Journal of Chromatography B, 879 (2011) 2437–2442

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Efficient and inexpensive method for purification of heparin binding proteins

Sumit Batra[a], Nilesh Sahi[a], Kristen Mikulcik[a], Heather Shockley[a], Camille Turner[b], Zachary Laux[b], Vivek D. Badwaik[a], Eric Conte[a], Dakshinamurthy Rajalingam[a,*]

[a] Department of Chemistry, Western Kentucky University, Bowling Green, KY 42101, United States
[b] The Carol Martin Gatton Academy of Mathematics and Science, Western Kentucky University, Bowling Green, KY 42101, United States

ARTICLE INFO

Article history:
Received 12 January 2011
Accepted 29 June 2011
Available online 6 July 2011

Keywords:
Heparin binding proteins
Fibroblast growth factor
Biopharmaceuticals
Protein purification
Inclusion bodies
Affinity chromatography
Thermal denaturation

ABSTRACT

Heparin binding (HB) proteins mediate a wide range of important cellular processes, which makes this class of proteins biopharmaceutically important. Engineering HB proteins may bring many advantages, but it necessitates cost effective and efficient purification methodologies compared to currently available methods. One of the most important classes of HB proteins are fibroblast growth factors (FGFs) and their receptors (FGFRs). In this study, we report an efficient off-column purification of FGF-1 from soluble fractions and purification of the D2 domain of FGFR from insoluble inclusion bodies, using a weak Amberlite cation (IRC) exchanger. FGF-1 and the D2 domain have been expressed in *Escherichia coli* and purified to homogeneity using IRC resin. This approach is an alternative to conventional affinity column chromatography, which exhibits several disadvantages, including time-consuming experimental procedures for purification and regeneration and results in the expensive production of recombinant proteins. Results of the heparin binding chromatography and steady state fluorescence experiments show that the FGF-1 and the D2 are in a native conformation. The findings of this study will not only aid an in-depth investigation of this class of proteins but will also provide avenues for inexpensive and efficient purification of other important biological macromolecules.

1. Introduction

Protein biopharmaceuticals are the fastest-growing category of new drugs as they currently target over 200 human diseases, including wound healing, cancer, heart disease, Alzheimer's disease, diabetes, multiple sclerosis, AIDS, and arthritis [1,2]. The impact of protein biopharmaceuticals on US healthcare and the economy is substantial and growing rapidly. However, proteins are a novel type of compound in comparison to traditional small molecules, and they present new and significant challenges for the realization of their full potential as therapeutic agents [3]. For example, the intrinsic property of proteins to adopt alternative conformations (such as during protein expression, folding, unfolding and aggregation during isolation) presents numerous challenges for their isolation/separation, and warrant sophisticated methodologies with multiple steps. These challenges result in higher costs for the production of recombinant proteins, which currently limits their effective application as biopharmaceuticals [4,5]. The advantages associated with using pure and homogenous biomolecules like proteins, enzymes, and nucleic acids, etc. in place of small organic molecules have attracted the attention of biochemists in devising new, economical and efficient techniques for expression, purification and characterization of these biomedically important biomolecules [1–5]. One important class of biomolecules are heparin binding proteins which consist of a wide range of protein families including epidermal growth factors (EGFs), fibroblast growth factors (FGFs), connective tissue growth factors (CTGFs), midkines, etc. [6]. Heparin and heparan sulfate glycosaminoglycans (HSGAGs) mediate a wide variety of complex biological processes by binding to this specific class of more than 200 extracellular proteins [7]. HSGAGs are linear polysaccharides composed of repeating glucosamine-hexuronic acid disaccharide units [8]. These protein/HSGAGs complexes are involved in various cellular processes such as apoptosis, cell cycle control, platelet activation, capacitation, acrosome reaction, sperm decondensation, wound repair, survival of neurons, tumours, and pathogenesis of various diseases [6–9].

One of the most important classes of heparin binding proteins are fibroblast growth factors (FGFs) which constitute a large family of structurally related proteins that are involved in many biological processes such as angiogenesis, cell growth, embryogenesis, differentiation, and wound healing [10,11]. FGFs are also used as biopharmaceutical drugs to improve wound healing

Abbreviations: FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; EGF, epidermal growth factor; CTGF, connective tissue growth factors; HSGAG, heparan sulfate glycosaminoglycans; Ni–NTA, nickel–nitrilotriacetic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; IPTG, isopropyl β-D-1-thiogalactopyranoside; SOS, sucrose octa sulfate.

* Corresponding author. Tel.: +1 270 745 2136; fax: +1 270 745 5361.
E-mail address: rajalingam.dakshinamurthy@wku.edu (D. Rajalingam).

