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Biochemical and Biophysical Research Communications 312 (2003) 229–234
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Overproduction and partial purification of the Norrie disease gene product, norrin, from a recombinant baculovirus

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Received 25 September 2003

Abstract

Abnormal vascularization of the peripheral retina and retinal detachment are common clinical characteristics of Norrie disease (ND), familial exudative vitreoretinopathy, Coats' disease, and retinopathy of prematurity. Although little is known about the molecular basis of these diseases, studies have shown that all of these diseases are associated with mutations in the ND gene. In spite of this, little is known about norrin, its molecular mechanism of action, and its functional relationship with the development of abnormal retinal vasculature. To obtain a large quantity of norrin for structural and functional studies, we have overproduced it in insect cells. For this purpose, a cDNA fragment (869 bp) was isolated from a human retinal cDNA library by amplification and was cloned into an expression vector. The purified plasmid was co-transfected with wild-type linearized Bac-N-Blue DNA into *S. frugiperda* Sf21 insect cells. The recombinant virus plaques were purified and clones were selected based on the level of recombinant protein expressed in Sf21 cells infected with a purified recombinant virus. From these, a high-titer stock was generated and subsequently used to prepare a fused protein on a large scale. The protein was partially purified by the process of immobilized metal affinity chromatography and the use of ion exchange chromatography

Keywords: Gene; Norrin; Baculovirus; Cloning; Expression; Purification; Transfection

Dedication

It is not possible to capture the spirit and interactions of Prof. I.C. Gunsalus (most commonly known to his colleagues as Gunny) with his colleagues by a single opinion. This is simply because his interactions are decidedly not uniform and always extreme. During the years 1974–1978, I was a postdoctoral fellow in his laboratory. It was the time when research on bacterial cytochrome P-450_{cam} was taking off. Gunny immediately established groups in physics, chemistry, biochemistry, and genetics. Each section was dominated by his demand for the quality and intensity of scientific inquiry. The expansion of his research in many directions enabled him to learn in detail what nature returns to his stated hypothesis of the system in which he is interested. In order to provide an unlimited supply of biological material, proteins were prepared from 25-L bacterial culture and purified with an automated system so that his demand for quality was not compromised before the true answer emerged. At the time of my leaving his laboratory in mid-1978, I asked him what kind of science will be interesting in the future? His reply was "There is nothing like studying the biochemistry of a pure protein." Today, after an enormous explosion in every branch of science and even in the genome era, protein biochemistry (proteomics) is still a demanding field to promote discovery. The following is a brief account of our current research dedicated to this remarkably talented pioneer biochemist who taught many of us how to "make sense" in biochemistry.

Introduction

Norrie disease (ND), also known as progressive oculoacousticocerebral dysplasia, is a bilateral X-linked

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0006-291X/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2003.09.223

recessive trait. It is characterized by bilateral retinal malformation, progressive mental deterioration, and auditory impairment [1,2]. In general, female carriers are healthy. It mostly affects males and has been observed in all ethnic groups. In all affected males bilateral blindness is typically observed at birth. Previous histological studies have demonstrated a marked generalized avascularity of the retina and severe retinal dysplasia [3].

DNA linkage analysis has mapped the ND gene to the short arm of the X chromosome, and a candidate gene has been isolated by positional cloning [4,5]. The ND gene consists of three exons and two introns. The first exon is an untranslated region of the gene. The nontranslated dinucleotide repeats region in exon 1 is found to control the expression of the ND gene [6]. The gene is found to contain microdeletion, inversion, and point mutations in affected ND patients [7]. The same gene is also mutated in two other clinically overlapping disorders such as familial exudative vitreoretinopathy (FEVR) and retinopathy of prematurity (ROP), [8]. Furthermore, the gene is expressed in several tissues, including the adult eye and brain, but not in the liver [9,10]. Targeted disruption of the ND gene in mice resulted in an overall disorganization of the ganglion cell layer, retinal vasculature changes, and several other ocular abnormalities similar to those observed in ND patients [11]. These results emphasize a possible role for norrin in the regulation of angiogenic processes in the retina.

