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Expression and Purification of the Central Domain from Vitronectin

Alexander C. Thurman
University of Tennessee - Knoxville

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Alexander C. Thurman, Nancy A. Horn, Cynthia B. Peterson

Abstract

Vitronectin is a key circulatory glycoprotein, exhibiting interactions with other proteins that regulate wound healing, cellular immunity, angiogenesis, coagulation, and fibrinolysis. Vitronectin structure/function depend on its localization within the circulation (fluid phase vs. vasculature). The conversion of vitronectin from a solution-phase (monomeric) to matrix-phase (oligomeric) form has been proposed to occur via intermolecular β-sheet propagation mediated by a β-propeller region within the central domain (~200 amino acids). In the present study, the vitronectin central domain was expressed as a small ubiquitin-related modifier (SUMO) fusion protein and purified via diethylaminoethyl (DEAE) and metal chelate (Ni) affinity chromatography. Cleavage of the SUMO fusion and purification of the central domain were successfully achieved. However, the purified central domain has exhibitied substantial self-aggregative properties that were unmitigated by the presence of the SUMO fusion. Thus, while the central domain was successfully isolated, its tendency for self-aggregation limits its biochemical applications.

Introduction

Vitronectin is a circulatory glycoprotein of established significance, having been implicated in such processes as wound healing, cellular immunity, angiogenesis, coagulation, and fibrinolysis. The structure and function of vitronectin are location-dependent: Soluble vitronectin monomers exist in circulation, insoluble oligomers in association with the extracellular matrix (ECM) of the vasculature. Soluble (monomeric) vitronectin has exhibited the capacity to bind several partners, including heparin,[1-3] plasminogen activator inhibitor-1 (PAI-1),[4-8] other proteases such as thrombin and urokinase-type plasminogen activator (uPA),[9] serpin-protease complexes,[9-11] and complement proteins,[12-14] whereas tissue-associated (oligomeric) vitronectin has been shown to interact with the ECM[15] and numerous cell surface receptors, including the uPA receptor (uPAR)[16-18] and the α_v integrin receptor subclass.[19-28] Vitronectin also promotes cell adhesion/migration in fibroblasts, endothelial cells, megakaryocytes, platelets, and certain cancer cell lines[29] and functions in organogenesis and neural development.[30,31]

Vitronectin mediates its most clinically relevant effects via interactions with PAI-1. Binding of PAI-1 to vitronectin stabilizes the active (inhibitory) conformation of the enzyme and significantly increases its half-life.[32,33] Vitronectin/PAI-1 interactions have been implicated in fibrotic lung disease,[34] kidney disease,[35] liver fibrosis,[36] atherosclerosis,[37] and angiogenesis,[38-40] and human alleles corresponding to increased PAI-1 expression are directly correlated with tumor severity and incidence of myocardial infarction/metabolic syndrome.[41-43] Conversion of vitronectin from a solution-phase to a matrix-phase protein is also hypothesized to occur via interactions with PAI-1. Subsequent conformational changes in vitronectin are suggested to expose a β-propeller region in the central domain – homologous to the four-bladed β-propeller fold present in another circulatory protein, hemopexin[44,45] – that induces oligomerization. Such β-propeller domains frequently participate in protein-protein interactions,[46,47] thus supporting the proposed model for vitronectin self-association.

In order to address the hypothesis that the self-association of vitronectin into a matrix-associated oligomeric form occurs via intermolecular β-sheet propagation, the vitronectin central domain (VNCD) will be expressed in isolation and tested for associative behavior. The mutagenesis and cloning of DNA corresponding to VNCD has already been accomplished in the laboratory. The goals for the present research will be to: 1. Optimize VNCD expression and purification; and 2. Use high-performance liquid chromatography (HPLC) to evaluate purification of this domain.

