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The major goals of this project are to purify hantavirus proteins (Jonsson) for X-ray structure determination (Arnold). By defining the three-dimensional structures of the N and RDRP proteins of hantaviruses, our studies will afford a means to model drugs that specifically interfere with important stages of viral replication. Particularly, disrupting one or more functions of the RDRP is expected to result in effective disease treatment with little toxicity to host cells. In addition, our research will yield a high through-put in vitro assay for identification of new antiviral drugs. Together, these studies should lead toward effective therapeutic measures for controlling and treating hantaviral infections.

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INTRODUCTION

The proposed work details a collaboration among Dr. Connie Schmaljohn at USAMRIID, Dr. Edward Arnold at the Center for Advanced Biotechnology and Medicine (CABM) at Rutgers University, and Dr. Colleen Jonsson at New Mexico State University (NMSU). The three laboratories cover a wide breadth of expertise in virology (Schmaljohn), biochemistry (Jonsson) and structural biology (Arnold). Dr. Schmaljohn's funding was handled through a separate office although she was in this proposal of effort.

The major goals of this project are to purify hantavirus proteins (Jonsson) for X-ray structure determination (Arnold). By defining the three-dimensional structures of the N and RDRP proteins of hantaviruses, our studies will afford a means to model drugs that specifically interfere with important stages of viral replication. Particularly, disrupting one or more functions of the RDRP is expected to result in effective disease treatment with little toxicity to host cells. In addition, our research will yield a high through-put in vitro assay for identification of new antiviral drugs. Together, these studies should lead toward effective therapeutic measures for controlling and treating hantaviral infections.

Dr. Jonsson' group has been involved in the expression and purification of the hantaviral N protein, and RDRP (both as enzymatically active fragments and as a complete protein). These efforts are key to our goals of defining the structure of the RDRP and developing an in vitro assay for drug screening. Toward the latter goal, we established conditions for RDRP activity by using infectious HTNV, and will apply those conditions to expressed RDRP to create an in vitro assay of enzyme function. Such information may serve as the basis for future investigations on other newly emerging viruses.

BODY

STATEMENT OF WORK --YEAR 1

1. Produce and purify to at least 95% homogeneity the Sin Nombre virus (SNV) nucleocapsid (N) protein (NMSU) - Year 1.

2. Define conditions for crystal production of SNV N protein (CABM) – Year 1.

3. Produce and purify to at least 95% homogeneity the Hantaan virus (HTNV) RNA dependent RNA polymerase core domain (RDRP) (NMSU) and other soluble portions of the polymerase protein (USAMRIID) - Years 1 and 2 .

4. Define conditions for crystal production of HTNV RDRP core domain (CABM) - Year 1.

5. Complete studies to develop an assay for endonucleolytic cleavage of host mRNA using infectious hantaviruses (USAMRIID) - Year 1.

Aim 1. Produce and purify to at least 95% homogeneity the Sin Nombre virus (SNV) nucleocapsid (N) protein (NMSU) - Year 1.

In the appendix, the following manuscript detailing the accomplishments for this Aim is provided.

Jonsson, C.B., Gallegos, J., Fero, P., Xu, X. and C. S. Schmaljohn. Expression, Purification and Characterization of the Sin Nombre Virus Nucleocapsid Protein in Escherichia coli. Accepted with revision.

Aim 2 and 4. Define conditions for crystal production of SNV N and HTNV RDRP proteins (CABM) – Year 1.

Samples of nucleocapsid protein appear monodisperse by gel filtration, anion exchange chromatography, and gel electrophoresis. The protein can be concentrated to between 10 and 20 mg/ml, which is an appropriate range for crystallization protocols. Limited crystallization experiments have been performed allowing the possible range of crystallization conditions to be narrowed down.

Crystallization procedures utilized the hanging drop method. Protein solutions were mixed with appropriate salts and precipitant solutions to induce crystal formation and growth. This required a trial of a large range of possible conditions. It can be difficult to determine appropriate crystallization conditions for protein molecules. Some proteins will crystallize over a wide range of conditions, while others may do so only in a very narrow range. Initially, we are using commercially available crystallization kits and methodologies. These are very widely used and successful as evidenced in many publications. They have been developed from surveys of successful conditions from thousands of publications, and the crystallization matrices are mathematically determined to cover many variables (such as pH and salt concentration) in a small number of experiments. We will continue to explore conditions to crystallize the N protein.

Aim 3. Produce and purify to at least 95% homogeneity the Hantaan virus (HTNV) RNA dependent RNA polymerase core domain (RDRP) (NMSU) and other soluble portions of the polymerase protein (USAMRIID) - Years 1 and 2.

Cloning, expression and purification of recombinant Hantaan RdRp— The Hantaan polymerase, 246 kDal RdRp, is one of the largest polymerases known among viruses. Such a large protein can often create technical difficulties in large-scale production and purification. Expression of the recombinant Hantaan RdRp in bacterial cells is low, although on occasion we have noted fairly large quantities accumulating in the cell (Jonsson, data not shown). One common and very successful approach to these problems is to divide a large protein into smaller functional domains. Fortunately, the structure-function relationship has been well characterized for numerous polymerases (1). Using this information, we have identified a region which contains the putative catalytic core, and hence, the major catalytic residues for replicative synthesis. The HTNV RdRp core represents an internal region of the protein that is highly conserved among

polymerases, the putative catalytic domain. The region spans nts 2579 to 3739 (cRNA, 44 kDal); encoding for 387 amino acids. The core functional domain was cloned in pET21b and expressed in BL21DE3 cells as a C-terminal hexahistidine fusion. Optimal expression of the RdRp core required fresh transformation of plasmid into BL21DE3 (data not shown). The core protein was purified by nickel affinity and SP sepharose FF chromatography. The protocol developed for the purification is given below. The protein was stable in bacterial cells as shown by the absence of proteolytic break down in western blot analysis. The yield from one liter of bacterial cells typical were approximately 3 mg with 80% purity.

Expression and Initial Purification of HTNV RdRp Catalytic Core—Escherichia coli BL21 DE3 cells, containing the catalytic core, were inoculated into 4 X 100 ml cultures containing Luria broth (LB) with ampicillin (amp) (200 μg/ml). The cultures were grown at 37°C for 16 hours at 200 rpms. The 100 ml cultures were then added to 4 X 1 liter LB containing amp 200 μg/ml and shaken at 200 rpms, 30°C, for 1 hr. The cultures were induced with 1mM isopropyl-b-D-thio-galactoside (IPTG) and shaken at 150 rpms, 30°C, for 80 min. Centrifugation at 5,000 g for 5 min yielded pellets, which were frozen at -80°C until used. The pellets were pooled and completely re-suspended in 200 ml solubilization buffer [300 mM NaCl, 10 mM cholamidopropyl-diamethylammonio-propanesulfonate (CHAPS), 20 mM 2-b-mercaptoethanol (BME), 10 mM imidazole, and 50 mM sodium phosphate buffer, pH 8.0]. 400 mg lysozyme was added to re-suspension mixture and dounced for 45 minutes on ice followed by sonication for 10 cycles (1 cycle= 1 min sonication/ 2 min on ice) or until completely clarified. The extract was centrifuged at 30,000 g for 60 min. The clarified extract was mixed with 14 ml 50% Ni-NTA Superflow resin and gently stirred at 4°C overnight. The resin was poured into a 0.22 μm disposable filter column (Fisher). The resin was washed with 35 ml wash buffer (300 mM NaCl, 20 mM 2-mercaptoethanol, 20 mM imidazole, 10 mM CHAPS, and 50 mM sodium phosphate buffer, pH 8.0). The protein was then eluted with 15 ml buffer containing 300 mM NaCl, 20 mM 2-b-mercaptoethanol, 250 mM imidazole, and 50 mM sodium phosphate buffer, pH 8.0. Elutant was placed in 3,500 MWCO snakeskin (Pierce) and dialyzed in buffer A (50 mM morpholino-ethanesulfonic acid (MES), pH 6.2, 200 mM NaCl, 1 mM dithiothreitol (DTT), and 5 % glycerol). Western blots were performed using polyclonal mouse sera to polyhistidine (diluted 1:2000) (Sigma; monoclonal—clone no. His-1). The secondary antibodies were alkaline phosphatase-conjugated anti-mouse IgG (diluted1:1000) (Promega), which were developed using Western Blue Reagent (Promega). In addition, protein concentrations were measured using the Bradford method (2) with BioRad Micro-Assay reagents as recommended.