Norrie disease protein (NDP) or norrin is a cysteine-rich protein and shares homology with mucins, the immediate early gene, and von Willebrand factor [7]. A signal peptide sequence of 24 amino acids at the N-terminal of the protein indicates that it is a secreted protein. No glycosylation sites were identified in the NDP. Three-dimensional modeling of NDP revealed a cystine knot tertiary structure present in growth factor families such as nerve growth factor (NGF), transforming growth factor β_2, and platelet derived growth factor BB (PDGF-BB). Moreover, in a COS cell transfection assay, it has been shown that norrin forms disulfide-bonded oligomers that are associated with an extracellular matrix upon secretion from cells [12]. To gain further insight into the mechanisms of the development of an abnormal retinal vasculature in diseases such as ND, FEVR, and ROP, we have overproduced norrin and begun to purify the recombinant protein.

Materials and methods

(a) Cloning of a cDNA for the human ND gene. For the purpose of overproducing it in insect cells, a cDNA fragment (869 bp) was isolated from a human retinal cDNA library by amplification with a pair of primers: 5′-AATGACAACCAGAAAGCTTC-3′ and 5′-GTGTAT GAGGGCCCACTTTTTCC-3′. The purified fragment was characterized by sequencing on both sense and antisense strands using the same primers as for the PCR as well as internal primers. As expected, the fragment was found to contain the entire coding sequence for norrin, including nontranslated 5′ (136 bp) and 3′ (325 bp) sequences reported earlier [4,5]. The purified fragment was subcloned into a vector (pCR II-TOPO) using TOPO TA cloning system according to the manufacturer's instructions (Invitrogen, CA). The presence of the insert was confirmed by screening white colonies using PCR followed by restriction analysis as well as DNA sequencing. A single colony containing the insert was picked up and a large-scale plasmid was prepared using standard procedures.

(b) Cloning into an expression vector. The cDNA insert containing the coding sequence for norrin was amplified by using internal primers, digested with *Sac*I/*Nco*I and was ligated to the *Sac*I/*Nco*I digested pBlueBac His 2B vector (Fig. 1A) and transformed into *E. coli* strain TOPO 10 according to the supplier's instructions (Invitrogen, CA). Approximately 10 colonies (blue) were analyzed for the presence of the insert, and one colony containing the insert was bidirectionally sequenced to confirm the correct orientation and sequence integrity of