Materials/Methods

Expression. The VNCD DNA sequence was inserted into the Champion pET SUMO Expression System (Invitrogen, see **Fig. 1**) and expressed in Rosetta 2(DE3)pLysS Competent Cells (Novagen). Rosetta cell constructs were grown in Terrific broth as 5% (50mL) seed cultures and incubated for ~16 hours at 30°C. Seed cultures were subsequently transferred to larger flasks (2.8L Fernbach flasks, 1L TB) and incubated with orbital shaking (300rpm, New Brunswick) at 20°C. When an OD (A_{600}) of ~4 was established, media was brought to 1% (w/v) glucose and 2mM IPTG to induce VNCD-SUMO fusion expression, and incubation was continued for ~16 hours with orbital shaking (300rpm) at 15°C. The final OD (A_{600}) of the cell cultures was ~6. Cell paste was harvested by centrifugation at ~16000g for 15 minutes at 4°C.

Cell Lysis. Approximately 90 grams of wet cell paste were suspended in 600mL of 2M urea, 0.02M Na_2HPO_4, 1% (v/v) Tween 80, pH 8.0 buffer containing 3 dissolved Complete Protease Inhibitor Cocktail Tablets (Roche). The suspension was sonicated on ice (with stirring) for 30 minutes (1 minute pulse, 30 second rest cycle). Following sonication, the lysate was centrifuged for 20 minutes at ~16000g (9500 rpm) at 4°C. The supernatant was decanted and collected, and 5 grams of Celpure P1000 (Advanced Minerals) per 100mL of lysate were added. The clarified lysate was filtered using a Buchner apparatus with a 0.45μm nylon membrane (MSI) and a glass fiber prefilter (Pall) pre-coated with a 1cm layer of Celpure P1000.

DEAE Chromatography. A 1.5cm×6cm DEAE Sepharose Fast Flow column (GE Healthcare) was washed and equilibrated with 2M urea, 0.02M Na_2HPO_4, pH 8.0 buffer at 4°C. The filtered/clarified lysate was loaded using a peristaltic pump at a flow rate of

1mL/min. The column was washed with 2M urea, 0.02M Na_2HPO_4, pH 8.0 buffer until the absorbance profile – monitored with an inline UV detector (Pharmacia) – returned to baseline. The column was developed and product eluted with a 400mL linear gradient from 2M urea, 0.02M Na_2HPO_4, pH 8.0 to 2M urea, 0.02M Na_2HPO_4, 1 M NaCl, pH 8.0 at 3mL/min. Fractions from the dominant eluting peak were collected, pooled (see **Fig 2.**), and analyzed by SDS-PAGE and western blotting.

Metal Chelate (Ni) Affinity Chromatography I. A 1.5cm×8cm Chelating Sepharose Fast Flow column (GE Healthcare) was charged with 0.1M $NiSO_4$. The column was then washed with 10 column volumes of water followed by 10 column volumes of 2M urea, 0.02M Na_2HPO_4, 0.3M NaCl, 0.005M imidazole, pH 8.0 buffer at 4°C. The DEAE column pool (320mL) was loaded at a flow rate of 3mL/min and washed with 2M urea, 0.02M Na_2HPO_4, 0.3M NaCl, 0.005M imidazole, pH 8.0 buffer until the absorbance returned to baseline. The column was developed with a linear gradient from 2M urea, 0.02M Na_2HPO_4, 0.3M NaCl, 0.005M imidazole, pH 8.0 to 2M urea, 0.02M Na_2HPO_4, 0.3M NaCl, 0.3M imidazole, pH 8.0. 10mL fractions were collected, analyzed by SDS-PAGE and western blotting, and pooled accordingly (see **Fig. 3**).

SUMO Cleavage. 30mL of proteolysis solution was prepared by combining 19.96mL metal chelate affinity eluate (~21mg VNCD-SUMO fusion protein), 400 units (40µL) SUMO Protease 1 (LifeSensors), 400µL 150mM DTT solution (2mM DTT in 30mL), and 9.6mL DI H_2O. This solution was incubated at 30°C for 1 hour and subsequently transferred to 4°C for overnight incubation (~12 hours). Successful cleavage was confirmed by SDS-PAGE and western blotting (see **Fig. 4**).