SP sepharose Purification of Catalytic Core—Dialyzed protein was filtered through a 0.45 μm syringe filter into a sterile container. The sample was loaded on a 1 ml SP sepharose FF cation exchange column using a BioRad Dualflow HPLC system. The column was washed with 5 ml buffer A. The protein was eluted using a gradient from 100% buffer A to 100% buffer B (buffer A with 1M NaCl) in a volume of 10 ml. 1 ml fractions were collected and subject to SDS-PAGE, and immunoblot reacted with a polyclonal anti-histidine antibodies as described above.

Aim 5. Complete studies to develop an assay for endonucleolytic cleavage of host mRNA using infectious hantaviruses (USAMRIID) - Year 1.

Primer-independent polymerase activity of virions—A rapid membrane assay was developed to characterize the polymerase activity of purified virions. The conditions established will be instrumental in adapting the recombinant purified RDRP to the cap-snatching assay. The reaction, performed in a 96-well plate, measures the incorporation of ^{32}P-UTP into nascent RNA without the addition of template or primer to the reaction. The virion provides the RdRp and the viral genomic RNA template or vRNA. Reactions were terminated and slotted onto DEAE membrane to ascertain the level of new synthesis. As the reaction conditions have not been previously optimized for any negative strand RdRP from this family *in vitro*, the concentrations of salt and NTP as well as the type and concentration of metal ion were determined. The ionic strength of the polymerase reaction was examined from 40 mM to 140 mM NaCl. Reactions containing 60 and 80 mM NaCl reproducibly showed the highest levels of activity.

A key component of viral polymerase reactions is the metal ion (3). Previously, polymerase activity was demonstrated with a combination of 5 mM $MnCl_2$ and 1.5 mM $MgCl_2$ (4). We examined the presence of $MnCl_2$ in the reaction conditions as described above and $MgCl_2$ (data not shown). Reactions were assembled as described above, however, the final NaCl concentration was 80 mM and metal was omitted. The concentration of $MnCl_2$ was titrated from 0 to 12.5 mM. Typically, reactions containing 7.5 mM $MnCl_2$ showed the greatest level of synthesis. Little to no synthesis was observed when reactions were assembled with $MgCl_2$ alone (data not shown). This result is consistent with earlier observations (4).

Finally, the concentration of NTP was examined with the membrane assay. In these experiments, reactions were assembled as described above using 80 mM NaCl, 2.5 mM $MgCl_2$ and 2.5 mM $MnCl_2$. The ribonucleotides CTP, ATP, and GTP were omitted from the reaction buffer and titrated at 0.25, 0.50, 0.75 and 1.00 mM. Of the concentrations tested, reactions conducted at 0.25 mM CTP, ATP and GTP were routinely found to give the highest levels of synthesis.

KEY RESEARCH ACCOMPLISHMENTS

- Method for purification of soluble, homogenous N protein from E. coli
- Five additional clones for RDRP expression in E. coli
- Expression of three RDRP clones in E. coli
- Purification protocol for RDRP clones in E. coli
- Development of an assay for endonucleolytic cleavage of host mRNA using infectious hantaviruses

- Defined conditions for crystal production of SNV N protein

REPORTABLE OUTCOMES

Manuscripts

Jonsson, C.B., Gallegos, J., Fero, P., Xu, X. and C. S. Schmaljohn. Expression, Purification and Characterization of the Sin Nombre Virus Nucleocapsid Protein in Escherichia coli. Accepted with revision.

Xu, X., Severson, W.E., Villegas, N., Schmaljohn, C.S. and C. B. Jonsson The RNA binding activity of the Hantaan N protein maps to a central, conserved region. In preparation.

Jonsson, C.B., Gallegos, Severson, W.E., and C. S. Schmaljohn. Characterization of the Polymerase Activity of Virion and Recombinant Hantaan Virus RNA Dependent RNA Polymerase. In preparation.

Abstracts

C. Jonsson. Third International Virus Assembly Meeting, Seeon, Germany, April 1st - 5th 2001. The nucleocapsid or N protein of hantaviruses encapsidates both viral genomic RNA (vRNA) and the antigenomic RNA (cRNA), but not viral mRNA. Previous work has shown that the N protein has preference for vRNA and this suggested the possibility of a *cis*-acting signal that could be used to initiate encapsidation for the S-segment. To map the *cis*-acting determinants, several deletion RNA derivatives and synthetic oligoribonucleotides were constructed from the S-segment of the Hantaan virus (HTNV) vRNA. N protein-RNA interactions were examined by UV crosslinking, filter binding assays, and gel electrophoresis mobility shift assays to define the ability of each to bind HTNV N protein. The 5′ end of the S-segment vRNA was observed to be necessary and sufficient for the binding reaction. Modeling of the 5′ end of the vRNA revealed a possible stem loop structure (SL) with a large single-stranded loop. We suggest that a specific interaction occurs between the N protein and sequences within this region to initiate encapsidation of the viral genomic RNAs.

American Society of Virology, 20th Annual Meeting, July 2-25, Madison, WI (Poster). Purification and Characterization of the Catalytic Core Domain of the RNA Dependent RNA Polymerase from Hantaan Virus. Juan-gabriel Gallegos, William Severson, Connie Schmaljohn, Lisa Leon, and Colleen Jonsson. New Mexico State University, Department of Chemistry and Biochemistry. Virology Division, USAMRID.

The Hantaan virus (HTNV) RNA Dependent RNA Polymerase (RdRp) catalyzes the transcription and replication of negative-sense, single-stranded, L, M, and S viral RNAs

as well as the transcription of antigenomic or complementary RNAs and messenger RNAs. The RdRp is encoded by the L-segment and has an apparent molecular weight of 246 kDa. Comparing deduced amino acid sequences of the RdRp of HTNV with those of other RNA virus polymerases reveals a region of highly conserved residues between aa 847-1234, which may constitute a catalytic core domain; i.e., a region that is important for catalysis, metal binding, and RNA template-primer interactions. As a first step toward devising an *in vitro* system for studying hantavirus replication, we cloned this predicted domain into the bacterial expression vector pET 21b and expressed it as a hexahistidine fusion protein in BL21 DE3 *E. coli* cells. We purified the expressed polypeptide by nickel affinity and Sepharose cation exchange chromatography and analyzed it by SDS-PAGE and immunoblotting. We detected a polypeptide of the expected size of approximately 44 kDa. We tested the functionality of the core product by using an assay that measures elongation of a primer-template RNA by incorporation of a single α-^{32}P-ATP. Analysis of reaction mixtures by gel electrophoresis and autoradiography revealed a product one nucleotide longer than the template, thus demonstrating enzymatic functionality of the cloned fragment.
Supported by DOD grant, DAMD 17-00-1-0513.

American Society of Virology, 20th Annual Meeting, July 2-25, Madison, WI (Talk).

Cis-ACTING SIGNALS IN THE ENCAPSIDATION OF HANTAAN VIRUS S-SEGMENT vRNA BY ITS N PROTEIN.