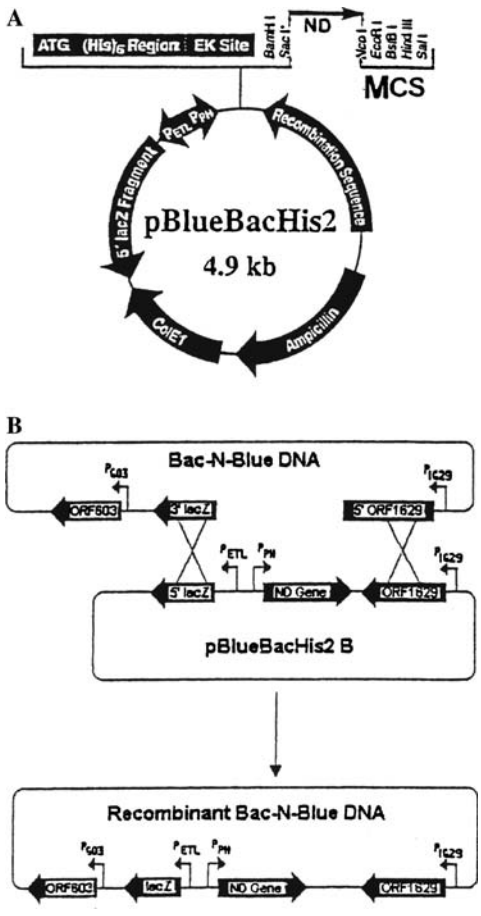

Fig. 1. Construction of recombinant plasmid carrying wild-type ND gene. (A) The features of the pBlueBac His2B vector (obtained from Invitrogen) containing the wild-type ND gene cloned into *Sac*I and *Nco*I sites. The foreign gene was under the control of the polyhedrin promoter. Proteins expressed from this vector are fused at the N-terminus to a tag of six tandem histidine residues and an enterokinase cleavage site. (B) Where recombination of pBlueBacHis 2B vector occurs with Bac-N-Blue DNA. Upon co-transfection and recombination, the essential sequences are restored (C-terminal sequences), blue occlusion body negative recombinant plaques are formed, and these are viable recombinant viruses. MS, multiple cloning sites; EK, enterokinase recognition site; Pph, polyhedrin promoter; and P_{ETL}, early to late promoter.

the cDNA insert in the pBlueBac His 2B vector. A large-scale plasmid was prepared using this clone.

(c) Transfection. The purified plasmid was co-transfected with wild-type linearized Bac-N-Blue DNA (wild-type baculovirus) into *S. frugiperda* Sf21 insect cells (Invitrogen, CA) (Fig. 1B). The recombinant virus plaques were purified as described by others [13]. Recombinant clones were selected based on the level of recombinant protein expressed in Sf21 cells infected with purified recombinant virus as measured by SDS–PAGE and Western blots of soluble extract (using polyhistidine antibody). Briefly, 100 μl of viral stock from 6 clones was used to infect 1×10^6 cells seeded in Graces complete media (Invitrogen) and harvested 48 h postinfection. The cell pellet was separated from the supernatant by centrifugation at 2500 rpm for 5 min. A negative control (cells only) and a wild-type baculovirus control were also generated for use in the Western blot analysis. Each sample was prepared for SDS–PAGE analysis to examine expression of the recombinant protein. To each harvested pellet, 100 μl of NP-40 lysis buffer (150 mM NaCl, 1% IGEPAL CA-630, 50 mM Tris–HCl, pH 8.0) containing two protease inhibitors (Pepstatin and Leupeptin) was added. The samples were vortexed for 5 s, and 25 μl of 4× NuPAGE sample buffer (0.05 M Tris–HCl, pH 6.8, 20% glycerol, 10% SDS, 0.1% bromophenol blue, 5% 2-mercaptoethanol) was added and the samples were boiled for 5 min. A 25 μl aliquot was loaded per lane on a 4–12% gradient NuPAGE Bis-Tris SDS–PAGE. On the same gel, a positive control, positope (Invitrogen, a recombinant protein specifically engineered to contain 6 histidine tag for detection with antibody), and a protein standard were included (Blue plus 2, Invitrogen). The gels were electrophoresed until the dye front reached the bottom of the gel. The gel was transferred to a 0.2 μm PVDF (polyvinylidine difluoride) membrane and the membrane was blocked with 5% nonfat dry milk overnight, incubated with 2% milk plus primary antibody anti His G/HRP for 2 h. To detect the N-terminal His tag, the PVDF membrane was placed between two sheets of transparency films and 1.5 ml of each of detection reagents (Amersham Pharmaceuticals) were combined and poured over the blocked PVDF membrane. A film was then exposed to the membrane for 1–10 min.

(d) Generation of high-titer stock. This is accomplished by using the recombinant virus clone. The cells used in the high-titer stock were grown to a density of $2–3 \times 10^6$ cells/ml and were infected with virus which had been amplified for 5 days in Sf21 cells seeded in T-25 flask. The infection was allowed to proceed until greater than 70% cell lysis was obtained. The high-titer stock was harvested and cell debris was pelleted by centrifugation for 20 min at 4000 rpm. The pellet was then discarded and the high-titer stock was titered using a standard plaque assay procedure. The titer was found to be 1.6×10^8 pfu/ml.