Metal Chelate (Ni) Affinity Chromatography II. A 1cm×15cm Chelating Sepharose (Ni) Fast Flow column was prepared and equilibrated as described above. The digested protein was loaded and the column washed with 35mL of 2M urea, 0.02M Na_2HPO_4, 0.3M NaCl, 0.005M imidazole, pH 8.0 buffer. The column was further developed with 35mL steps of 0.125M, 0.250M, 0.325M, and 0.5M imidazole in identical base buffer (2M urea, 0.02M Na_2HPO_4, 0.3M NaCl, pH 8.0). Step fractions were collected and analyzed by SDS-PAGE and western blotting (see **Fig. 5** and **Fig. 6**).

SDS-PAGE. SDS-PAGE (12% acrylamide) was performed according to established protocol.[48]

Western Blotting. 12% acrylamide SDS-PAGE gels were blotted onto nitrocellulose membrane (0.45µm pore size, Bio-Rad) pre-wetted in 25mM Tris, 250mM Glycine, 15% (v/v) methanol transfer buffer by the filter paper sandwich method using a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) for 15 minutes at 15V. The blotted membrane was blocked in 50mL of 7.5% non-fat milk in PBS for 30~60 min. 50mL of fresh 7.5% non-fat milk in PBS was added and the membrane was probed with a 1:2000 dilution of murine Monoclonal Anti-polyHistidine-Peroxidase (Sigma A7058-1VL) and incubated for 30~60 min. The blot was then washed three times with 30~50mL 0.1% (w/v) Tween 20 in PBS. HRP-conjugated antibody was detected using 4-chloro-1-naphthol.

Results

Rosetta cells were chosen for VNCD expression due to the presence of the pRARE plasmid, which encodes tRNAs for mammalian codons that are seldom present in other *E. coli* cell lines.[49] VNCD was also expressed as a His-tagged SUMO fusion protein to enhance primary expression and solubility (see **Fig. 1**).[50] Unfortunately, the SUMO fusion alone was unable to overcome the inherent aggregative properties of the central domain; thus, the presence of urea was required throughout the purification process.

Immediately following cell lysis and centrifugation, the cell lysate was clarified with Celpure 1000 and subjected to DEAE chromatography (see **Fig. 2**) to minimize incidental proteolysis. The dominant eluting DEAE peak was collected, pooled, and subjected to metal chelate (Ni) affinity chromatography to provide separation based on the poly-His tag present on the SUMO portion of the VNCD-SUMO fusion (see **Fig. 3**).

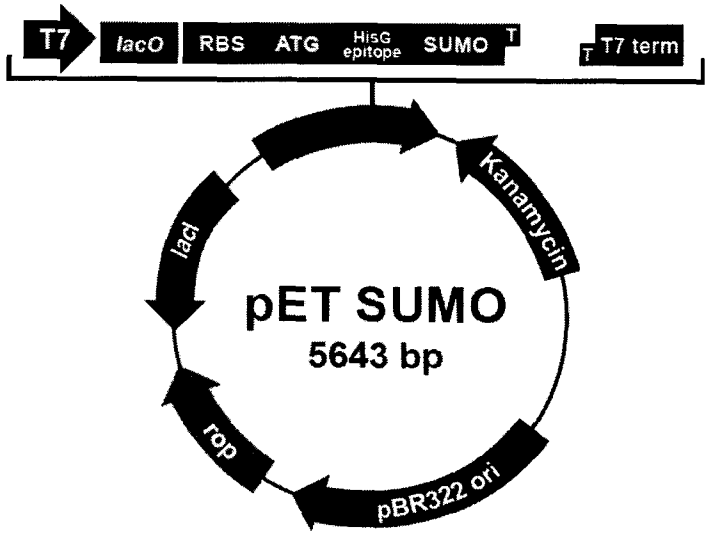

Fig. 1. Champion pET SUMO plasmid map (Invitrogen).