William E. Severson, Xiaolin Xu, and Colleen B. Jonsson
Graduate Program in Molecular Biology and the Department of Chemistry and Biochemistry, New Mexico State University, Las Cruces, NM

The nucleocapsid or N protein encapsidates both viral genomic RNA (vRNA) and the antigenomic RNA (cRNA), but not viral mRNA. Previous work has shown that the N protein has preference for vRNA and this suggested the possibility of a *cis*-acting signal that could be used to initiate encapsidation for the S-segment. To map the *cis*-acting determinants, several deletion RNA derivatives and synthetic oligoribonucleotides were constructed from the S-segment of the Hantaan virus (HTNV) vRNA. N protein-RNA interactions were examined by UV crosslinking, filter binding assays, and gel electrophoresis mobility shift assays to define the ability of each to bind HTNV N protein. The 5′ end of the S-segment vRNA was observed to be necessary and sufficient for the binding reaction. Modeling of the 5′ end of the vRNA revealed a possible stem loop structure (SL) with a large single-stranded loop. We suggest that a specific interaction occurs between the N protein and sequences within this region to initiate encapsidation of the viral genomic RNAs.

Presentations

1. C. Jonsson. Third International Virus Assembly Meeting, Seeon, Germany, April 1st - 5th 2001.
2. C. Jonsson. Fifth International Meeting on HFRS, HPS and hantaviruses, Annecy, France, June 12-17, 2001. (Keynote Speaker)

3. J. Gallegos, W. Severson, C. Schmaljohn, L. Leon, S. Romero, P. Frazer and C. Jonsson, Purification and Characterization of the Catalytic Core Domain of the RNA Dependent RNA Polymerase from Hantaan Virus. American Society of Virology, 20th Annual Meeting, July 2-25, Madison, WI (Poster)
4. W. Severson, Xiaolin Xu, and Colleen B. Jonsson, American Society of Virology, 20th Annual Meeting, July 2-25, Madison, WI (Severson, talk)

Funding applied for based on work supported by award

Jonsson, C. PI, NIH R01, October 2000, Encapsidation of the Hantaan vRNA by its N protein

CONCLUSIONS

In the past year, a rapid method for purification of sufficient amounts of soluble, homogenous N protein from E. coli has been developed. This protocol will allow progress toward the crystallization of the N protein for crystallization studies. Light scattering experiments of the purified protein found it to be monodisperse. Methods to remove contaminating cellular RNA were developed and also lay expectations that the protein will be more amenable to crystallization. We have also defined the RNA binding domain for the N protein. The functional information will be extremely useful upon completion of the crystal structure.

A subclone of the RDRP was expressed and purified. Because we were not satisfied with the level of expression, we generated five additional clones of the RDRP expression in E. coli. One of these clones appears to give a greater level of expression and have good solubility. We are currently in the process of scaling the purification of this clone to isolate sufficient quantities for crystallization and characterization.

Development of an assay for endonucleolytic cleavage of host mRNA using infectious hantaviruses is being performed in Connie Schmaljohn's laboratory. The reaction conditions to use for the assay have been well characterized. We are currently searching for a suitable substrate for the reaction. We expect to be testing recombinant protein for cap-snatching activity in the following year.

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APPENDICES

Purification and Characterization of the Sin Nombre Virus Nucleocapsid Protein Expressed in *Escherichia coli*

Colleen B. Jonsson[1*], Juan Gallegos[1], Phillip Fero[2], William Severson[1], Xiaolin Xu[3], and Connie S. Schmaljohn[2]

Running head: SNV N protein: expression, purification and characterization

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ABSTRACT

Sin Nombre virus is a member of the *Hantavirus* genus, family *Bunyaviridae*, and is an etiologic agent of hantavirus pulmonary syndrome. The hantavirus nucleocapsid (N) protein plays an important role in the encapsidation and assembly of the viral negative-sense genomic RNA. The Sin Nombre (SNV) N protein was expressed as a C-terminal hexahistidine fusion in *Escherichia coli* and initially purified by nickel affinity chromatography. We developed methods to extract the soluble fraction and to solubilize the remainder of the N protein using denaturants. Maximal expression of protein from native purification was observed after a 1.5 h induction with IPTG (2.4 mg/L). The zwitterionic detergent, CHAPS, did not enhance the yield of native purifications, but increased the yield of protein obtained from insoluble purifications. Both soluble and insoluble materials, purified by nickel affinity chromatography, were also subjected to Hi Trap SP Sepharose fast flow (FF) chromatography. Both soluble and insoluble proteins had a similar A_{280} profile on the sepharose FF column, and both suggested the presence of a nucleic acid contaminant. The apparent dissociation constant of the N protein, purified by nickel affinity and SP Sepharose FF chromatography, and the 5′-end of the viral S-segment genome were measured using a filter binding assay. The N protein-vRNA complex had an apparent dissociation constant of 140 nM.

INTRODUCTION

Hantaviruses cause two illnesses in humans, hemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS) (1). Hantaan virus (HTNV) and Sin Nombre virus (SNV) are the major causative agents of HFRS and HCPS, respectively. Hantaviruses have a negative-sense, single-stranded RNA genome that consists of three segments, S, M and L (2,3). Transcription of each vRNA by the viral-encoded RNA dependent RNA polymerase (RdRp) yields a complementary RNA (cRNA), which in turn is used as a template to generate vRNA by the RdRp. In contrast to the hantaviral mRNAs, which do not associate with the viral nucleocapsid protein (N) (4), the vRNA and cRNA replicative templates are encapsidated by N. The interactions of the hantaviral RNAs and N are not well defined. Gott *et. al.* showed the presence of nonspecific RNA binding domain in the C-terminus of the HTNV N protein (5). In another study, the HTNV N bound preferentially to the viral genome rather than to nonviral RNA (6). More extensive biochemical studies of RNA-protein or protein-protein interactions will require sufficient quantities of highly purified, soluble, monodispersed N protein, that is free of contaminating ribonucleases, nucleic acids, and RNA binding proteins. Toward this goal, we have investigated methods to recover hantaviral N from a bacterial expression system in a form suitable for biochemical analysis.

Our previous purification strategy, as well as that of others, relied on using denaturing methods to recover soluble expressed N from bacteria (5,6). Because renaturing does not always result in native protein, large losses in functional N are common. Comparing yields of HTNV and SNV N after denaturing and renaturing shows that the SNV N protein is more readily recovered, and probably is more stable (6). No obvious reason for the difference in ease of recovery of soluble SNV N versus HTNV N

can be deduced from examining the amino acid sequences of the two proteins. The most notable difference is the substitution of 7 Gly residues in the HTNV N protein with Asn, Ala, Ser or Asp, in the SNV N protein. All of these substituted amino acids are more hydrophilic than Gly (7), and if they are located on the surface of the protein, they could facilitate the interaction of the protein with an aqueous environment. Insight into the molecular basis for the differences awaits a three-dimensional structure that could provide information on the solvent accessibility of these residues.

Herein, we report studies leading to the development of a rapid soluble extraction protocol and chromatography method for recovery and purification of the SNV N protein expressed in *E. coli*. We further test this method for suitability for preparing N proteins of three other hantaviruses.

MATERIALS AND METHODS

Reagents and Expression Vectors

Restriction enzymes and Vent polymerase were purchased from New England Biolabs (Beverly, MA). T4 DNA ligase and kinase were purchased from Gibco-BRL (Grand Island, NY). All chemicals were purchased from Sigma (St. Louis, Mo.). The plasmid expression vectors, pSEO-N pET-1 and pPUU-N pET-1, (gift of Brian Hjelle, MD, University of New Mexico) contain the open reading frames (ORF) for the Seoul virus 80/39 (SEOV) and Puumala virus P360 (PUUV) N proteins (8). HTNV N protein was expressed from pHTNV-N as described previously (6).

