RESEARCH ARTICLE

Expression and Purification of Recombinant Von Willebrand Factor A1A2A3 Domains

Esra Seyran [1,2]
Rohana Liyanage [1]
Jackson Lay [1]

Authors' addresses:
1 University of Arkansas. Department of Chemistry and Biochemistry Fayetteville AR, USA.
2 Cumhuriyet University, Faculty of Science, Department of Molecular Biology and Genetics, Sivas, Turkey.

Correspondence:
Esra Seyran
Cumhuriyet University, Faculty of Science, Department of Molecular Biology and Genetics, Sivas, Turkey.
Tel.: +90 538 870 2471
Fax: +90 346 219 1011
e-mail: esraseyran@gmail.com

Article info:
Received: 2017
Accepted: 2017

ABSTRACT

In order to initiate the formation of a platelet plug Von Willebrand Factor (VWF) must be assembled into large multimers. VWF undergoes post translational modifications by dimerizing through multiple intermolecular disulfide bonds between carboxyl terminal ends of the protein and once in Golgi by forming interdimer disulfide bonds. The resulting multimers range in size between 500 to 20000 kDa. The protein dimerizes and the dimers then form a variety of disulfide crosslinked multimers with as many as 80 monomeric units, weighing more than 20 million Daltons. Studying such an enormous molecule poses special challenges. The separate domains within the VWF subunit exhibit specific properties, involving interactions with other molecules. Binding sites that are independent of multimer assembly but important for the hemostatic function are located in the A1A2A3 domains of VWF. We expressed the A1, A2, and A3 domains of von Willebrand factor in a single polypeptide using *Pichia pastoris* expression system. Proteins with disulfide bonds, requiring post translational modifications and glycosylation can be produced in their correctly native folded states with full function from *Pichia pastoris*. We purified the A1A2A3 domain using ethanol, ammonium sulfate precipitation and ion exchange chromatography. Our efforts in solubilizing the purified protein were unsuccessful more likely due to the unusual adhesive nature of the A1A2A3 domain of the VWF.

Key words: Von Willerband Factor, VWF A1A2A3 Domains, *Pichia pastoris*, Protein Purification

Introduction

Von Willebrand factor (VWF) is a large glycoprotein, found in plasma and platelets and synthesized by megakaryocytes and endothelial cells. The pre-pro-protein is 2813 amino acids and the mature monomer is 2050 amino acids. VWF monomer contains multiple copies of four types of domains called A, B, C and D-type domains. Binding sites that are independent of multimer assembly but important for the hemostatic function are located in the A1A2A3 domains of VWF (Sadler, 1998).

Expression of the A1, A2, and A3 domains individually in E. coli has been successful, but attempts to express all three in a single polypeptide in *E. coli* have failed. We attempted to express, purify, and characterize the A1, A2, and A3 domains of von Willebrand factor in a single polypeptide in order to study a model more realistic than a simple peptide. The lack of domains responsible for the multimer formation would make it more practical to study and less complex than multimers ranging in size from 2-20 million Daltons. Proteins with post translational modifications with disulfide bonds and requiring glycosylation are very difficult to obtain using E. coli expression, *Pichia pastoris* has been used successfully to produce a number of difficult to express proteins in large amounts (White et al., 1994). Formerly the portion of the human gene expressing the A1, A2, and A3 domains was subcloned into an E. coli vector and homologously recombined into a site in Pichia pastoris. Unfortunately, this failed to express, then a synthetic gene was optimized in codon usage and homologously recombined into Pichia (Talla, 2007).

Materials and Methods

Growth and maintenance of P. pastoris strains

The codon optimized A1A2A3 domain of VWF was expressed in the Pichia pastoris expression system (Cereghino et al., 2002). The protease A, carboxypeptidase Y and protease B1 deficient SMD1168 strain of P. pastoris was used for the production of the recombinant protein (Cregg et al., 1993). The use of protease-deficient strains such as SMD1168 has been found to improve both yield and quality of the recombinant protein. The SMD1168 strain of Pichia pastoris is defective in the histidine dehydrogenase gene (his4), which allowed the selection of transformants based on their ability to grow on histidine-deficient medium. P. pastoris strains were typically