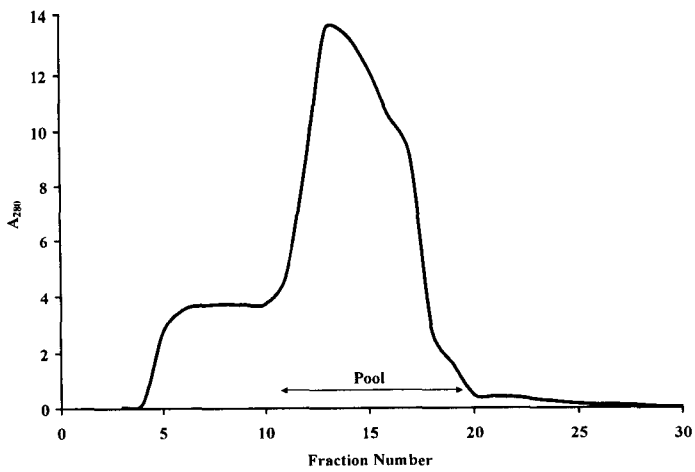

Fig. 2. DEAE column elution profile. Pooled fractions (11-19) are indicated.

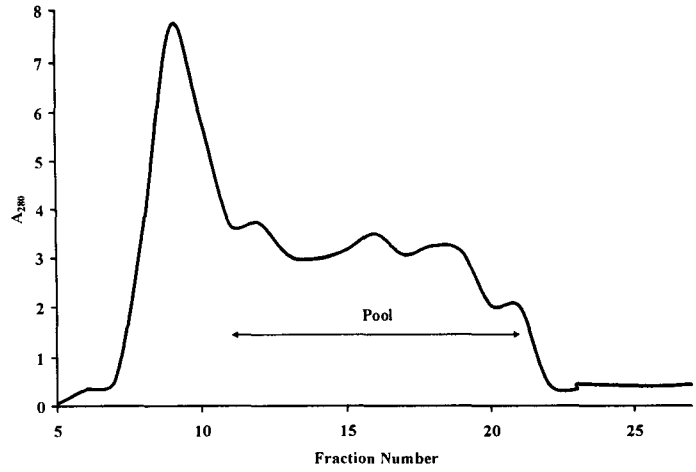

Fig. 3. Nickel column elution profile (0.005M-0.30M imidazole gradient). Pooled fractions (11-21) are indicated.

Following metal chelate (Ni) affinity purification, the pooled eluate was subjected to proteolytic cleavage of the SUMO domain from VNCD by the addition of SUMO Protease 1. Butt *et al.* had previously shown SUMO Protease 1 to be active at urea concentrations similar to those used in this work.[51] Successful cleavage was confirmed by the generation of three distinct bands post-digestion (SDS-PAGE, see **Fig. 4**). The lowest of these bands was western positive with murine αpoly-His, while the upper two bands were western positive with Bunny-12 (polyclonal αvitronectin). The bottom band is proposed to be the cleaved SUMO product; the top band, fully intact VNCD (~20523Da); and the middle band, VNCD cleaved incidentally by unknown protease(s) during purification. The SUMO cleavage product and SUMO protease were removed via a second round of metal chelate (Ni) affinity chromatography with an imidizole step gradient. This final purification step was confirmed by SDS-PAGE (see **Fig. 5**) and western blotting (αpoly-His, see **Fig. 6**).