1570-0232/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2011.06.047

caused by metabolic diseases such as obesity, diabetes, infection, chronic liver failure, malnutrition, and second-degree burns [10–12]. FGFs exert their biological activity by binding to tyrosine kinase receptors (FGFRs) on the cell surface in the presence of HSPGs [12]. The signaling involves ligand-induced receptor dimerization and autophosphorylation, followed by a downstream transfer of the signal [13]. However, in the absence of cell surface proteoglycan, heparan sulfate results in a dramatic decrease in FGFR activity for proliferation, mitogenesis, wound healing, and neuro-vascularization [12–14]. FGFRs contain an extracellular domain, a short transmembrane domain, and a cytoplasmic tyrosine kinase domain. The extracellular domain consists of three structural subdomains named D1, D2, and D3. The D2 subdomain contributes to the ligand (FGF) binding [12–14].

Previously, various methods have been developed in order to express, purify and characterize FGF-1 and different domains of the FGFR, which emphasizes the importance of these biomolecules for biopharmaceutical and research purposes (i.e., to understand the structural-functional relationship of these proteins) [15–21]. Conventionally, synthetic heparin affinity and different types of metal affinity chromatography methods were used to purify most of the heparin binding proteins including FGF-1 and the D2 domain of the FGFR [16–21]. FGF-1 is expressed in a soluble form in a bacterial host and has been shown to be purified by expensive heparin affinity chromatography [16–19]. FGFRs purification and refolding involves multiple steps because the overexpressed protein was invariably trapped in inclusion bodies [20,21]. While conventional heparin/metal affinity column purification methods are effective, they exhibit several disadvantages including time-consuming experimental procedures for purification, and regeneration (a requirement for expensive affinity resins) and difficulties in maintenance of the resins due to bacterial degradation [15–21]. These disadvantages associated with previously reported procedures results in the high cost of commercially available purified proteins which have many biopharmaceuticals applications.

In the present study we devised an inexpensive and efficient off-column purification method to be used as an alternative that overcomes the disadvantages associated with conventional affinity chromatography methods. This new technique is based on ion exchange chromatography; where, inexpensive Amberlite weak cation exchange resin was used in place of a heparin/Ni–NTA resins to achieve a pure, and homogeneous protein (98%).

Amberlite ion exchange resin was first used for protein purification to remove pectinmethylesterase from pectinpolygalactonurase [22]. Since then Amberlite cation exchange resins have been used to successfully purify different types of proteins, which include growth hormones, cytochrome c, different types of proteases and several other proteins [22–36]. In this new method, both the soluble protein (i.e., FGF-1) and protein which is expressed in the inclusion bodies (i.e., the D2 domain of FGFR) were purified using this off-column method using weak cation exchange resins. The authenticity of the purified protein and its biological activity and nativity were characterized by gel electrophoresis, heparin affinity chromatography, and steady state fluorescence spectroscopy. This present study paves a way for the inexpensive and efficient purification protocol for heparin binding protein pharmaceuticals.

2. Materials and methods

2.1. Materials

Escherichia coli [BL21(DE3)] was purchased from Invitrogen. Heparin and Ni–NTA sepharose were obtained from GE Health Science. Amberlite IRC 50 was purchased from Acros organics. All other chemicals used in this study were of high quality analytical grade.

2.2. Methods

2.2.1. Construction and expression of FGF-1 and D2 domain of FGFR

Human FGF-1 (GenBank # X59065) and D2 domain of human FGFR (GenBank # AK026508) from full length FGFR, cDNAs were made by procedures as previously described and cloned into a pJExpress414 vector (DNA2.0) and transformed into BL21(DE3) *E. coli* cells for the overexpression [37]. The gene sequences were optimized using DNA2.0 codon bias algorithm to maximize the yield of the proteins, prior to cloning [38,39]. *E. coli* cells transformed with pJExpress414 containing the FGF-1 and D2 domain insert were grown in 100 mL Luria broth (LB) medium separately. Protein induction was achieved by the addition of IPTG (0.5 mM/L) when the absorbance of the growing culture had reached about 0.6 at 600 nm. The culture was incubated at 37 °C for additional 6 h and the cells were harvested and lysed by sonication. The expression and solubility of both FGF-1 and D2 domain were analyzed by SDS-PAGE. Typically protein samples were prepared for SDS-PAGE analysis by precipitating the proteins using 15% (w/v) of tricholoracetic acid (TCA) from 500 μL of samples [40]. The acid treated proteins solutions were incubated at 4 °C for 20 min. The precipitated proteins samples were pelleted down by centrifugation at 12,000 rpm for 10 min. The pellet was then dissolved in 15 μL of 8 M urea followed by the addition of 15 μL of sample loading buffer. The samples were then analyzed by loading 20 μL in each well of SDS-PAGE.