(e) Expression of recombinant protein in high-five insect cells. A recombinant baculovirus clone from high-titer viral stock was screened for expression of the protein in high-five insect cells. Briefly, the spinner flask containing 250 ml high-five cells used for the time course were grown to a density of 3×10^6 cells/ml. A 1.5-ml baseline sample (0 h) was taken and the content of the spinner flask was then infected with high-titer stock at various multiplicity of infection (MOI). The cells were grown in serum-free media and harvested at 24, 48, 72, and 96 h postinfection. The cell pellet was separated from the supernatant by centrifugation at 2500 rpm for 5 min. A negative control (cells only) was also generated for use in Western blot analysis. Each sample was prepared for SDS–PAGE analysis to examine the expression of the recombinant protein as described above.

(f) Large-scale expression (1 liter). High-five cells were grown in serum-free media, in a 2.8-liter culture flask for 15 passages at 27 °C. The viability of the cells was examined throughout the growth phase by trypan blue exclusion assay. The cells were maintained at a density between 1.0 and 3.6×10^6 cells/ml. The cells were infected with recombinant baculovirus at an MOI of 1.0. Approximately 13.1 ml of viral stock with a titer of 1.6×10^8 pfu/ml was used to infect 2.1×10^9 total cells. Prior to harvesting, a sample was taken to determine cell count and viability. The harvest at 96 h postinfection was prepared by centrifugation at 2500 rpm for 20 min at 4 °C. The pellet was washed with 1× PBS, centrifuged at 4 °C for 5 min at 2500 rpm. The supernatant was discarded and the cell pellet was stored at −80 °C until further use.

(g) Partial purification of recombinant fusion protein. First, we attempted to locate the protein and confirm that the 6× His tag was fused to the N-terminus of the protein. For this purpose approximately 200 μl of pellet was resuspended with 800 μl of lysis buffer (150 mM NaCl, 1% NP-40, and 50 mM Tris–HCl, pH 8.0) containing the protease inhibitors leupeptin and pepstatin at concentrations of 0.5 μg/ml each. The resuspended cells were vortexed and sonicated three times for 3 s each at medium intensity with a microtip. The sample was then centrifuged in a microfuge for 15 min, and the supernatant was aspirated. The supernatant from the lysed pellet and the original supernatant from the baculoviral fermentation were then analyzed by Western blot using Invitrogen's chromogenic and chemiluminescent detection kits. The blots were probed with anti-His G-AP (for N-terminal 6× His). To isolate a large quantity of protein, we then attempted to purify it on an Ni-NTA affinity column under various pH conditions (ranging from 6.5 to 9.5). An aliquot of the supernatant prepared above was loaded on a Ni-NTA column equilibrated with buffer A (150 mM NaCl, Tris–HCl, pH 8.0). The column was washed with 3 column volumes of buffer A and the bound protein was eluted