Construction of the SNV N Protein Expression System

SNV, strain CC107, S-ORF was amplified by PCR from SNVS/pCRII (9) with two DNA oligonucleotide primers, SNV S-Nhe, 5′-TCACTGGATTCCATATG-GCTAGCACCTCAAAGAATGC and SNV S-Xho- 5′-TCACTGGATTCTTA-CTCGAGAAGCTTAAGTGGTTCCTGGTTAGAAATTTC. Primers were synthesized with an ABI 394 DNA/RNA Synthesizer. The SNV S-5′ ORF contained a *Nhe*I restriction enzyme digestion site at the 5′ end (underlined) and the SNV-3′ ORF contained a *Xho*I digestion site at the 5′-end (underlined). The PCR product and the pET21b expression vector were digested with *Nhe*I and *Xho*I (New England Biolabs). The products were separated by agarose electrophoresis and purified with the QIAquick gel extraction kit (Qiagen) according to manufacturer's protocol. The SNVS-ORF and pET21b were ligated with T4 DNA ligase (Gibco-BRL). Clones of SNVN/pET21b were

selected by restriction enzyme mapping and confirmed through DNA sequencing using the LiCOR 4200 IR^2 Automated Sequencer. SNVN/pET21b was transformed into competent *E. coli* BL21 (DE3) cells (Novagen) for expression studies.

Expression and Purification of SNV N Protein

In general, *E. coli* BL21 (DE3) cells harboring the SNVN/pET21b and other hantavirus N proteins were grown overnight in 200 ml of Luria-Bertani (LB) medium containing 200 μg/ml of ampicillin. After 16-19 h, cells were diluted 1:20 in LB containing 200 μg/ml of ampicillin, and grown for 1 h at 30°C at which time isopropyl-thio-galactoside (IPTG) was added to a final concentration of 0.6-0.8 mM to induce expression of the protein. The methods that follow are based on a harvest of an 800 ml bacterial culture grown in a 2-liter flask. Soluble and insoluble extractions were performed and subjected to both metal chelate affinity chromatography and ion exchange chromatography. In general, fractions were collected and 20 μl of each fraction was examined for SNV N protein by SDS-PAGE or separation by 4-12% NuPAGE Novex Bis-Tris Gels (Invitrogen) and western blot analysis. Western blots were performed using polyclonal rabbit sera to SNV N (diluted 1:4000) mouse sera to polyhistidine (diluted 1:2000) (Sigma; monoclonal—clone no. His-1). The secondary antibodies were alkaline phosphatase-conjugated anti-mouse or anti-rabbit IgG (diluted 1:1000) (Promega), which were developed using Western Blue Reagent (Promega). In addition, protein concentrations were measured using the Bradford method (10) with BioRad Micro-Assay reagents as recommended.

Purification Using Nickel Nitriloacetate Agarose Under Native Conditions

After induction with IPTG for 1.5-5 hours, 800 mls of the culture were harvested and resuspended in 50 ml of ice cold solubilization buffer, SB [50 mM sodium phosphate pH 8.0, 300 mM NaCl, 10 mM imidazole, 20 mM β-mercaptoethanol, protease cocktail inhibitor (Boehringer Mannheim), and 0.50 mg lysozyme with or without CHAPS, 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate]. The suspension was dounced for 30 min on ice. Following lysis, the material was sonicated five or six times on ice, with a cycle consisting of 1 min on and 2 min off, in a Branson Sonifier until the material clarified. Soluble and insoluble materials were separated by centrifugation at 30,000 g for 1 h. Insoluble material was saved and placed at –80°C for subsequent extraction. To the soluble fraction, 1 ml of a 50% suspension of nickel nitriloacetate agarose (Qiagen, Chatsworth, CA) was added per liter of starting material, and stirred at 4°C for 2 h to overnight. The material was added to a column and washed with 10 column volumes of wash buffer, WB [50-100 mM sodium phosphate buffer pH 8.0, 300 mM NaCl, 20 mM imidazole, 20 mM β-mercaptoethanol (BME)]. The N protein was eluted from the resin with elution buffer, EB [50 mM sodium phosphate buffer pH 8.0, 300 mM NaCl, 250 mM imidazole, 20 mM β-mercaptoethanol]. Fractions were either stored at –80°C or directly placed into 500 ml of dialysis buffer [40 M Hepes pH 8.0, 200 mM NaCl, 0.1 mM dithiothreitol (DTT)] for 2-4 h with two changes of buffer.

Purification Using Nickel Nitriloacetate Agarose under Non-native Conditions.

Insoluble material was resuspended on ice in 50 ml denaturing buffer, DB [50 mM sodium phosphate buffer pH 8.0, 0.5 M NaCl, 20 mM BME, 8M urea, with or

without 10 mM CHAPS]. The suspension was dounced and sonicated as described above. The extract was gently shaken at room temperature for an additional 30 min, and then centrifuged at 30,000 g for 1 h. The supernatant was applied to a 1 ml column pre-equilibrated with buffer DB, pH 8.0. The column was washed with 10 column volumes of DB, pH 8.0, DB, pH 6.3 and DB, pH 5.9. The N protein was eluted with 10 column volumes of DB, pH 4.5. One ml fractions were collected and 10 µl of each fraction was examined for SNV N protein by SDS-PAGE and western blot analysis. Fractions containing N were dialyzed over a 5 day period into a final buffer with 20 mM Hepes, pH 8.0, 1 mM EDTA, 1 mM DTT, 500 mM NaCl, 5 % glycerol, with urea concentrations decreasing daily (day 1 – 4.0 M, day 2 – 2.0 M, day 3 – 1.0 M, day 4 – 0.5 M, Day 5 – 0 M).

Purification of SNV N Protein under Native Conditions with SP Sepharose Chromatography

Material isolated from soluble or insoluble/refolded fractions were subjected to an overnight dialysis into 50 mM MES pH 6.2, 200 mM NaCl. Fifty ml of the dialyzed material was then bound to a 1 ml Pharmacia Hi Trap SP sepharose fast flow column preequilibrated with 50 mM MES pH 6.2, 200 mM NaCl at a flow rate of 0.5 min/ml. A 10 ml gradient from 200 mM to 1 M NaCl was run by the FPLC at a flow rate of 1.0 ml/min. One ml fractions were collected and 20 µl of each fraction was examined for SNV N protein by SDS-PAGE and western blot analysis as described above.

Filter Binding Assay of SNV N protein

The filter binding assays were as described previously (6) using synthetic RNA and purified N protein. The oligoribonucleotide corresponding to the 5′-end of the SNV S-segment vRNA (5'-UAgUAgUAgACACCUUgAAAAgCAAUCAAgAAUUUACUU-3') was synthesized and HPLC purified by Integrated DNA Technologies, Inc (Coralville, IA). Synthesis was performed on a 1μM scale. The synthetic RNA was labeled at the 5' terminus with [γ-^{32}P] ATP and T4 polynucleotide kinase (New England Biolabs), and purified on Quick Spin^{TM} Columns (Roche). SNV N protein was serially diluted in binding buffer (40 mM HEPES pH 7.4, 100 mM NaCl, and 5% glycerol) to give a final concentration range of 5.6×10^{-9} to 5.6×10^{-6} M. Apparent dissociation constants (K_d) were calculated by fitting a nonlinear binding curve to the empirical data using the Origin program (MicroCal). The apparent K_d corresponds to the concentration of N protein required to obtain half-saturation; assuming the complex obeys a simple binding bimolecular equilibrium. We assumed the plateau in the percent binding of the RNA represents complete binding of the RNA, to allow the calculation at half-saturation.

Dynamic Light Scattering of SNV N protein

SNV N protein was examined by a DynaPro LSR (Protein Solutions Inc.) to determine its molecular weight. Measurements were made in the buffers in which the proteins were eluted from the column.

RESULTS AND DISCUSSION

Optimization of Soluble Extraction and Purification Conditions for the SNV N Protein

Small scale (800 ml) bacterial cultures were used to qualitatively define the optimal extraction and nickel affinity purification conditions for the SNV N protein. Three extraction/running buffers were examined: buffer 1— 100 mM sodium phosphate, pH 8.0, 300 mM NaCl, 30 mM imidazole; buffer 2—100 mM sodium phosphate, pH 8.0, 300 mM NaCl, 10 mM imidazole, 5% glycerol; buffer 3—100 mM sodium phosphate, pH 8.0, 1M NaCl, 10 mM imidazole, 5% glycerol. Buffer 1 resulted in the greatest yield of soluble SNV N protein. Comparison of buffer 1 with an identical buffer with 10 mM imidazole showed that the higher concentration of imidazole did not reduce background binding of nonspecific proteins; therefore, the imidazole was lowered to 10 mM in subsequent experiments.