2.2.2. Purification of recombinant FGF-1

Purification of FGF-1 was achieved by applying clear soluble fraction of bacterial cell lysate on an IRC column packed in a 15 mL centrifuge tube. It was allowed to stand for 45 min and then centrifuged at 12,000 rpm for 10 min and the supernatant was collected. The resin was then extensively washed, with 10 mM Tris buffer (pH 7.4) and thrice with 10 mM Tris buffer (pH 7.4) containing 50 mM NaCl. After each wash, samples were centrifuged at 12,000 rpm for 10 min and the supernatant fractions were then collected separately. FGF-1 was further eluted by incubating the resin with 10 mM phosphate buffer (pH 7.2) containing 1.5 M NaCl for 30 min. The incubated resin was further centrifuged at 12,000 rpm for 10 min and the supernatant was collected as a separate fraction. The temperature was maintained at 4 °C throughout the purification. The protein was further desalted and concentrated by ultrafiltration in 10 mM phosphate (pH 7.2) containing 100 mM NaCl. The concentration of the protein was estimated on the basis of the extinction coefficient value (Σ_{280} = 17,420 M^{-1} cm^{-1}) calculated from the amino acid sequence of FGF-1 [41]. The homogeneity and authenticity of the protein was assessed by using SDS-PAGE, MALDI-TOF mass analysis, heparin binding affinity and steady state fluorescence.

2.2.3. Purification of D2 domain of FGFR

Purification of the D2 domain was achieved by denaturation of the inclusion bodies (insoluble fraction of bacterial cell lysate) by the use of 5 mL of denaturation buffer (8 M urea, 10 mM Tris buffer) resulting on 2 mg/mL protein. The suspension was then centrifuged for 10 min at 12,000 rpm and the supernatant was loaded onto the IRC column packed in a 50 mL centrifuge tube. Refolding of the protein was induced by decreasing the urea concentration using 10 mM Tris buffer (pH 7.4), in a stepwise linear gradient from 8 to 0 M over a period of 2 h. After refolding, the protein was incubated for 60 min, centrifuged at 12,000 rpm for 5 min and collected. The resin was then washed thrice with 10 mL of 10 mM phosphate

buffer (pH 7.4), centrifuged, and the fractions were collected separately. Further elution was done by incubating the resin with 10 mM phosphate (pH 7.2) containing 1.5 M NaCl for 40 min. The incubated resin was further centrifuged and collected as a separate fraction. The protein was then desalted and concentrated by ultra filtration in 10 mM phosphate (pH 7.2) containing 100 mM NaCl. The refolding and purification procedures were carried out at 4 °C. The concentration of the protein was estimated on the basis of the extinction coefficient value (Σ_{280} = 24,075 M^{-1} cm^{-1}) calculated from the amino acid sequence of the D2 domain [41]. Purified D2 domain was assessed for homogeneity and authenticity by using SDS-PAGE, MALDI-TOF mass analysis, heparin binding affinity and steady state fluorescence.

2.2.4. Heparin affinity chromatography

The purified protein (FGF-1 or D2 domain) was loaded onto heparin sepharose column and incubated for 90 min. The column was then washed with 10 mM phosphate buffer (pH 7.2). Further washings were carried out with 10 mM phosphate buffer containing 0.5 M NaCl (pH 7.2). Finally, the protein was eluted with the elution buffer containing 1.5 M NaCl. The temperature was maintained at 4 °C throughout the purification. The authenticity of the protein was checked by SDS-PAGE.

2.2.5. Steady state fluorescence

Fluorescence experiments were performed on a PerkinElmer spectrofluorimeter. The excitation wavelength was set at 280 nm, and bandwidths for excitation and emission were set at 2.5 nm and 10 nm, respectively. Intrinsic fluorescence measurements were made at a protein concentration of 50 μM. For the thermal denaturation experiments, the protein and sucrose octasulfate (SOS-a structural analog of heparin) were mixed in a 1:1 ratio in 10 mM phosphate buffer (pH 7.2) containing 100 mM NaCl. The requisite temperature(s) in the thermal denaturation experiments was attained using a Quantum Northwest temperature controller system. Necessary background corrections were made in all spectra.