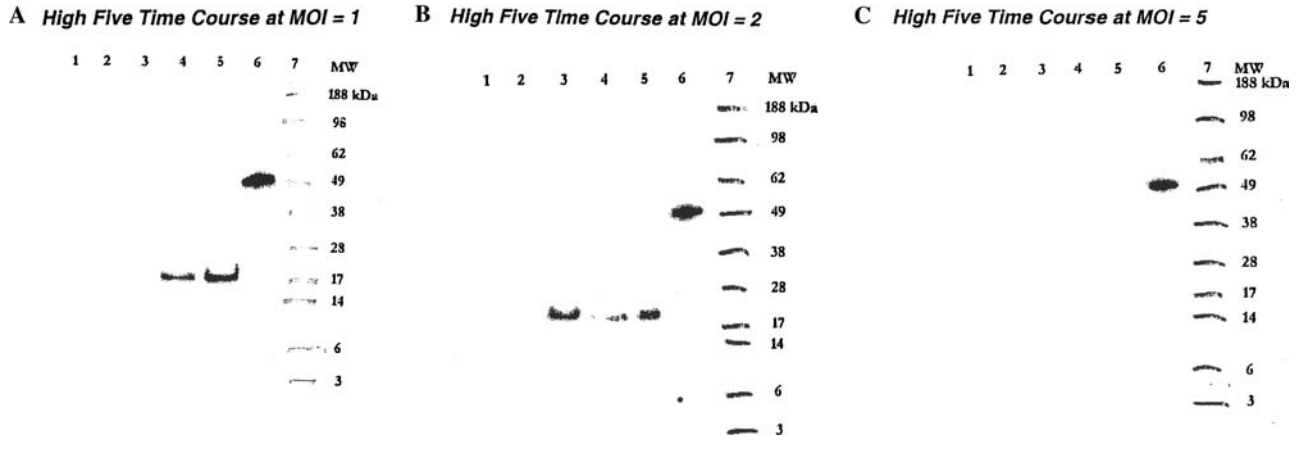

Fig. 2. Chemiluminescence detection of expression of recombinant norrin at various multiplicity of infection (MOI). High-five cells (3×10^6/ml) were infected with high-titer viral stock and harvested at 24, 48, 72, and 96 h postinfection (lanes 2–5 in each panel). The harvested pellet was lysed and analyzed by Western blot using anti-His G/HRP. Lane 1, cells only (preinfection); lane 6, positive control (positope); lane 7, molecular weight markers.

with a 20 column volume gradient from 0 to 1.0 M imidazole in buffer A. The input, flow-through, and eluted fractions were then analyzed using SDS–PAGE and Western blot. In addition, strong anion (Hi Trap Q) and cation (Hi Trap SP) exchangers equilibrated with 50 mM Tris–HCl, pH 8.0, were explored.

Results and discussion

To obtain an unlimited amount of norrin for structural and functional studies, we have been able to successfully overproduce the recombinant protein in a baculovirus expression system. As can be seen in Fig. 2, a protein of approximately 17 kDa (nearly expected size) was expressed at MOI = 1 (A, lanes 4 and 5) which reacted with anti-His antibody. On the other hand, at MOI = 5, there is no expression of the recombinant protein (panel C). The expressed protein is not secreted into the growth media and is present in the cell pellet as evidenced by Western blot analysis (Figs. 3A and B). The protein detected in the pellet runs as an approximately