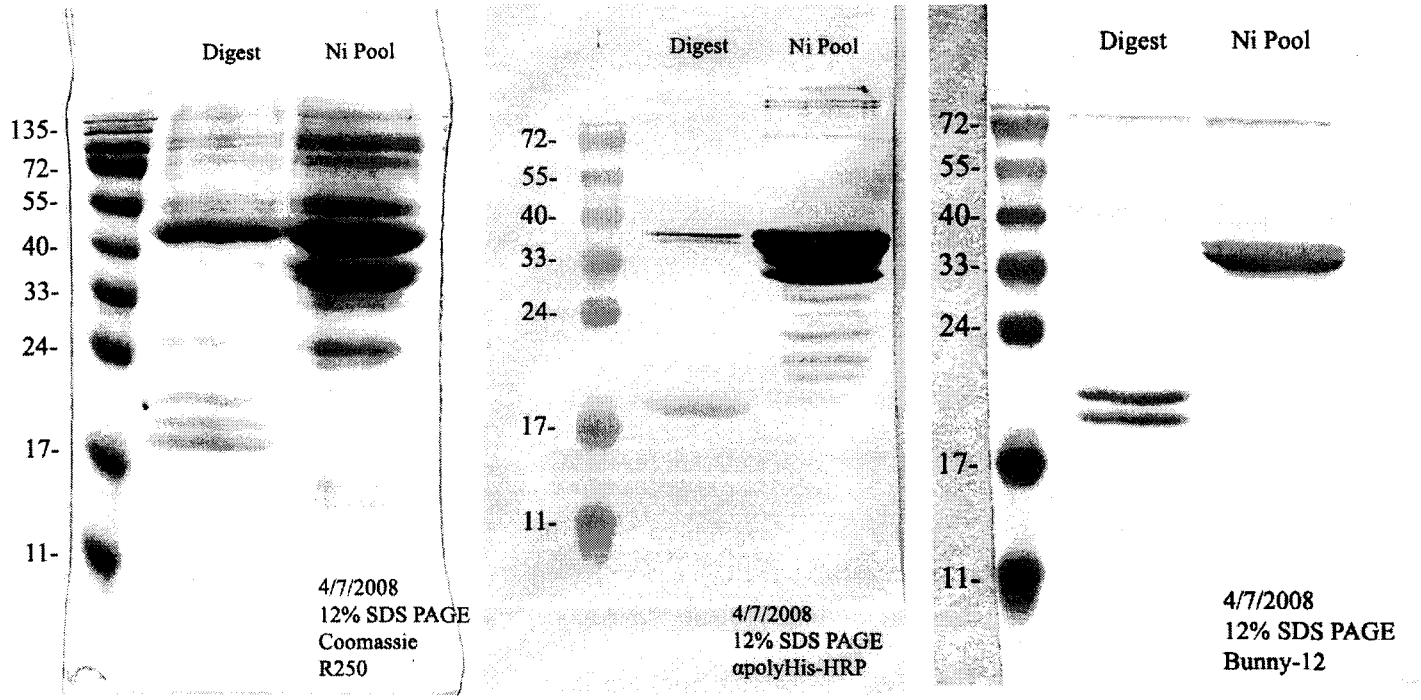

Fig. 4. Comparison of samples pre- and post-SUMO Protease 1 digest. Shown are a 12% acrylamide gel (samples stained with Coomassie Blue R-250, left), a western blot probed with murine αpoly-His conjugated to HRP (center), and a western blot probed with Bunny-12 (polyclonal αvitronectin, right).