3. Results and discussions

3.1. Overexpression and purification of the FGF-1

SDS-PAGE of the bacterial cells induced by IPTG showed that most of the protein was expressed in soluble form (Fig. 1A). The purification of the recombinant FGF-1 was facilitated by using an Amberlite weak cation exchange resin IRC. FGF-1 was bound to IRC resin quite strongly and the unbound contaminating protein was eliminated by washing the resin three times with 10 mL of wash buffer [10 mM Tris buffer (pH 7.4)]. Other impurities which were nonspecifically bound to IRC resin were further removed by washing the column with 10 mM Tris buffer (pH 7.4) containing 50 mM NaCl. FGF-1 was eluted with 10 mM phosphate buffer (pH 7.2) containing 1.5 M sodium chloride. SDS-PAGE of the purified FGF-1 sample yielded single intense band corresponding to a molecular mass of ~16 kDa with about 98% purity (Fig. 1B). The authenticity of the protein was further confirmed by MALDI-TOF mass spectrum analysis (Fig. S1A). The final yield of the pure FGF-1 was 30 mg/L of the bacterial culture. This is comparable to the conventional heparin column purification method, which yielded 32 mg/L (Supplementary material, Fig. S2A).

3.2. Overexpression and purification of the D2 domain

SDS-PAGE of the bacterial cells induced by IPTG showed that most of the expressed protein was trapped as inclusion bodies (Fig. 2A). The pellet containing the D2 domain when dissolved in 8 M urea showed an intense band corresponding to a molecular mass of about 13 kDa and accounted for about 95% of total protein recovered from the cell lysate (Fig. 2A). The refolding of the recombinant D2 domain was facilitated by incubating the denatured fraction of the protein with affinity IRC resin followed by refolding using a linear gradient (8 M to ~0 M) of 10 mM Tris buffer (pH 7.4) containing 50 mM NaCl. Further purification was facilitated by washing the resin with 60 mL of wash buffer [10 mM phosphate buffer (pH 7.2) containing 50 mM of NaCl (Fig. 2B)]. Repeated washing of the column with the wash buffer aided the removal of any residual denaturant. The D2 domain was eluted with 10 mL phosphate buffer (pH 7.2) containing 1.5 M sodium chloride to yield a single intense band (purity ~98%) on SDS-PAGE corresponding to a molecular mass of about 13 kDa, which was further confirmed by MALDI-TOF mass spectrum analysis (Fig. S1B). The final yield of the pure D2 domain was 24 mg/L of the bacterial culture. This is comparable to the conventional multiple step on-column purification method, which yielded 20 mg/L (Fig. S2B).

Amberlite weak cation exchange resin (IRC 50) is composed of copolymerized methacrylic acid and divinylbenzene. This resin contains 3.5 mmol of carboxylate groups per mL (3.5 M) of 400 mesh resin. The remaining groups in smaller amounts are

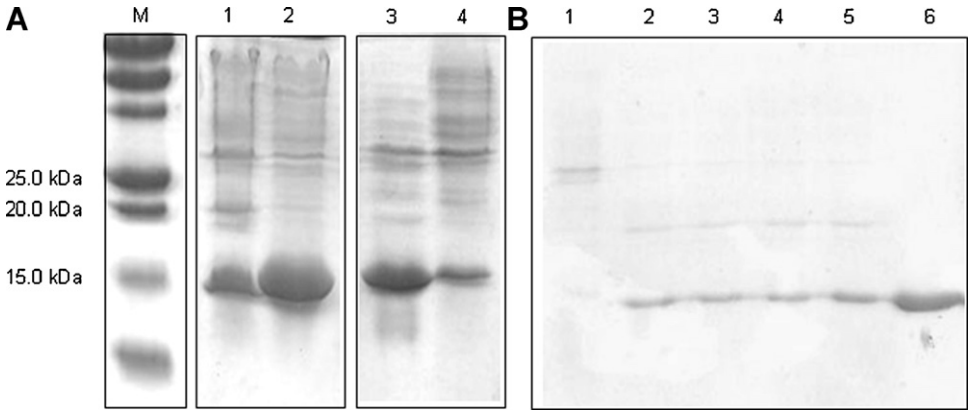

Fig. 1. (A) SDS-PAGE depicting the expression and purification of FGF-1 from *E. coli*. Lane M, represents the molecular weight marker; lane 1, uninduced sample; lane 2, induced sample; lane 3, lysate of induced sample soluble fraction; lane 4, lysate of induced sample insoluble fraction. (B) SDS-PAGE of fractions collected with buffer containing varying concentration of sodium chloride. Lane 1, represents flow through; lane 2, depicts protein bands contained in fraction eluted in 10 mM Tris buffer; lanes 3–5, depicts protein bands contained in fractions eluted in 10 mM Tris buffer containing 50 mM NaCl; and lane 6 depicts protein band contained in fractions eluted in 10 mM phosphate buffer containing 1.5 M NaCl.

