Protein Expression and Purification 139 (2017) 49–56

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

Recombinant expression of Ixolaris, a Kunitz-type inhibitor from the tick salivary gland, for NMR studies

V.S. De Paula [a, b], F.H.S. Silva [b, c], I.M.B. Francischetti [d], R.Q. Monteiro [e], A.P. Valente [b, c, *]

[a] Campus Xerém, Universidade Federal do Rio de Janeiro, Rio de Janeiro, 25245-390, Brazil
[b] Centro de Biologia Estrutural e Bioimagem, Rio de Janeiro, 21941-920, Brazil
[c] Instituto de Bioquímica Médica, Centro Nacional de Ressonância Magnética Nuclear Jiri Jonas, Universidade Federal do Rio de Janeiro, 21941-920, Brazil
[d] Vector Biology Section, Laboratory of Malaria and Vector Research (LMVR), National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), Bethesda, MD, United States
[e] Instituto de Bioquímica Médica, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, 21941-902, Brazil

ARTICLE INFO

Article history:
Received 8 June 2017
Received in revised form
29 June 2017
Accepted 17 July 2017
Available online 19 July 2017

Keywords:
Anticoagulant
Ixolaris
Factor Xa
Tissue factor
Tick saliva
NMR

ABSTRACT

Ixolaris is an anticoagulant protein identified in the tick saliva of *Ixodes scapularis*. Ixolaris contains 2 Kunitz like domains and binds to Factor Xa or Factor X as a scaffold for inhibition of the Tissue Factor (TF)/Factor VIIa (FVIIa). In contrast to tissue factor pathway inhibitor (TFPI), however, Ixolaris does not bind to the active site cleft of FXa. Instead, complex formation is mediated by the FXa heparin-binding exosite. Due to its potent and long-lasting antithrombotic activity, Ixolaris is a promising agent for anticoagulant therapy. Although numerous functional studies of Ixolaris exist, three-dimensional structure of Ixolaris has not been obtained at atomic resolution. Using the pET32 vector, we successfully expressed a TRX-His_6-Ixolaris fusion protein. By combining Ni-NTA chromatography, enterokinase protease cleavage, and reverse phase HPLC (RP-HPLC), we purified isotopically labeled Ixolaris for NMR studies. 1D ^{1}H and 2D $^{15}N-^{1}H$ NMR analysis yielded high quality 2D $^{15}N-^{1}H$ HSQC spectra revealing that the recombinant protein is folded. These studies represent the first steps in obtaining high-resolution structural information by NMR for Ixolaris enabling the investigation of the molecular basis for Ixolaris-coagulation factors interactions.

1. Introduction

Ixolaris is an exogenous tissue factor pathway inhibitor (TFPI) characterized from the salivary gland of the tick *Ixodes scapularis* [7]. Ixolaris has two Kunitz domains and, in contrast to human TFPI, its second Kunitz domain binds to the heparin-binding exosite (HBE) of factor Xa (FXa) (unlike TFPI, which binds to the active site) while its first domain binds to FVIIa active site in complex with tissue factor (TF) [7]. Another remarkable feature that differentiates Ixolaris from human TFPI is the ability to interact with FX, the zymogen form of FXa, possibly through a precursor state of the HBE [18]. Interestingly, a number of exogenous coagulation inhibitors that interact with the zymogen forms of coagulation enzymes have been characterized, including Nitrophorin-2 and Bothrojaracin, which recognize FIX and prothrombin (FII), respectively [15,17]. In addition, Nematode Anticoagulant Protein c2 (NAPc2) interacts with high affinity with either FXa or FX [27]. Although displaying no primary sequence homology with Ixolaris, NAPc2 exhibits a similar anticoagulant mechanism of action: it binds to FX/FXa exosite and further recognizes FVIIa/TF active site [2,19,27]. Further, monoclonal antibodies and short-peptides (5–20 residues) have also been developed as inhibitors of the FVIIa/TF complex [6,21].