Purification of the SNV N Protein Following IPTG Induction

Induction periods of 1.5 or 5.0 hr were tested for influence on SNV N protein yield from 3200 ml cultures of bacteria. N protein was extracted and purified by nickel affinity chromatography under native conditions as described in Materials and Methods. Fractions from each of the purifications were examined by Coomassie blue staining (Fig. 1 A and B) as well as western blot analysis (data not shown). The N protein had an apparent molecular weight slightly greater than its predicted molecular weight of 49 kDa (11). The total yield of protein in fractions 1-4 from the 1.5 hr induction was 7.6 mg or 2.4 mg/L, while the yield from the 5 hr induction was 3.3 mg or 1.0 mg/L (Table 1).

Thus approximately twice as much total protein was recovered following the shorter induction period.

Expression and Purification of the SNV N Protein in the absence of CHAPS

The zwitterionic detergent, CHAPS, has been frequently used to enhance the solubility of proteins recovered during both native and denaturing extraction protocols (12). And thus, our initial extraction and running buffers included this detergent. To determine if CHAPS enhanced the solubility and yield of the SNV N protein, we extracted bacterial cells and performed nickel chromatography using the same native conditions as described above for the time course study, but without the addition of CHAPS. Fractions from each of the purifications were examined by Coomassie blue staining (Fig. 2) as well as western blot analysis (data not shown). The total yield of protein obtained in fractions 1 through 4 was 7.7 mg (2.4 mg/L). This yield was similar to that observed when CHAPS was included in the extraction buffer (Table 1), although inclusion of CHAPS resulted in a higher proportion of the total protein in fraction 2 of the elutant (Fig. 1A and B). In contrast to these results, inclusion of 10 mM CHAPS in the denaturing protocol did enhance SNV N recovery (Table 1 and Fig. 3 A, B). Overall, the native extraction protocol, irrespective of the inclusion of CHAPS, yielded a greater amount of N protein than the denaturing methods; however, the denaturing protocol (Fig. 3A, B) yielded a much more homogenous and pure preparation of N than the native extraction (Fig. 1 and 2).

Expression and Purification of the other hantavirus N Proteins using Methods Developed for the SNV N.

In previous studies, we were unable to purify HTNV N protein expressed in E. coli using native conditions, although we could recover some protein (approximately 1.5 mg per liter of culture) from the insoluble fraction using denaturing conditions (6,12). To determine if the methods that we devised for extraction and purification of soluble SNV N would be applicable to other hantavirus N proteins expressed in *E. coli*, we tested them for recovery of expressed N of three hantaviruses that cause hemorrhagic fever with renal syndrome: HTNV, Puumala virus (PUUV) and Seoul virus (SEOV). Proteins were extracted with the nondenaturing buffer containing CHAPS and isolated by nickel affinity chromatography. Eluted fractions were separated by electrophoresis on gradient gels and visualized by Coomassie blue staining (Fig. 4 A-C). The total yield of PUUV or SEOV V N proteins from one liter of culture was 1.3 mg/L or 0.45 mg/L, respectively (Table 1). The yield of HTNV protein was difficult to compare to the other N proteins because of higher background, nevertheless, the amount visualized by Coomassie blue staining was much greater than we previously observed using denaturing and refolding methods.

Purification of the SNV N protein by SP sepharose fast flow chromatography

To further refine our purification protocol for SNV N, we subjected material recovered by nickel affinity chromatography from both the soluble and insoluble-refolded fractions to chromatography on a 1 ml Pharmacia SP sepharose FPLC column with a gradient of 200 mM to 1 M NaCl (Fig. 5A). Before loading on the FPLC column, the

nickel affinity column fractions were pooled and dialyzed overnight against 50 mM MES pH 6.2, 200 mM NaCl. This dialysis step enhanced the binding of the protein on the SP sepharose matrix. From both the native and refolded protein preparations, two peaks were evident in the FPLC column A_{280} profile. Fig. 5A and B show the profiles for the N protein recovered following refolding. Examining the proteins in those peaks by SDS-PAGE revealed little N protein in the first peak (Fig. 5B, lanes 1 and 2). The A_{260}:A_{280} ratio of this peak was 1.7, which strongly suggests the presence of nucleic acids in these fractions. The second peak, which eluted at 9 min in 660 mM NaCl, had most of the N protein (both in refolded and soluble preparations) (Fig. 5B lanes 3 and 4). This data suggests that the refolded material has properties similar to the native material. At present, we estimate the isolated N protein has 85% or greater homogeneity. A summary of the yields from the NTA and SP Sepharose FF are presented in Table 2.

Light scattering analysis of N protein showed the N protein isolated from the nickel affinity resin and the SP sepharose were 96 and 87% monodispersed, respectively (data not shown). As reflected in the results stated above, the molecular weight of the N protein isolated by the nickel affinity resin, 87 kDa, was greater than the predicted molecular weight, 49 kDa. And supports the presence of a nucleic acid contaminant. Light scattering showed the N protein isolated by SP sepharose chromatography (Fig. 5B lanes 3 and 4) had a molecular weight of 54 kDa.

RNA Binding Activity of the SNV N Protein

To determine if the extraction and purification protocol that we devised resulted in SNV N protein suitable for biochemical studies, we tested the purified protein in an RNA binding assay. Based on earlier findings suggesting that the terminal nucleotides of the hantaviral S-segment are involved in encapsidation and nucleocapsid assembly (13), we prepared a 39-base synthetic oligoribonucleotide for the binding assay. This oligonucleotide, designated SNV-vRNA 1-39, corresponds to the 5'-end of the nascent S-segment vRNA. Filter binding experiments performed with increasing concentrations of purified SNV N protein and a constant amount of SNV-vRNA were used to generate a binding isotherm. The apparent dissociation constant (K_d), calculated as half-maximum binding was approximately 140 ± 30 nM. This interaction shows a similar binding affinity reported for the HTNV N-protein vRNA (1-39) complex (132 ± 9) (13). These data indicate that the purified SNV N protein is of sufficient quality and purity to substitute for authentic viral protein in filter binding assays.

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FIGURE LEGENDS

FIG 1. Influence of IPTG induction time on SNV N yield. 3.2 L cultures of bacteria expressing SNV N were extracted at 1.5 hr (A) or 5.0 hr (B) after adding IPTG. Proteins were extracted with CHAPS and purified by nickel affinity chromatography and analyzed by electrophoresis on 4-12% Bis-Tris Gels, and Coomassie blue staining. L- column load, F- column flow-through, W- column wash, 1-4, fractions eluted from column, M-molecular weight markers. An arrow points to the N protein.

FIG 2. Native Purification of the SNV N Protein by Ni-NTA chromatography without CHAPS. A representative purification is shown from cellular material extracted without CHAPS. L- column load, F- column flow-through, W- column wash, 1-4, fractions eluted from column, M-molecular weight markers. An arrow points to the N protein. These samples were subjected to separation by 4-12% NuPAGE Novex Bis-Tris Gels.

FIG 3. Denaturing Purification of the SNV N Protein in the presence and absence of CHAPS by NTA chromatography. Purifications are shown from insoluble cellular material extracted with CHAPS (A) and without CHAPS (B): L- column load, 1-4, fractions eluted from column, M-molecular weight markers. An arrow points to the N protein. These samples were subjected to separation by 4-12% NuPAGE Novex Bis-Tris Gels.