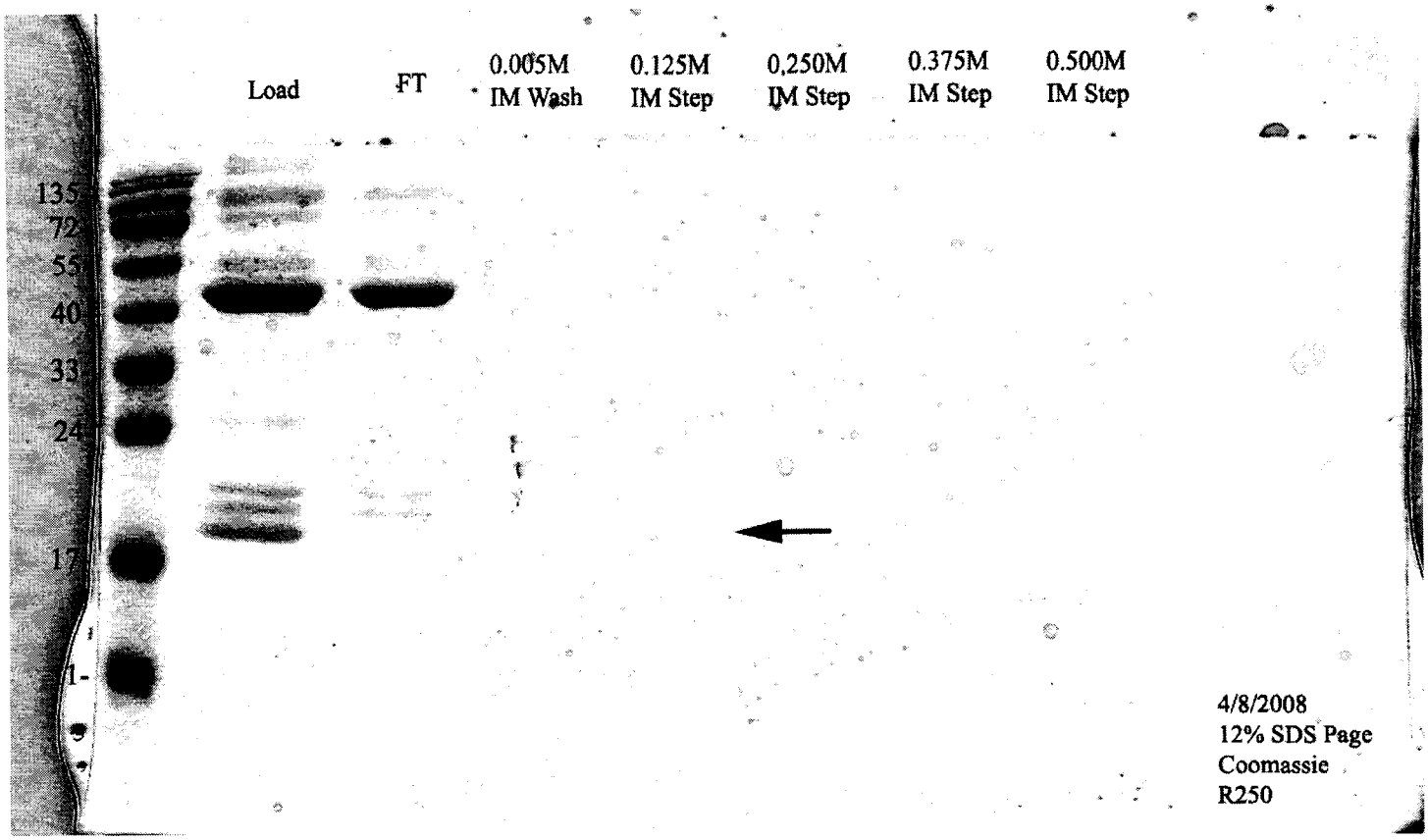

Fig. 5. 12% acrylamide gel of samples (stained with Coomassie Blue R-250) post-metal chelate (Ni) affinity chromatography (imidizole steps). The indicated band represents the cleaved SUMO product and SUMO protease, which both contain a poly-His tag.