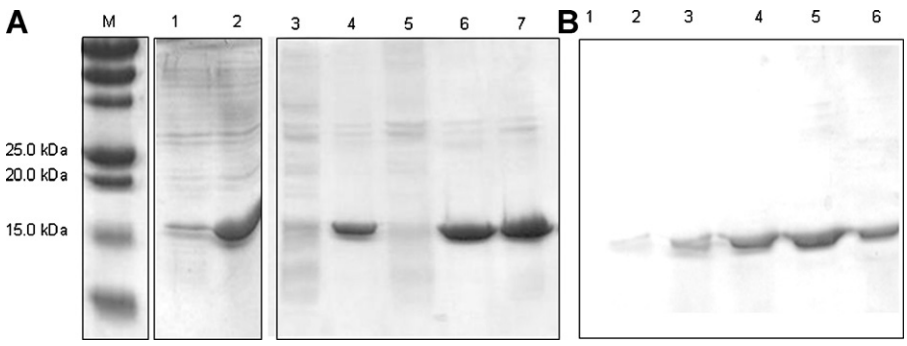

Fig. 2. (A) SDS-PAGE depicting the expression and purification of the D2 domain of FGFR from *E. coli*. Lane M, represents the molecular weight marker; lane 1, uninduced sample; lane 2, induced sample; lane 3, lysate of induced sample soluble fraction; lane 4, lysate of induced sample insoluble fraction; lane 5, supernatant from 2 M NaCl wash of the insoluble lysate; lane 6, insoluble lysate after 2 M NaCl wash; and lane 7, lysate in 8 M urea soluble fraction. (B) SDS-PAGE of fractions eluted in buffer containing varying concentration of sodium chloride. Lane 1–3, depicts protein bands contained in fractions eluted at 10 mM phosphate buffer; and lane 4–6, depicts protein bands contained in fractions eluted in 10 mM phosphate buffer containing 1.5 M NaCl.

methyl and phenyl (crosslinkers) groups. Thus, this stationary phase contains both hydrophilic charged sites (carboxylate) and hydrophobic sites (methyl and phenyl) which provide both affinity and hydrophobic binding site(s) for the proteins. We have found the off column purification method using IRC resin to be efficient without the disadvantages associated with conventional heparin/Ni–NTA sepharose column chromatography. The efficiency of this new method was based on a quicker purification procedure time, which is approximately 1/3rd of conventional heparin chromatography (in the case of proteins expressed in soluble form). Also noteworthy is the lower cost of the resin as compared to other affinity sepharose resins. The presented method has also been proven useful in cases where proteins were expressed in inclusion bodies. Also the time required for refolding the protein was reduced by 1/4th and the refolding occurred on the same column, thereby making this technique cost effective by eliminating the requirement of another column. The maintenance and regeneration of the IRC resin was very convenient, and was achieved by simply incubating the resin in 0.1 M HCl and washing it several times with distilled water. Implementation of this new protocol will in turn reduce the cost of commercially available purified protein, by eliminating the need for sophisticated instruments used to purify proteins by conventional methods (Table S1). Overall, this off-column purification strategy using IRC resin is economical, efficient and easy to maintain.

3.3. Biological activity of recombinant FGF-1 and D2 domain

It is important to verify whether the recombinant protein remains biologically active, after the off-column purification. In order to test the biological activity of the recombinant FGF-1 and D2 domain, we observed it's binding with heparin using heparin affinity chromatography [15–18]. Both FGF-1 and D2 domain were loaded onto the heparin column [previously washed with 10 mM phosphate buffer (pH 7.2)] separately. No protein was eluted when washed with both 10 mM phosphate buffer (pH 7.2) or with 10 mM phosphate buffer (pH 7.2) containing 0.75 M NaCl. However, both FGF-1 and D2 domain eluted as a single peak in 10 mM phosphate buffer (pH 7.2) containing 1.5 M sodium chloride and yielded a single intense band on SDS-PAGE corresponding to a molecular mass of about 16 kDa and 13 kDa, respectively (Fig. 3A and B). These results clearly suggest that both the recombinant FGF-1 and D2 domain purified using the off-column strategy are in their biologically active confirmation as confirmed through their strong binding with heparin.