To better understand the molecular basis of Ixolaris interaction with FX/FXa and FVIIa/TF, structural studies are needed to unravel the regions responsible for Ixolaris binding to its coagulation factors ligands. The full-length cDNA of Ixolaris was expressed previously and purified at homogeneity [7]. In this previous study, recombinant Ixolaris was obtained in insect cells and showed to inhibit FVIIa/TF–induced FX activation with an inhibitory concentration of 50% (IC_{50}) in the picomolar range [7]. However, several aspects have to be taken into account to obtain Ixolaris from high-five insect cells including: (i) Ixolaris presents 3 putative N-linked glycosylation sites, at Asn65, Asn98, and Asn136. The most

* Corresponding author. CNRMN-Cenabio-Centro de Biologia Estrutural e Bioimagem, Universidade Federal do Rio de Janeiro, 21941-902, Brazil.
E-mail address: valente.anap@gmail.com (A.P. Valente).

http://dx.doi.org/10.1016/j.pep.2017.07.012
1046-5928/

abundant form (~95%) of the recombinant Ixolaris from insect cells was the glycosylated form, (ii) no therapeutic protein is currently produced using this system as this would compromise *in vivo* bioactivity and potentially induce allergenic reactions, and (iii) obtaining protein samples for structural characterization by NMR are hampered due to the high costs of incorporating stable isotopes into insect cells [14]; [23].

Because of these issues, an improved expression system and purification protocol was needed to generate Ixolaris protein suitable for structural characterization by NMR. *E. coli* is one of the most frequently used hosts for the production of heterologous proteins due to its well-studied genetics, rapid growth and ease of high cell density fermentation on inexpensive cultivation media [5]. Currently, about 30% of biopharmaceuticals are produced in bacterial systems [24,29].

In the present study, we described an efficient strategy to obtain the recombinant Ixolaris as a fusion protein with Thioredoxin (TRX) in *E. coli* to generate non-glycosylated protein. The pET32 vector uses TRX as the fusion partner, which can increase expression level, stability and solubility [10]. The His-tag at the N-terminal can be used for nickel affinity purification. An enterokinase protease recognition site is present between TRX and Ixolaris, which is crucial for the release of Ixolaris from the TRX fusion protein. In this study we have also decided to pursue two constructs composed only of Kunitz-domains I and II sequences (namely K1 and K2) to further study the structural features and the mechanism of inhibition of blood coagulation of each domain separately. After successful soluble expression using pET32 approach, we purified both full length and the Kunitz-domains K1 and K2 constructs. Analyses of the ^{15}N-labeled Ixolaris reveal that it exhibits high quality two-dimensional (2D) heteronuclear single quantum coherence (HSQC) spectrum suitable for structural studies. These studies represent the first steps in obtaining structural information for the Ixolaris, which will shed light on the molecular mechanism underlying the specificity of interaction between Ixolaris and its targets in the blood coagulation system.

2. Material and methods

2.1. Generation of expression constructs of Ixolaris

Codon optimized DNA of Ixolaris (full sequence) and the isolated Kunitz domains were synthesized and cloned into the expression vector pET32a into the 5′ *Nco*I and 3′ *Bam*HI restriction sites by Genscript. Two nucleotides were added right after *Nco*I site (CCA TGG GT) to avoid frame shift. The resulting plasmids encode a N-terminally hexahistidine-tagged fusion protein of TRX and Ixolaris constructs, with an enterokinase protease recognition site in between. The final proteins after purification and enterokinase cleavage will have 2 additional residues at the N-terminus (Alanine and Metionine), which are leftover from the cloning process. The full sequence of Ixolaris (namely Full) corresponds to residues 1–140. The Kunitz domain 1 (namely K1) corresponds to residues 1–75, and Kunitz domain 2 (namely K2) corresponds to residues 69–140. The recombinant constructs were confirmed by DNA sequencing using T7 primers.