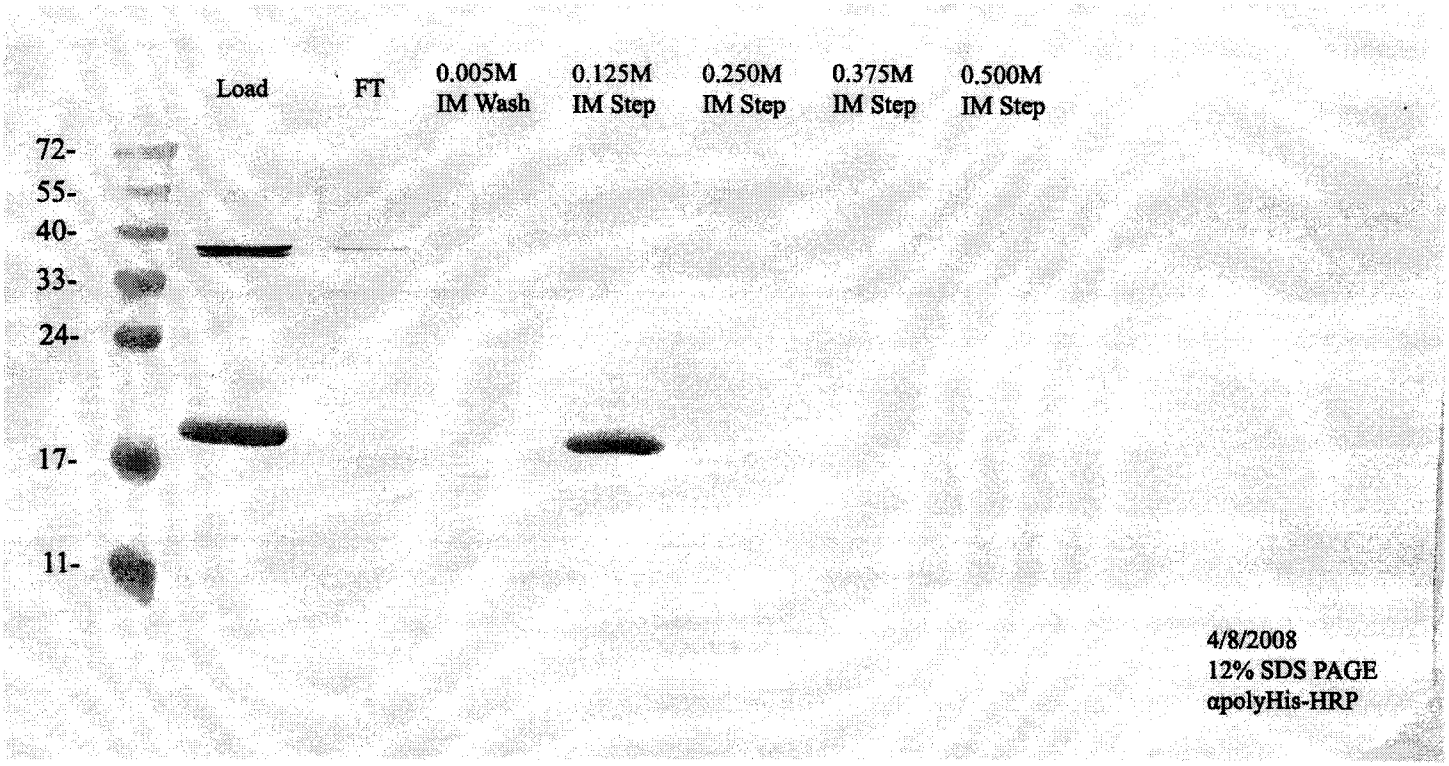

Fig. 6. Western blot of samples post-metal chelate (Ni) affinity chromatography probed with murine αpoly-His conjugated to HRP. The western positive band in the 0.125M IM Step lane represents the cleaved SUMO product and SUMO protease, which both contain a poly-His tag.

Discussion

Following the second round of metal chelate (Ni) affinity chromatography, reversed phase HPLC allowed for the resolution of the two remaining VNCD bands (data not shown). Characterization of these purified samples via MALDI-TOF mass spectrometry necessitated the removal of urea from the sample buffer; thus, attempts at characterization were unsuccessful due to the self-aggregative properties of VNCD in the absence of urea. Of note is the reversibility of VNCD aggregation: The removal of urea promotes aggregation, while its reintroduction effects resolubilization (data not shown). Future experiments might entail VNCD expression in a eukaryotic system in order to achieve relevant post-translational modifications, as normal glycosylation has been shown to enhance vitronectin solubility.[52]

Ultimately, the expression of VNCD as a VNCD-SUMO fusion was not sufficiently able to overcome VNCD β-propeller-mediated oligomerization for the production of soluble monomers. However, it is the opinion of the author that although the purified product is largely unsuitable for biochemical assays/analyses due to its propensity for self-aggregation at high protein concentrations, certain low-concentration assays/analyses may be feasible since self-aggregation at low protein concentrations could be negligible.

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