grown on YPD-agar plates (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose and 2% (w/v) agar) or YPD medium (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose). One liter solution of YPD media was prepared by dissolving all the ingredients in 900ml of reverse osmosis water and sterilizing by autoclave process, the dextrose was dissolved in 100ml of reverse osmosis water and autoclaved separately. After cooling the YPD solution to 55 ºC, 100 ml dextrose solution ascetically transferred and the medium plated in sterile Petri plates. To prepare cells for long term storage, a single colony selected from YPD-agar plates was used to inoculate a 10 ml solution of YPD in a sterile baffled flask. The culture allowed to shake (250 rpm) at 30 ºC for 24h. The cells were collected by centrifugation (Sorvall, GS-3 rotor) for 10 min at 3000 rpm and resuspended in a 1ml solution of YPD containing 15% glycerol. The cultures were then flash-frozen in liquid nitrogen and stored at -80 ºC. These 'frozen stocks' were used to inoculate fresh cultures (Cregg et al., 1985; Hearn and Daly, 2005; Jahic et al., 2006; O'Leary et al., 2004).

Protein Production

Cultures of Pichia pastoris were grown in BMGY, composed of 3.4g/L yeast nitrogen base without amino acids and ammonium sulfate, 1% (w/v) ammonium sulfate, 1% (w/v) casamino acids, 2ml/L biotin solution, 2% (v/v) 1M potassium phosphate buffer (pH 6.0, adjusted with potassium hydroxide) and 1% (v/v) glycerol. The prepared BMGY solution sterilized by filtration through a 0.22µm PES filter (Corning Incorporated). The biotin solution was prepared by dissolving 0.2g/L of biotin in 0.02M potassium hydroxide solution, then sterilized by filtration through a 0.22µm PES filter (Corning Incorporated) and stored in aliquots at -20 ºC. Prior to inoculation, 2L culture flasks covered with four layers of cheese cloth and two layers of aluminum foil then sterilized by autoclaving at 121 ºC for 20 min. The sterile BMGY solution was transferred aseptically into the 2L culture flasks. The amount of media in the flasks was usually 1/5th of the flask volume, 4L of BMGY was equally distributed into 12 sterile 2L culture flasks. A starter culture of P. pastoris was prepared by inoculating 10ml of BMGY in a sterile baffled flask with the frozen stock culture and allowed to incubate at 30 ºC with shaking (250 rpm) for 12h. The media in the 2L culture flasks was inoculated with the 10 ml starter culture. After 24h of growth, the aluminum foil on the 2L culture flasks was removed. After this point forward, the flasks were covered with only cheese cloth. This allows better aeration and contamination is not a concern in actively growing yeast cultures. After 48h of total growth in the 2L culture flasks, the cells were harvested by centrifugation at 3000 rpm for 10 minutes. The supernatant was discarded and the cells were resuspended in BMMY. Since mutS phenotype of Pichia was utilized, the volume of BMMY used was half the volume of BMGY utilized during the growth phase. BMMY has the same composition as BMGY except 2% methanol was used as a replacement for 1% glycerol. The cells resuspended in BMMY were returned to the 2L culture flasks and covered with cheese cloth and incubated with shaking (250 rpm) at 30 ºC. Induction periods was 36h. Cells were harvested by centrifugation at 3000 rpm for 60 min. The cells were discarded and 5mM disodium EDTA to final concentration was added to the supernatant. The supernatant containing the secreted protein may be frozen at – 20ºC for several weeks before purification.