FIG 4. Purification of PUUV, SEOV and HTNV N Protein by NTA chromatography. Purifications are shown for cellular material extracted with CHAPS for PUUV (A) and SEOV (B) and HTNV N protein (C); L- column load, F- column flow-through, W- column wash, 1-4, fractions eluted from column, M-molecular weight markers. An arrow points to the N protein. These samples were subjected to separation by 4-12% NuPAGE Novex Bis-Tris Gels.

FIG 5. SP sepharose chromatography of SNV N protein. (A). 100 ml of refolded N protein was loaded onto a 1 ml Pharmacia SP Sepharose column. The column profile shown was run from 200 mM to 1 M NaCl. (B). Fractions from the two major peaks shown in the chromatogram in (A) are presented: Peak 1 -fractions 1 and 2 (Lanes 1 and 2); Peak 2- fractions 5 and 6 (Lanes 3 and 4). These samples were subjected to separation by 12% SDS-PAGE.

TABLE 1

Summary of Ni-NTA purification yields for various N proteins.

Hantaviral N Protein	Induction (hours)	Yield (mg/L)	CHAPS Added	Extraction Method[a]
SNV	1.5	2.4	No	N
	1.5	2.4	Yes	N
	5.0	1.0	No	N
	1.5	0.09	Yes	D
	1.5	0.07	No	D
SEOV	1.5	0.45	Yes	N
PUUV	1.5	1.3	Yes	N
HTNV	1.5	ND	Yes	N

Note; ND, not determined

[a] N- native, D-denaturing

TABLE 2

Soluble Purification of the SNV N Protein

Step	Volume (ml)	Total Protein (mg)	Estimated Purity (%)
Culture Medium	3200	ND	ND
NTA	20	3.4	≈70%
SP Sepharose	3	1.8	>85%