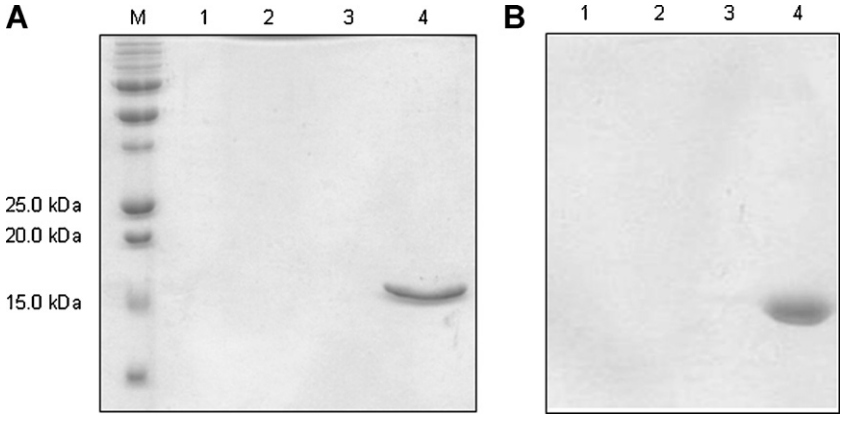

Fig. 3. (A) SDS-PAGE depicting heparin affinity chromatography of FGF-1. Lane M, represents molecular weight marker; lane 1, represents flow through; lane 2, depicts protein bands contained in fraction eluted in 10 mM phosphate buffer; lane 3, depicts protein bands contained in fraction eluted in 10 mM phosphate buffer containing 0.75 M NaCl; and lane 4 depicts protein bands contained in fractions eluted in 10 mM phosphate buffer containing 1.5 M NaCl. (B) SDS-PAGE depicting heparin affinity chromatography of the D2 domain. Lane 1, represents flow through; lane 2, depicts protein bands contained in fraction eluted in 10 mM phosphate buffer; lane 3, depicts protein bands contained in fractions eluted in 10 mM phosphate buffer containing 0.75 M NaCl; and lane 4, depicts protein bands contained in fractions eluted in 10 mM phosphate containing 1.5 M NaCl.

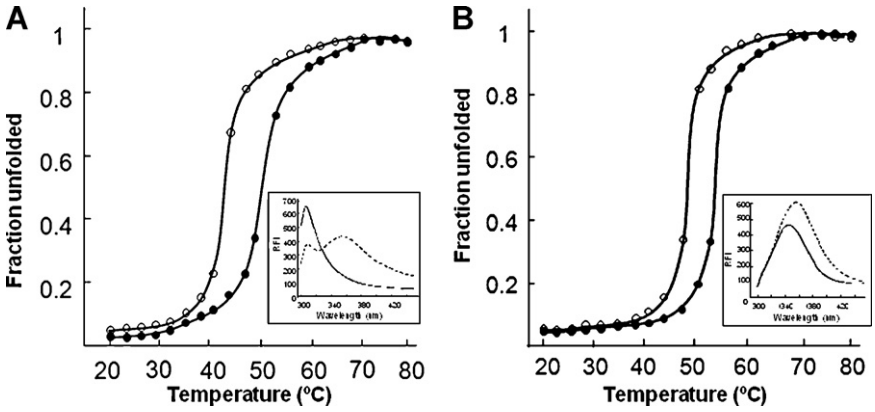

Fig. 4. (A) Thermal induced unfolding of FGF-1 in the presence (closed circle) and absence (open circle) of SOS. The ligand (SOS) binds to the FGF-1 and stabilizes the protein. The inset figure shows the emission spectra of FGF-1 in its native state (continuous line) and in the denatured state(s) state (broken line). (B) Thermal induced unfolding of D2 domain in the presence (closed circle) and absence (open circle) of SOS. The ligand (SOS) binds to the D2 domain and stabilizes the protein. The inset figure shows the emission spectra of the D2 domain in its native state (continuous line) and in the denatured state(s) state (broken line).

3.4. Biophysical characterization of recombinant FGF-1 and D2 domain

We further assessed the confirmation of the recombinant FGF-1 and D2 domain using fluorescence spectroscopy. The fluorescence measurement serves as an excellent probe to characterize the thermodynamic stability of proteins in the absence and presence of a ligand by monitoring the tertiary structural changes that occur in the proteins under different environmental conditions (i.e., solution condition) [42,43]. FGF-1 contains a single well-conserved tryptophan at position 121 in the primary sequence [42,43]. The emission spectrum of the protein is dominated by a tyrosine emission peak at ~308 nm in its native state (Fig. 4A, inset). However, in the completely unfolded state (in 8 M urea), FGF-1 exhibits an emission spectrum dominated by tryptophan fluorescence at ~350 nm (Fig. 4A, inset). These spectral features are ideal to monitor the denaturant induced unfolding of the protein. Equilibrium thermal denaturation of FGF-1 was performed to assess the conformational stability of FGF-1 upon binding to sucrose octasulfate (SOS-a structural analog of heparin) [20]. The T_m (the temperature at which 50% of the molecules are in the native state) of the protein increases in the presence of the SOS by about ~6 °C (from ~44 to 50 °C), suggesting that thermodynamic stability of the protein is enhanced upon binding to the SOS (Fig. 4A). These results clearly indicate that the recombinant FGF-1 is in a stable, folded and in a biologically active conformation.