Protein Purification

The proteins are least soluble when at their isoelectric point thus the pH of supernatant adjusted to 5.2 using concentrated glacial acetic acid. The pI of A1A2A3 domains of VWF was calculated to be 5.4. Solid ammonium sulfate was added to the supernatant to a final molar concentration of 2.5M, initially. The supernatant was centrifuged (Sorvall, GS-3 rotor) at 5000 rpm for 10 min. Pellets corresponding to 2.5M ammonium sulfate precipitation was discarded then solid ammonium sulfate is added to the supernatant to a final molar concentration 5M. The solution was allowed to equilibrate for 1h at 4 ºC. The supernatant was centrifuged (Sorvall, GS-3 rotor) at 5000 rpm for 10 min, the pellet resuspended in 50mM MES, 1mM EDTA at pH 6.5. Then the final volume of cold ethanol adjusted to 80% and incubated at -20 ºC for 1 hour, then centrifuged (Sorvall, GS-3 rotor) at 5000 rpm for 10 min. The supernatant was discarded. The pellet resuspended in 50mM MES, 1mM EDTA at pH 6.5. Approximately 2.0 ml of the 80% ethanol precipitation pellet was dialyzed overnight at 4 ºC against 1L. 25mM MES, 1mM EDTA at pH 6.5, dialysis buffer was changed twice. Two milliliters of Sepharose fastflow Q (GE Healthcare) was poured into a 12 ml Bio-Rad disposable column. The column was equilibrated with 10ml of 25mM MES, 1mM EDTA at pH 6.5. Dialyzed 2 ml sample then passed over the bed followed by washing with an additional 2ml 25mM MES, 1mM EDTA at pH 6.5. To elute the protein the column was washed with 4ml of 1M NaCl, 25mM MES, 1mM EDTA at pH 6.5. The elution product was concentrated down to 1-2 ml volume using Amicon Ultra–15, regenerated cellulose (30000 kDa molecular weight cutoff) from Millipore corporation The sample was stored at -20 ºC or immediately subject to HPLC. The concentrated sample then injected into a Waters HPLC system for reverse phase chromatography containing Atlantis dC18, 5µm particle, 300 A° 4.6x250mm column held at 58°C. Upon injection, a gradient is run at a flow rate 1 ml/min from 10 to 90% acetonitrile, 01% trifluroacetic acid over 37 minutes.

Western Blot Analysis

Protein samples were resolved by 12.5% SDS–PAGE under reducing conditions using duplicate gels. One gel was Coomassie Blue stained, the other was used for electrophoretic transfer (Trans-Blot SD Semi-Dry Cell apparatus, Bio-Rad) of the eluted and or fractionated proteins onto PVDF membranes. The membranes were incubated for one hour at room temperature in 3% blocking reagent to prevent non- specific binding. Then, the membrane was incubated overnight at 4 ºC with 1:1000 dilution of primary antibody (Rabbit polyclonal to von Willebrand Factor), then 1:2000 dilution of HRP conjugated secondary antibody (Donkey polyclonal to Rabbit IgG). Membranes were washed in PBST for one hour at room temperature and signals developed following the (Bio-Rad) chemiluminescent kit protocol.

Deglycosylation

The protein was treated with Deglycosylation Enzyme Mix (Endo-α-N-Acetylgalactosaminidase, PNGase F, Neuraminidase, β1-4 Galactosidase, β-N-Acetylglucosaminidase) (New England Biolabs, Ipswich, MA). Protein deglycosylation enzyme mix was supplied in 50 mM NaCl and 20 mM Tris-HC l at pH 7.5. Protein deglycosylated by the addition of 10 Units of enzyme mix followed by incubation at 37°C for four hours.

Mass Spectrometry Analysis

The identity of the protein was confirmed using MALDI-TOF. Samples were analyzed at the Statewide Mass Spectrometry Laboratory located at the University of Arkansas. Two microliters of deglycosylated protein sample was mixed with an equal volume of 1M 2, 5-dihydroxybenzoic acid (DHB) in 90% methanol containing 0.1% formic acid and spotted onto a Bruker MTP 384 stainless steel MALDI target. MALDI-TOF analysis was performed using a Bruker Reflex III MALDI-TOF mass spectrometer (Bruker Daltonik GMBH, Bremen, Germany). Mass spectrometer operated in positive ion reflectron mode.

Digestion of Recombinant VWF A1A2A3

Protein samples were deglycosylated using Deglycosylation Enzyme Mix (New England Biolabs) as described previously. Sequencing grade Trypsin or Pepsin (Promega, WI) prepared according to manufacturer's instructions. Protein was taken into the buffers listed in the second column of Table 1. Buffer exchange was performed according to the manufacturer's instructions using Amicon Ultra–15, regenerated cellulose (10000 kDa molecular weight cutoff) centrifugal filters. Reduction and Alkylation carried as part of our efforts to digest the protein. Protein sample in 25 mM ammonium bicarbonate subjected to reduction and alkylation prior to digestion with recombinant trypsin. Sample reduced for 1 hr with 10 mM 1,4- dithiothreitol (DTT) followed by alkylation with 20 mM 2-iodoacetamide in dark for 20 min at 37°C. Excess iodoacetamide was quenched by an addition of DTT.