2.2. Expression of TRX-His$_6$-Ixolaris

Competent *E. coli* Rosetta-gami B (DE3) cells were transformed with pET32a-Full, pET32a-K1 and pET32a-K2 and selected on LB agar containing 100 μg/mL ampicillin and 34 μg/mL chloramphenicol. A single colony was picked and used to inoculate an overnight 50 mL culture in LB broth (Sigma) at 37 °C with shaking at 200 rpm. Overnight culture was then used to inoculate 600 mL sterile LB in 2 L baffled flasks and incubated at 37 °C with continuous shaking at 200 rpm until optical density (OD_{600}) reached 0.7. Subsequently, protein expression was induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 20 h under agitation (200 rpm) at 15 °C. The cells were harvested by centrifugation at 6000*g* at 4 °C for 30 min. Cell pellet were stored at −20 °C prior to protein purification. ^{15}N-labeled proteins were grown in M9 minimal medium ($Na_2HPO_4 \cdot 12H_2O$ 17 g/L, KH_2PO_4 3 g/L, NaCl 0.5 g/L, $CaCl_2 \cdot 2H_2O$ 14.7 g/L, thiamine 1 g/L, glucose 4 g/L) supplemented with $^{15}NH_4Cl$ (1 g/L). The M9 medium was also supplemented with Celtone® Base Powder [U-^{15}N] 0.5 g/L (Cambridge Isotopes Laboratories), yeast nitrogen base without aminoacids 1.7 g/L (SIGMA) and MEM Vitamin Solution (SIGMA).

2.3. Purification of TRX-His$_6$-Ixolaris

The pellet cells were resuspended in lysis buffer [50 mM Tris–HCl (pH 7.6), 500 mM NaCl, 10 mM Imidazole, 10 μg/mL lysozyme and 1 mM phenylmethanesulfonyl fluoride (PMSF)] and disrupted by sonication at 30% amplitude (10 cycles of 30 s on followed by 60 s off intervals). The cellular debris was separated by centrifugation at 6000*g* for 30 min at 4 °C. Soluble protein in the supernatant of cell lysate was purified by a Ni^{2+}-affinity chromatography step using a His-Trap HP 5 mL column (GE Healthcare) previously equilibrated in buffer A (50 mM Tris–HCl pH 7.6, 500 mM NaCl, 10 mM imidazole). The column was washed with buffer A to remove non His$_6$-tagged proteins. TRX-His$_6$-IXO constructs were eluted from the column with a linear imidazole gradient varying from 0 to 100% buffer B (50 mM Tris–HCl pH 7.6, 500 mM NaCl, 500 mM imidazole). Fractions containing the protein, as identified by 12% Tris-Glycine SDS-PAGE, were pooled and subsequently dialyzed in buffer C (50 mM Tris–HCl buffer pH 7.6, 500 mM NaCl) to remove the imidazole. The proteins were incubated with 5 U enterokinase protease (Invitrogen) at 4 °C for 18 h to achieve complete cleavage, as determined by 12% Tris-Glycine SDS-PAGE. The protein solutions were loaded onto a His-Trap HP 5 mL column (GE Healthcare) previously equilibrated with buffer A. The Full, K1 and K2 proteins were recovered in the flow-through, while the TRX-His$_6$ remains bound to the resin and were further eluted with buffer B. The proteins were further purified to homogeneity by reverse phase chromatography using a C_8 column PRP-3 (Hamilton 7.0 mm × 305 mm) with a linear gradient of acetonitrile (10–90%) containing 0.1% TFA. The resulting samples were freeze-dried under vacuum. The identity and purity of Ixolaris was confirmed with MALDI-TOF mass spectrometry. The protein concentration was determined spectrophotometrically by measuring the absorbance at 280 nm, and comparing it to the theoretical molar extinction coefficient of 8855 M^{-1} cm^{-1} for K1, 11,710 M^{-1} cm^{-1} for K2, and 20,565 M^{-1} cm^{-1} for Full Ixolaris.

2.4. Analytical size exclusion chromatograph

The K1, K2 and Full Ixolaris samples were applied to a Superdex 75 10/300 column (GE Healthcare) equilibrated with buffer D (20 mM sodium phosphate 7.2, 100 mM NaCl). The column was attached to an AKTA prime HPLC system, with detection conducted at 214 nm. Calibration of the gel filtration column was performed with the following proteins: cytochrome C (12.2 kDa) and ubiquitin (8.6 kDa).

2.5. NMR spectroscopy

NMR spectra of Full Ixolaris, K1 and K2 were acquired at 25 °C and 35 °C on a Bruker Avance III 800 MHz spectrometer (Bruker Biospin, USA) equipped with an inverse-detection triple resonance