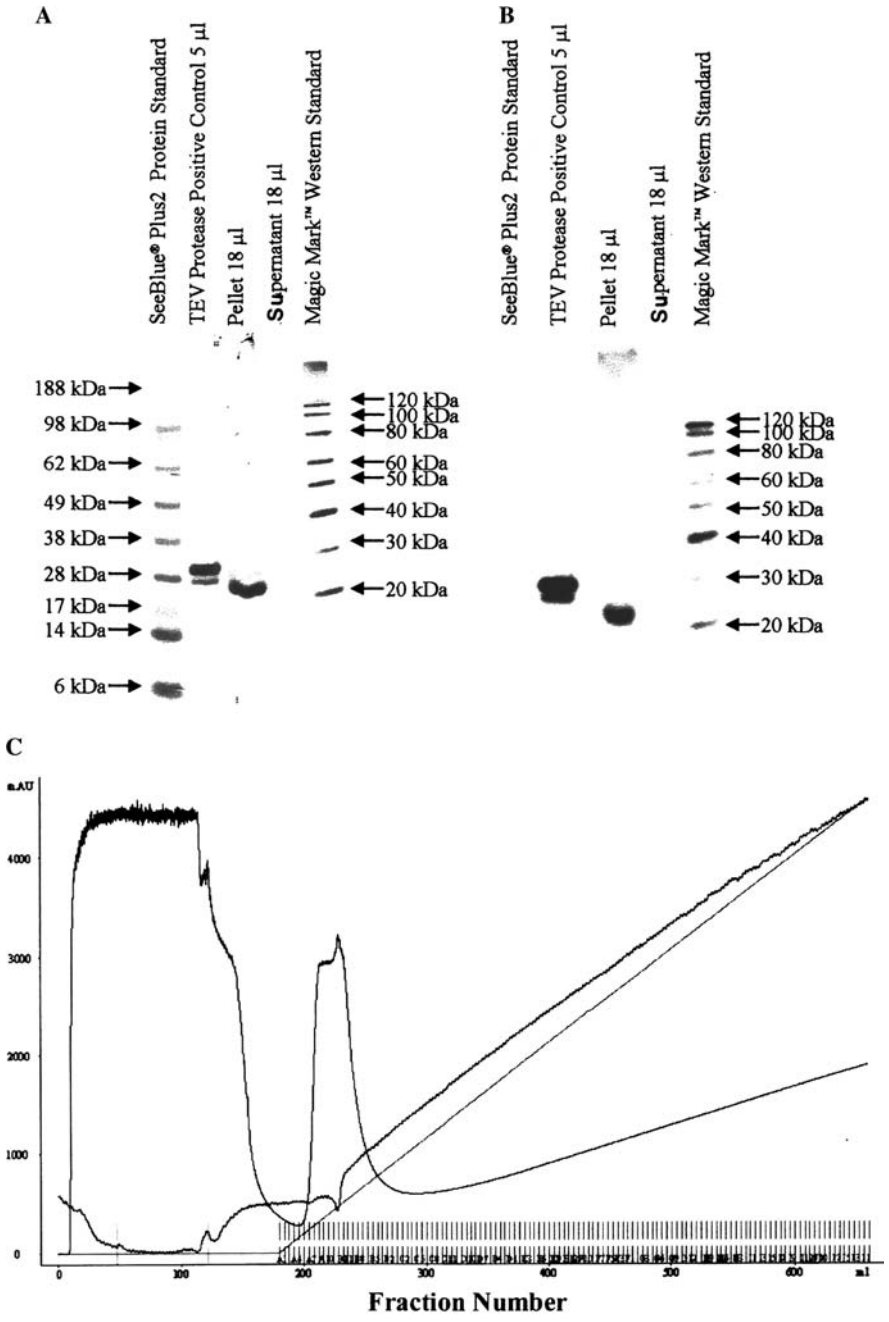

Fig. 3. Western blot analysis of supernatant and pellet from the baculoviral fermentation. (A and B) Chromogenic and chemiluminescent detection, respectively. The protein of interest is present in the pellet and not in the supernatant. (C) The elution profile of Ni-NTA affinity chromatography. A gradient of 0.0 to 1.0 M imidazole was applied to elute the bound protein.

21 kDa protein, which could be due to partial denaturation by the SDS in the loading buffer. Affinity chromatography with Ni-NTA column under native conditions suggests that a small amount of the protein of interest did bind to the column and eluted by 250 mM imidazole. (Fig. 3C and Figs. 4A and B). However, the majority of protein of interest did not bind the column and passed-through (Figs. 4A and B). This could be due to insufficient cell lysis or inaccessible 6× His tag. In such cases, other lysis methods such as homogenization with and without detergents or French press and moving the tag to the C-terminal end may help making it more soluble and accessible. Additionally, in both cases it is clear that the flow-through and eluted protein fractions contain a large amount of contaminating proteins. Moreover, a second antibody reactive protein of approximately 38 kDa has also been present in the same fractions containing the 17-kDa protein which is likely to represent the oligomer of the norrin. The difference in molecular weight in Figs. 2 and 3 could be due to the presence of contaminating proteins or partial denaturation under the conditions employed. When the affinity chromatography experiment was repeated under various pH conditions, no additional purification was achieved