Note; ND, not determined

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Figure 1A

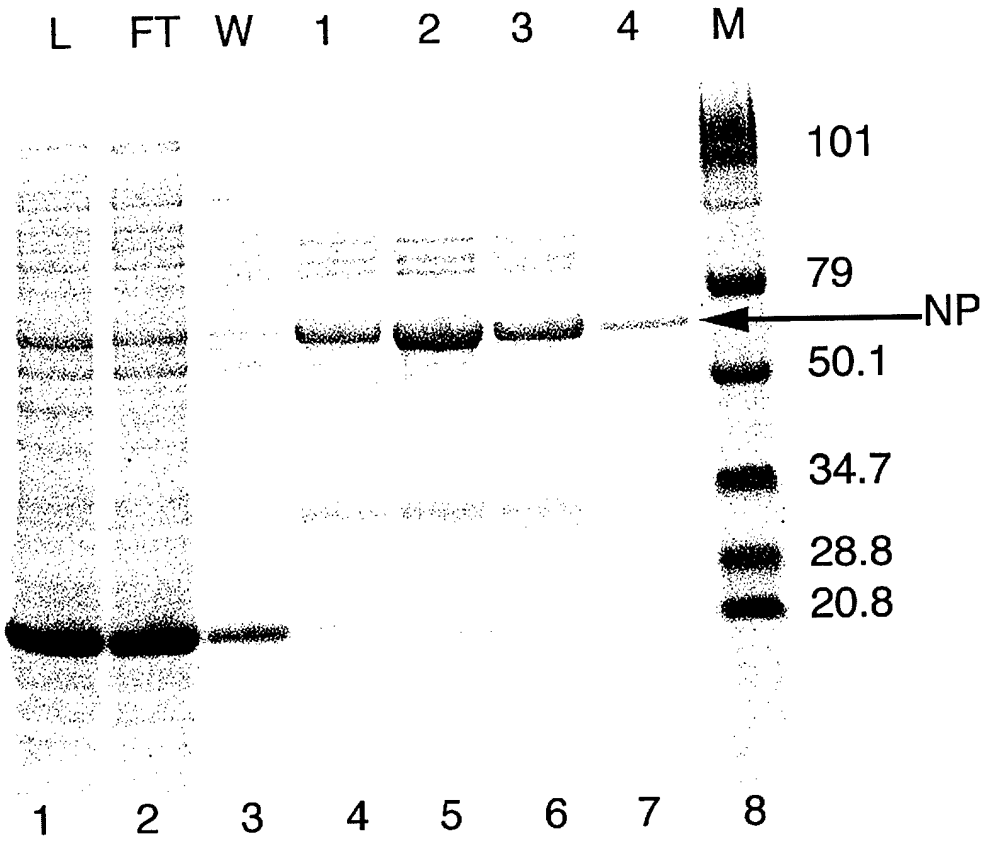

Figure 1B

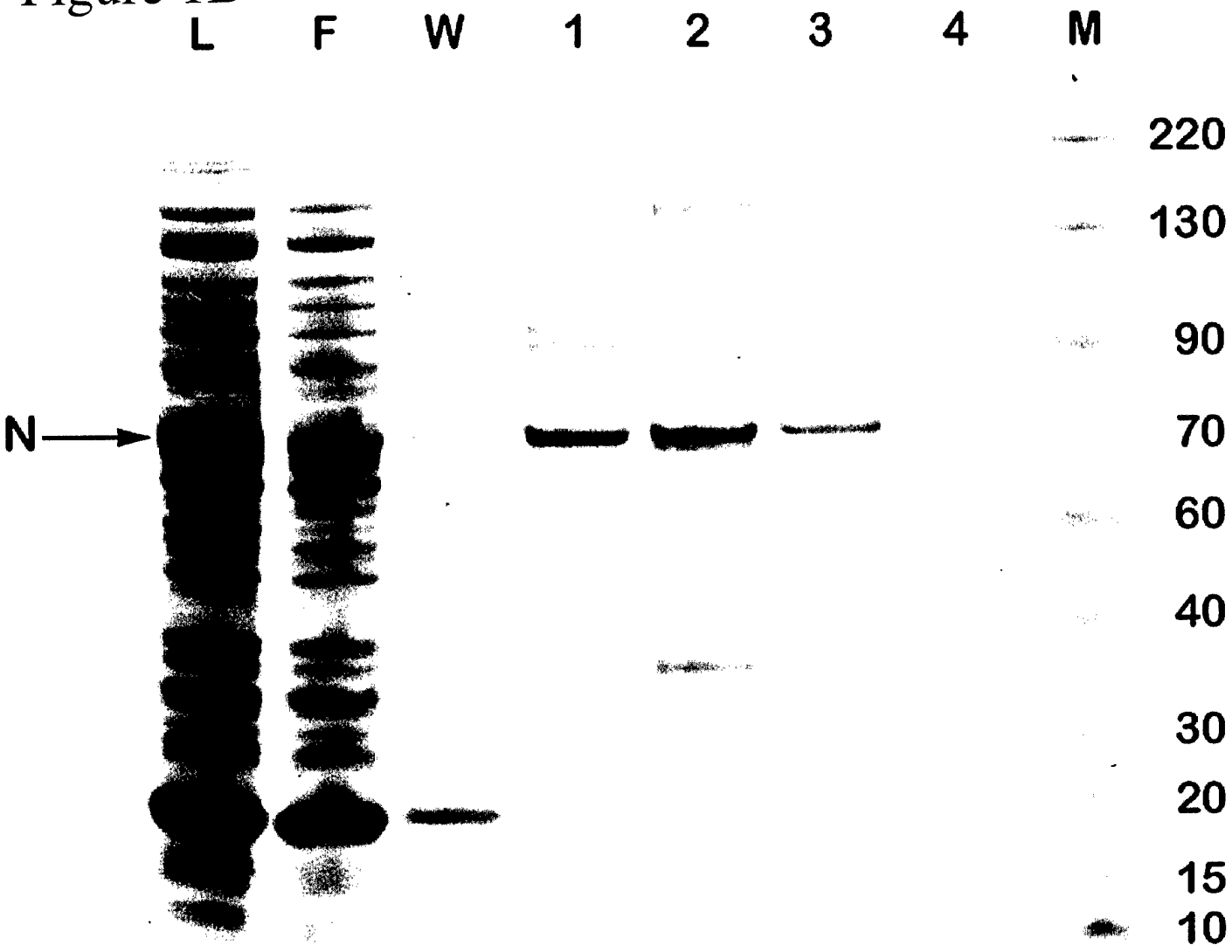

Figure 2A

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Figure 2B

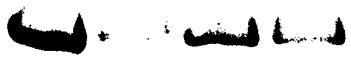


Figure 3A

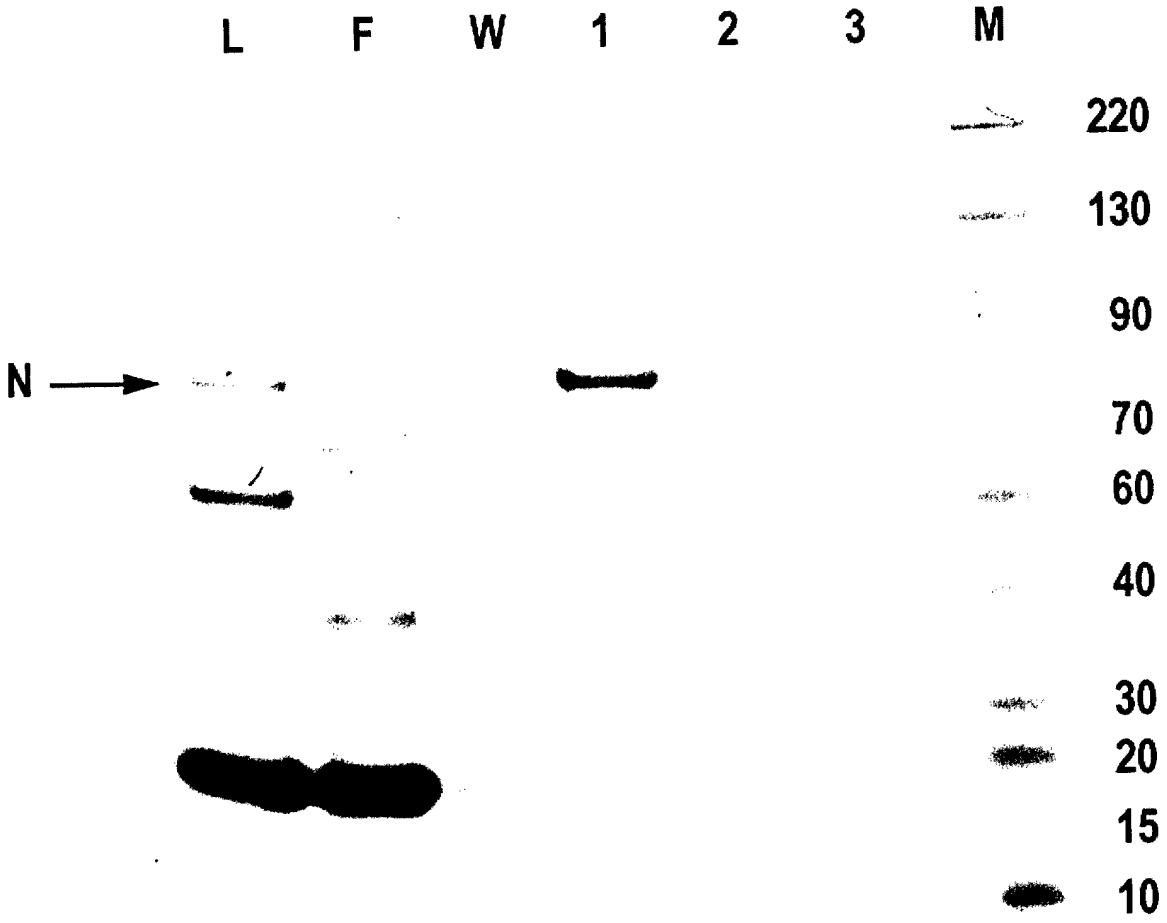


Figure 3B

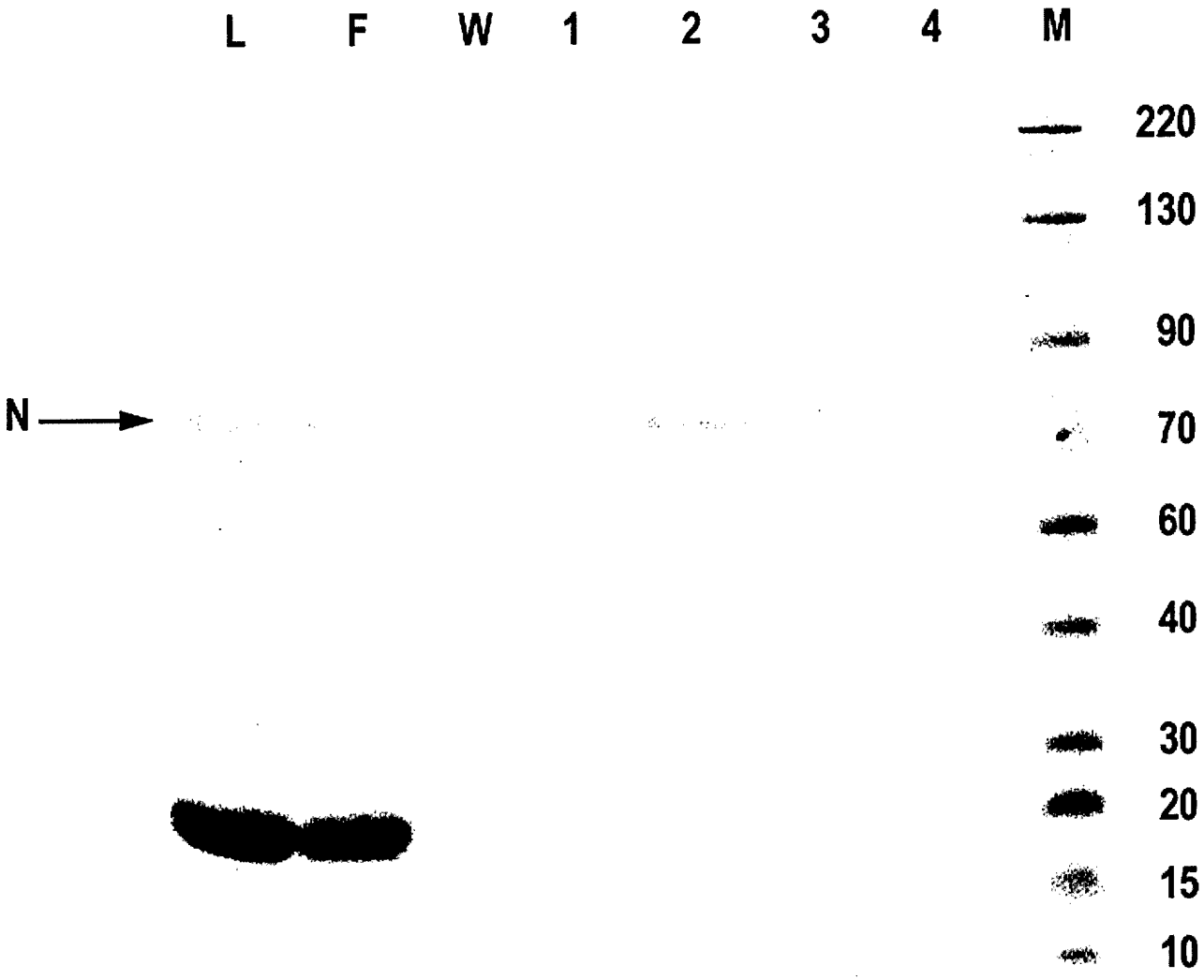


Figure 3C