We also assessed the confirmation of the recombinant D2 domain using fluorescence spectroscopy. The D2 domain contains three tryptophan residues located at positions 155, 190 and 213 in the primary sequence [20]. Therefore, measurement of the intrinsic tryptophan fluorescence would serve as an excellent probe to monitor the tertiary structural changes that occur in the protein under different conditions. The fluorescence spectrum of the D2 domain in its native state showed an emission maximum around ~338 nm suggesting that the tryptophan residues are buried in the interior of the well organized native tertiary structure of the protein (Fig. 4B, inset). However, in the completely unfolded state (in 8 M urea), the D2 domain exhibits an emission spectrum dominated by tryptophan fluorescence at ~350 nm (Fig. 4B, inset). These spectral features are ideal to monitor the denaturant induced unfolding of the protein. Equilibrium thermal denaturation of D2 domain was performed to assess the conformational stability of D2 domain upon binding to SOS. The T_m (the temperature at which 50% of the molecules are in the native state) of the protein increases in the presence of the SOS by about ~5 °C (from ~48 to 53 °C), suggesting that thermodynamic stability of the protein is enhanced upon binding to the SOS (Fig. 4B). These results clearly indicate that the recombinant D2 domain is in a stable, folded and in a biologically active conformation.

4. Conclusions

We have successfully purified two heparin binding proteins namely FGF-1 (from the soluble fraction) and the D2 domain of FGFR (from the insoluble fraction) in a biologically active conformation using Amberlite IRC resin and thereby devised an efficient and economical method for the purification of this biologically important class of proteins compared to conventional affinity chromatography methods (Table S1). The findings of the present study will facilitate research towards understanding the regulation of heparin binding proteins mediated biological activities and will also aid in the production of similar biological macromolecules at a significantly reduced cost compared to conventional affinity column chromatography for various biopharmaceuticals applications.

Acknowledgments

The project was supported in part by Department of Chemistry, Advanced Materials Institute, Biotechnology Center, Ogden College of Science and Engineering, Office of Sponsored Programs, and Applied Research and Technology Program at Western Kentucky University, NIH Grant Number P20 RR-16481 from the IDeA Networks of Biomedical Research Excellence (INBRE) Program of the National Center for Research Resources, and National Science Foundation Research Experience for Undergraduates (REU) grant number EAR-1004655.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2011.06.047.

References

[1] D.J.A. Crommelin, G. Storm, R. Verrijk, L. de Leede, W. Jiskoot, W.E. Hennink, Int. J. Pharm. 266 (2003) 3.
[2] BIO, 2007. Biotechnology Industry Organization. www.bio.org.
[3] S. Frokjaer, D.E. Otzen, Nat. Rev. 4 (2003) 298.
[4] R. Krishnamurthy, M.C. Manning, Curr. Pharm. Biotechnol. 3 (2002) 361.
[5] S. Hermeling, D.J.A. Crommelin, H. Schellekens, W. Jiskoot, Pharm. Res. 21 (2004) 897.
[6] M. Nagaoka, H.L. Jiang, T. Hoshiba, T. Akaike, C.S. Cho, Ann. Biomed. Eng. 68 (2010) 683.