Reduced and alkylated peptides were then digested with trypsin for 24 h at 37°C, desalted, and concentrated using C18 ZipTips (Millipore) as recommended by manufacturer's protocol and spotted on to the MALDI target plate for analysis.

Table 1. *Digestion Conditions.*

Sample	Buffer	Reduction/Alkylation	Denaturant/Surfactant	Enzyme
10µl.(0.9mg/ml)	PBS	TCEP	-	Trypsin
HPLC fraction	25mM NH4HCO3	-	-	Trypsin
HPLC fraction	25mM NH4HCO3	10 mM DTT, 20 mM Iodoacetamide	-	Trypsin
In-Gel Digestion	25mM NH_4HCO_3	10 mM DTT, 20 mM Iodoacetamide	-	Trypsin
10µl.(0.9mg/ml)	25mM MES,1M NaCl	-	-	Trypsin
10µl.(0.9mg/ml)	25mM NH_4HCO_3	10 mM DTT, 20 mM Iodoacetamide	-	Trypsin
10µl.(0.9mg/ml)	25mM NH4HCO3	10 mM DTT, 20 mM Iodoacetamide	6M Urea	Trypsin
10µl.(0.9mg/ml)			60% Acetonitrile	Trypsin
10µl.(0.9mg/ml)	PBS	-	0.1%Tween 20	Trypsin
10µl.(0.9mg/ml)	Sodium Acetate	-	-	Pepsin
10µl.(0.9mg/ml)	Sodium Acetate	10 mM DTT, 20 mM	-	Pepsin

Results and Discussion

The expression of recombinant A1A2A3 in Pichia was determined using Western Blot assays (Figure 1.)

Figure 1. *Western Blot from the crude supernatant.*

The chromatogram (Figure 2.) was obtained when the ammonium sulfate and ethanol precipitation product was eluted using 1M NaCl 50 mM MES 10mM EDTA, pH 6.5, 100µl was injected in a 10 to 90 % acetonitrile gradient. 1 ml/min fractions were collected flash-frozen in liquid nitrogen and lyophilized using SpeedVac, reconstituted with 50 µl with deionized water and sonicated. Fractions corresponding to 15 to 21 minutes were submitted for mass spectrometry analysis. The following mass spectrum (Figure 3.) was obtained from