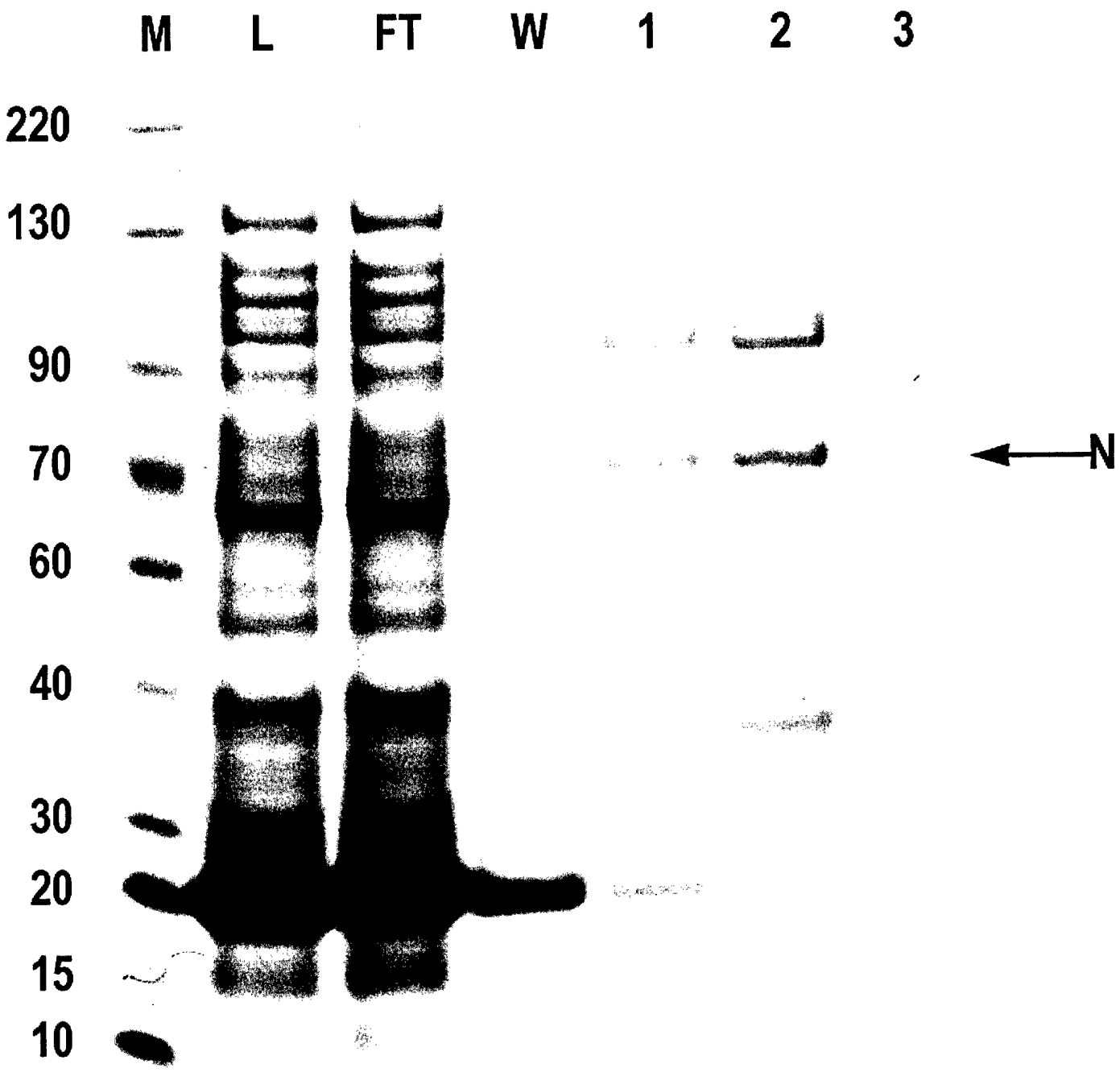


Figure 4

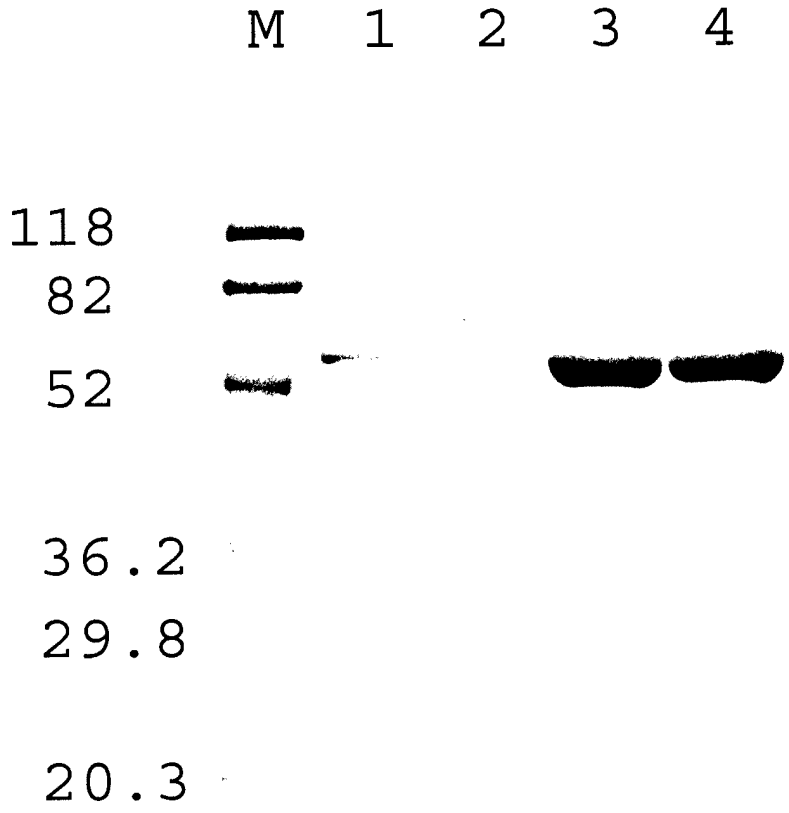
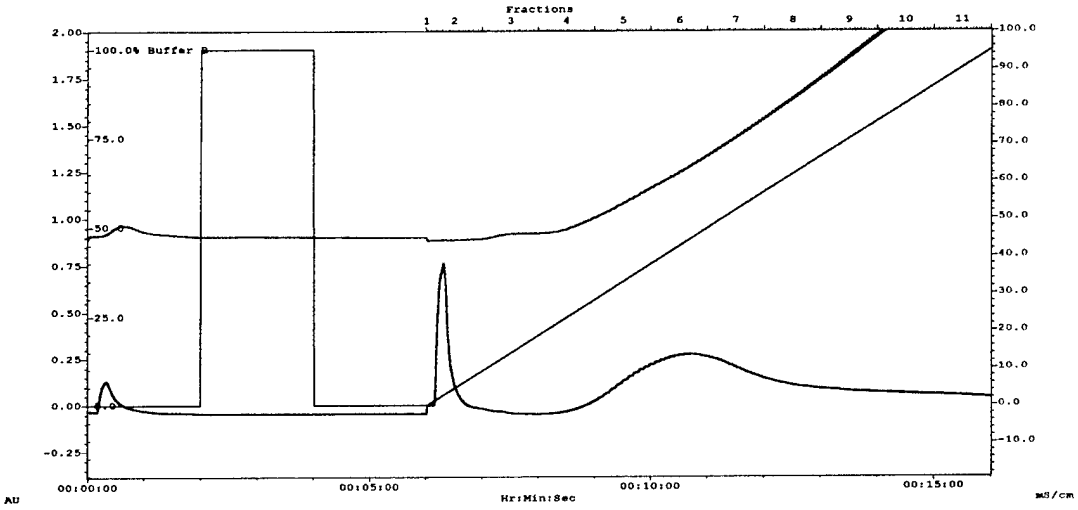


Figure 5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2.00
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1.50
1.25
1.00
0.75
0.50
0.25
0.00
-0.25
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
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Hr:Min:Sec
AU
mS/cm