[7] R. Raman, G. Venkataraman, S. Ernst, V. Sasisekharan, R. Sasisekharan, Proc. Natl. Acad. Sci. U.S.A. 100 (2003) 2357.
[8] K.F. Williams, C.L. Chu, M. Fannon, J.A.B. Thomas, M.A. Nugent, Ann. Biomed. Eng. 36 (2008) 2134.
[9] M. Zakrzewska, E. Marcinkowska, A. Wiedlocha, Crit. Rev. Clin. Lab. Sci. 45 (2008) 91.
[10] W. Wei, Q. Liu, Y. Tan, L. Liu, X. Li, L. Cai, Hemoglobin 33 (2009) 370.
[11] S. Barrientos, O. Stojadinovic, M.S. Golinko, H. Brem, M. Tomic-Canic, Wound Repair Regen. 16 (2008) 585.
[12] V. Kumar, M.D.I. Hassan, A.K. Tomah, T. Kashav, J. Nautiyal, S. Singh, T.P. Singh, S. Yadav, J. Biosci. 34 (2009) 899.
[13] P. Sharma, D. Rajalingam, T.K. Kumar, S. Singh, Biophys. J. 94 (2008) L71.
[14] B. Xie, E. Tassi, M.R. Swift, K. McDonnell, E.T. Bowden, S. Wang, Y. Ueda, Y. Tomita, A.T. Riegel, A. Wellstein, J. Biol. Chem. 281 (2006) 1137.
[15] B.M. Loo, J. Kreuger, M. Jalkanen, U. Lindhal, M. Salmivitra, J. Biol. Chem. 276 (2001) 16868.
[16] B.M. Loo, M. Salmivirta, J. Biol. Chem. 277 (2002) 32616.
[17] W.L. McKeehan, X. Wu, M. Kan, J. Biol. Chem. 274 (1999) 21511.
[18] X. Li, C. Tu, H. Peng, S. Yuan, F. Wu, Hua Xi Yi Ke Da Xue Xue Bao 30 (1999) 249.
[19] M.E. Gasparian, P.A. Elistratov, N.I. Drize, I.N. Nifontova, D.A. Dolgikh, M.P. Kirpichnikov, Biochemistry (Mosc) 74 (2009) 221.
[20] K.W. Hung, T.K.S. Kumar, Y.H. Chi, I.M. Chiu, C. Yu, Biochem. Biophys. Res. Commun. 317 (2004) 253.
[21] E.K. Ryu, K.J. Cho, J.K. Kim, N.J. Harmer, T.L. Blundell, K.H. Kim, Protein Express. Purif. 49 (2006) 15.
[22] M. Jutisz, M. Theoleyre, A. Colonge, R. Courrier, C. R. Hebd. Seances Acad. Sci. 257 (1963) 991.
[23] N.F. González-Cadavid, P.N. Campbell, Biochem. J. 105 (1967) 427.
[24] A.V. Troitskii, A.M. Lysenko, Biokhimiia 40 (1975) 1282.
[25] D.J. Etherington, Biochem. J. 153 (1976) 199.
[26] J.R. Giglio, A. Rossi, F.A. Leone, G. Chiericeto, J.C. Say, Biochem. J. 159 (1976) 29.
[27] K. Oda, T. Koyama, S. Murao, Biochim. Biophys. Acta 571 (1979) 147.
[28] Y. Hayashi, S. Miwa, K. Lee, K. Ksohimura, A. Kamel, K. Hamahata, M. Fujiwara, Anal. Biochem. 168 (1988) 176.
[29] S.M. Mozersky, R. Bradley, J.M. Straatsma, A. McFarline, Arch. Ophthalmol. 66 (1961) 534.
[30] M. Morrison, H.B. Hamilton, E. Stotz, J. Biol. Chem. 228 (1957) 767.
[31] S. Yokoyama, A. Obayashi, T. Osamu, E. Ichinshima, Appl. Microbiol. 28 (1974) 742.
[32] J.W. Richter, D.E. Ayer, A.W. Bazemore, N.G. Brink, K. Folkers, J. Am. Chem. Soc. 75 (1953) 1952.
[33] N.K. Boardman, S.M. Partridge, Biochem. J. 59 (1955) 543.
[34] S.O. Andrade, M.R. Silva, Biochem. J. 64 (1956) 701.
[35] D.J. Strydom, Eur. J. Biochem. 69 (1976) 169.
[36] Z. Lei, R.F. Reiser, M.S. Bergdoll, J. Clin. Microbiol. 26 (1988) 1236.
[37] P.J. Dillon, C.A. Rosen, Biotechniques 9 (1990) 298.
[38] M. Welch, S. Govindarajan, J.E. Ness, A. Villalobos, A. Gurney, J. Minshull, C. Gustafsson, PLosOne 4 (2009) e7002.
[39] M. Welch, A. Villalobos, C. Gustafsson, J. Minshull, Methods Enzymol 498 (2011) 43.
[40] D. Rajalingam, C. Loftis, J.J. Xu, T.K. Kumar, Protein Sci. 18 (2009) 980.
[41] S.C. Gill, P.H.V. Hippel, Anal. Biochem. 182 (1989) 319.
[42] D. Rajalingam, K.M. Kathir, K. Ananthamurthy, P.D. Adams, T.K. Kumar, Anal. Biochem. 375 (2008) 361.
[43] D. Rajalingam, T.K. Kumar, R. Soldi, I. Graziani, I. Prudovsky, C. Yu, Biochemistry 44 (2005) 15472.