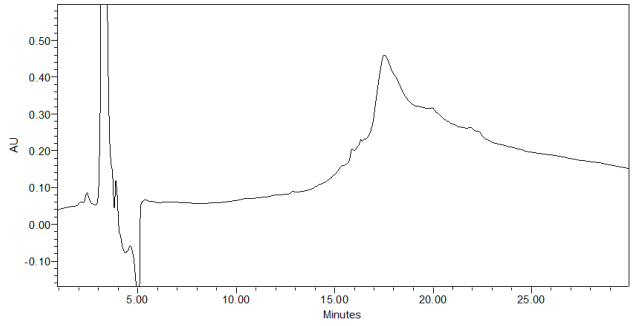

Figure 2. *The eluent was injected in a gradient of 10 to 90 % acetonitrile.*

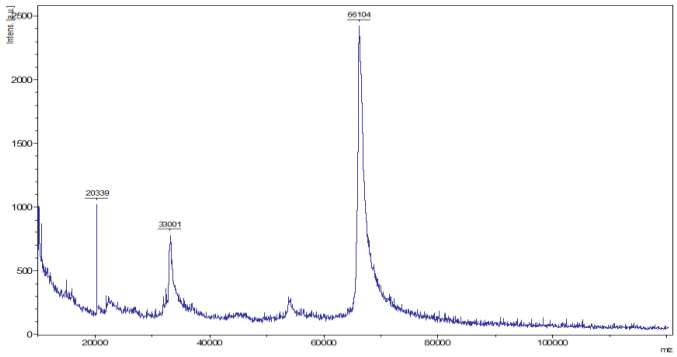

Figure 3. *HPLC fractions analyzed by MALDI-TOF.*

the HPLC fraction corresponding to 19th minute by MALDI TOF. The molecular weight of the protein estimated to be 66.1 ±0.3 kDa. The predicted molecular weight for the expected polypeptide is 66,101. When we examined 35µl of the sample on an SDS gel a single band corresponding to approximately 65 kDa was observed. (Figure 4.). The same sample developed a positive signal on Western blot at approximately at 65 kDa position (Figure 5.). Desalting of protein in 1M NaCl , 25mM MES, 1mM EDTA solution was crucial for the HPLC analysis however when the sample buffer exchanged into 25mM MES, 1mM EDTA with PBS following chromatogram where no signal observed was obtained (Figure 6.). When the sample buffer exchanged back to 1M NaCl, 25mM MES, 1mM EDTA

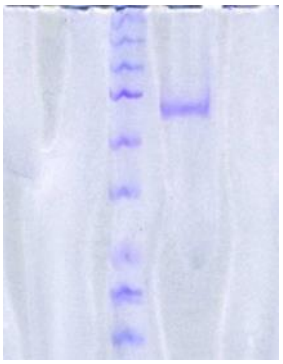

Figure 4. *12.5% SDS gel for the final purification of recombinant VWF A1A2A3 domains.*

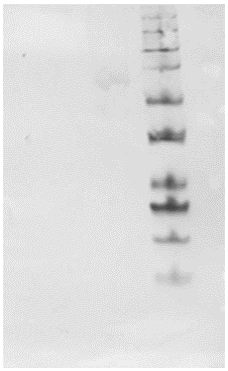

Figure 5. *Western Blot for the final purification of recombinant VWF A1A2A3 domains.*

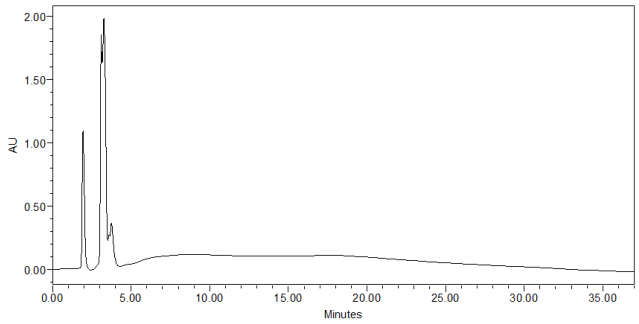

Figure 6. *Desalted sample was injected in a gradient of 10 to 90 % acetonitrile.*

the following chromatogram obtained (Figure 7.). N-terminal sequencing attempts to determine the N-terminal sequence of the protein with Edman degradation have failed. We were unable to digest the protein despite of all efforts summarized in Table 1. The A1A2A3 domain of VWF is adhesive in nature, contains binding sites for platelets, heparin and collagen. The purified recombinant A1A2A3 domain did not to solubilize in the absence of at least 1M NaCl or in standard buffer solutions such as phosphate buffered saline (PBS; 1.46 mM KH2P04, 9.9 mM Na2HP04, 2.68 mM KCl, 137 mM NaCl, pH 7.4). Protein purifications performed using described protocol, in various fermentation batches typically yielded 0.3 to 0.9 mg of recombinant protein, when the protein concentration determined by absorbance at 280 nm. We were unable to solubilize the recombinant protein in conditions that allow for subsequent studies. Solubility became our challenge more likely due to the lack of the domains that contributes to the solubility of the VWF under physiological conditions. In order to overcome this challenge, we tried to solubilize the protein in an acidic environment below the pI of the A1A2A3 domain, using Sodium acetate buffer at pH 4. We were unable to digest the protein under these conditions as well both using Trypsin and Pepsin enzymes. We also tried to denature the protein prior to digestion using urea, tween 20 and acetonitrile we were unable to digest the protein under these conditions probably due to the interfering effect of the denaturants with the enzymes. In the future efforts residues that could enhance solubility need to be included in the synthetic gene of the recombinant protein.

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