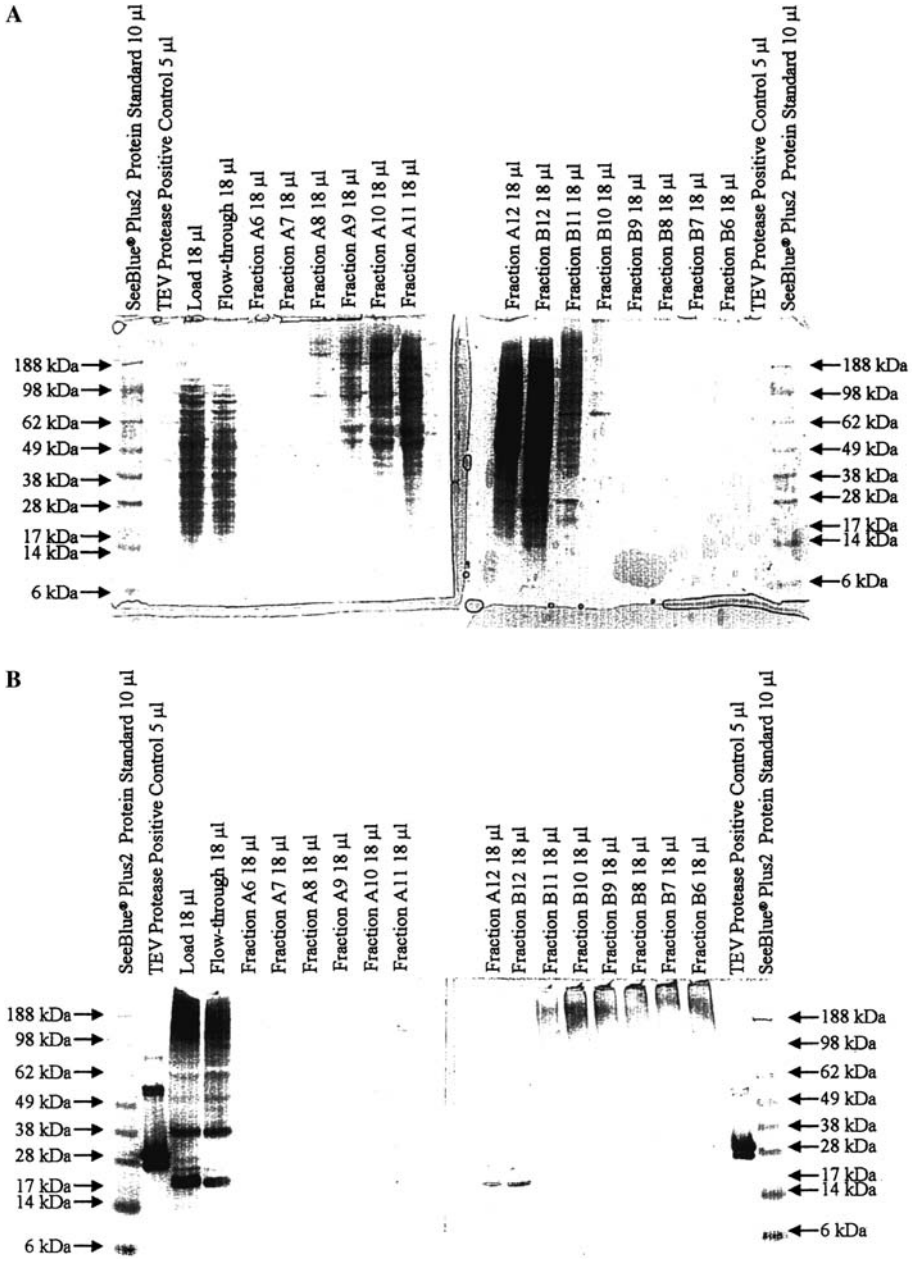

Fig. 4. SDS–PAGE analysis of proteins (A) and Western blot (B) of Ni-NTA affinity column fractions. The protein of interest did bind partially to the column and eluted in the gradient fractions but these fractions contain a large amount of contaminating proteins.

and the majority of the protein of interest did not bind the column. A similar result was also obtained when strong anion (Hi Trap Q) and cation (Hi Trap SP) exchangers were used for purification (data not shown). The majority of the protein of interest did not bind either column. Additional methods of purification are in progress and when a pure protein becomes available various biochemical and biophysical methods will be used to characterize this important protein to understand its functional role in normal as well as in the pathological retina.

Acknowledgments

We thank Dr. Anand Swaroop of University of Michigan for providing the retinal cDNA library. This study was supported in part by a grant from William Beaumont Hospital Research Institute (RI 02-01).

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