Bradford Reagent, catalog # E530) with BSA as the standard protein. The BSA concentration in the standard solution was determined by absorbance at 280 nm using $\square^{0.1\%}$ of 0.667 ml mg^{-1} cm^{-1}. Each sample with unknown protein concentration was replicated at least 3 times/assay. When guanidine hydrochloride was present in the sample, it was also present in the standards and blanks at the same concentration as in protein being assayed.

SDS-Polyacrylamide Gel Electrophoresis: Standard protocol (samples without guanidine)-Each sample, 20 □l, was resuspended in 20 □l of reducing Laemmli sample buffer [2X solution: 125 mM Tris(HCl) pH 6.8, 20% glycerol (v/v), 4% SDS (w/v), 5% □-ME (v/v), and 0.005% bromophenol blue (w/v)], and heated for 10 minutes at 90-100 °C.

Samples in guanidine buffer-The total protein in a 20 □l aliquot was precipitated with 500 □l 10% trichloroacetic acid and collected by centrifugation. The pellets were resuspended in 36 □l of Laemmli sample buffer with 4 □l of 1 M Tris and heated for 10 minutes at 90-100 °C.

Proteins were separated on 4-20% polyacrylamide gradient mini-gels (Invitrogen Corp., catalog # EC60255; 1 mm thick; 15 wells/gel) in 25 mM Tris base, 192 mM glycine, and 0.1% SDS (w/v) with 135 V constant voltage. The gels were stained with Coomassie brilliant blue.

References

Buchner, J., I. Pastan, and U. Brinkmann, A method for increasing the yield of properly folded recombinant fusion proteins: single-chain immunotoxins from renaturation of bacterial inclusion bodies. Anal. Biochem. (1992) 205:263-70.

Clark, E.D.B., Refolding of recombinant proteins: Curr. Opin. Biotechnol. (1998) 9:157-63.

Results:

VR2332 Protein	Inclusion body total protein (mg/2 liters culture)	Ni-NTA-purified total protein (mg/2 liters culture)	Refolded total protein (mg/60 mg Ni-NTA-purified protein)

nsp2p	256	105	30.2
GP5-3’	171	66	0
nucleocapsid	284	175.8	51.3

Discussion: High-level bacterial expression of three PRRS virus polypeptides was accomplished, and purification of three polypeptides, nsp2p, GP5-3’, and nucleocapsid, was carried to completion. The yield of nsp2p and nucleocapsid was within the expected range of protein yields from bacterial expression, and was higher than has been achieved in the lab of Dr. Murtaugh (personal communication). Likewise the recovery of refolded nsp2p and nucleocapsid proteins was very good however, recovery of refolded GP5-3’ was not detectable in assays of total protein or by SDS-PAGE. Nsp2p and nucleocapsid were delivered to Michael Murtaugh at the University of Minnesota. The yield of refolded GP5-3’ may be significantly improved by optimizing buffer components and equipment parameters.

Lay Interpretation: PRRS virus proteins that are immunogenic in pigs were expressed and purified. Nsp2p and nucleocapsid proteins are and will be available to PRRS researchers and can be used to detect exposure to PRRS virus, monitor immune responses, and to investigate mechanisms of immune protection against PRRS.