5-2011

Innovative Purification Protocol for Heparin Binding Proteins: Relevance in Biopharmaceutical and Biomedical Applications

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INNOVATIVE PURIFICATION PROTOCOL FOR HEPARIN BINDING PROTEINS: RELEVANCE IN BIOPHARMACEUTICAL AND BIOMEDICAL APPLICATIONS

A Thesis
Presented to
The Faculty of the Department of Chemistry
Western Kentucky University
Bowling Green, Kentucky

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science

By
Sumit Batra

May 2011

INNOVATIVE PURIFICATION PROTOCOL FOR HEPARIN BINDING PROTEINS: RELEVANCE IN BIOPHARMACEUTICAL AND BIOMEDICAL APPLICATIONS

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ACKNOWLEDGEMENT

IN THE NAME OF GOD THE MOST GRACIOUS AND THE MOST MERCIFUL

I am grateful to God the Almighty, for gracing me with strength to complete this thesis. I would like to take this opportunity to record my appreciation for those who had been so significantly influential and responsible for my achievement in order to complete this thesis. First and foremost, I would like to express my sincerest gratitude to my research supervisor Dr. Rajalingam Dakshinamurthy for his guidance, knowledge, constant support and detailed and constructive comments in assisting for the completion of my research. I attribute the level of my master's degree to his encouragement and effort, and without him this thesis, too, would not have been completed or written.

I would also like to thank Dr. Eric Conte our collaborator for his support and encouragement.

I would also like to thank Dr. Cathleen Webb, Department Head, Department of Chemistry, and my committee member Dr. Chad Snyder. They have been constantly helpful and I sincerely appreciate it. Also, I would like to thank the faculty and staff of the Department of Chemistry for their support in my graduate career at Western Kentucky University.

I wish to thank all my colleagues for their support.

Finally, I dedicate my thesis to my family Mr. Subhash Chander Batra, Mr. Ramesh Chand Batra, Mrs. Madhu Batra, Ms. Tanu Batra, Ms. Nitika Batra, Mr. Ashish Sareen and Ms. Pooja Patel who supported me the most during my challenging times here at Western Kentucky University.

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Sumit Batra May 2011 61 Pages

Directed by: Dr. Rajalingam Dakshinamurthy

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Heparin binding (HB) proteins mediates a wide range of important cellular processes, which makes this class of proteins biopharmaceutically important. Engineering HB proteins could bring many advantages, but it necessitates cost effective and efficient purification methodologies compared to the currently available methods. One of the most important classes of heparin binding protein is the fibroblast growth factors (FGFs) and its 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. This approach is an alternative to conventional affinity column chromatography, which exhibit several disadvantages, including time-consuming experimental procedures and regeneration and results in high cost for production of recombinant proteins. Authenticity of the purified proteins was verified by SDS-PAGE and MALDI mass spectrum analysis. Results of the heparin binding chromatography and steady state fluorescence experiments showed that the FGF-1 and the D2 are in a native biologically active 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.

INTRODUCTION

Proteins

Proteins, from the Greek word proteios, meaning, the class of organic compound that are present in and vital to every living cell. In the form of skin, hair, callus, cartilage, muscles, tendons and ligaments, proteins hold together, protect, and provide structure to the body of a multi-celled organism [1]. In the form of enzymes, hormones, antibodies, and globulins, they catalyze, regulate, and protect the body chemistry [2-6]. The word protein was first mentioned in a letter sent by the Swedish chemist Jöns Jakob Berzelius to Gerhardus Johannes Mulder on July 10, 1838. In the eighteenth century Antoine Fourcroy and others then recognized proteins as a distinct class of biological molecules. Proteins were distinguished by the molecules ability to coagulate or flocculate under treatments with heat or acid. During that time, examples of protein included albumin from egg whites, blood, serum albumin, fibrin, and wheat gluten. Dutch chemist Gerhardus Johannes Mulder carried out elemental analysis of common proteins and found that nearly all proteins had the same empirical formula. Mulder went on to identify the products of protein degradation such as the amino acid leucine for which he found a molecular weight of 131 Da [7-8].

Proteins are then discovered to be the large biological molecules with molecular weight upto few million Daltons [9]. Proteins consist of one or more polypeptides typically folded into a globular or fibrous form in a biologically functional way [10]. A polypeptide is a single linear polymer chain of amino acids bonded together by peptide bonds between the carboxyl and amino groups of adjacent amino acid residues [11]. The sequence of amino acids in a protein is defined by the sequence of a gene, which is

encoded in the genetic code [12]. There are only 20 standard amino acids that exist in living organism. Sometimes these amino acids are chemically modified in the protein after protein synthesis [11]. In total the number of different proteins, which it is possible to produce from 20 amino acids is enormous. For example for 10 amino acid sequence it is possible to have 20 different sequences, which is approximately equal to 10 trillions of different structures [9]. The study of proteins and their function is central to understanding both cells and organisms. There are a few reasons why proteins are important in biology which are; protein serve as a catalyst that maintain metabolic processes in the cell and also they serve as structural elements both within and outside the cell [7, 10-11].

A distinguishing property of protein is their ability to fold, which determines the active conformation of a protein [10]. Some proteins fold into highly rigid structure or called as “single structure”, and some proteins undergo large rearrangements in conformations [13]. This conformational change is often associated with “signaling event” which means when the protein comes in contact with other required molecule it changes into active conformation to carry out necessary biological functions [7, 14-16]. Active biological conformation is essential for proteins to carry out their function. For example, enzymes are proteins that catalyze biochemical reactions. The function of an enzyme relies on the structure of its active site, a cavity in the protein with a shape and size that enable it to fit the intended substrate very snugly [17]. It also has the correct chemical properties to bind the substrate efficiently. The active site also contains certain amino acids that are involved in the chemical reaction catalyzed by the enzyme [18]. Although not all proteins are enzymes, but still, all in some way rely on molecular

recognition in order to perform their functions. Transport proteins such as hemoglobin must recognize the molecules they carry, receptors on the cell surface must recognize particular signaling molecules, and transcription factors must recognize particular DNA sequences and antibodies must recognize specific antigens [5-6, 19-20]. The functional integrity of the cell depends critically on protein-protein interactions, particularly on the formation of multi-protein complexes [21-22].

Protein Biosynthesis

Protein biosynthesis sometimes referred to, as translation is a very complex multistep process that involves firstly synthesis of amino acids and transcription, which are then used for translation [7, 23]. Protein biosynthesis differs in prokaryotes and eukaryotes [13]. The processes by which proteins are synthesized biologically have become one of the central themes of molecular biology.

The sequence of amino acid residues in a protein is controlled by the sequence of the DNA as expressed in messenger RNA at ribosome [23, 11, 13]. Protein synthesis is the creation of proteins using DNA and RNA. In short the RNA is transcribed in the nucleus and after completion of this process is transported to cytoplasm and translated by the ribosomes [10-11, 13]. Proteins can often be synthesized directly from genes by translating mRNA. When a protein is harmful and needs to be available on short notice or in large quantities, a protein precursor is produced. A pro-protein is an inactive protein containing one or more inhibitory peptides that can be activated when the inhibitory sequence is removed by proteolysis during posttranslational modification [25-26]. Figure 1 represents the series of events occur during protein synthesis.

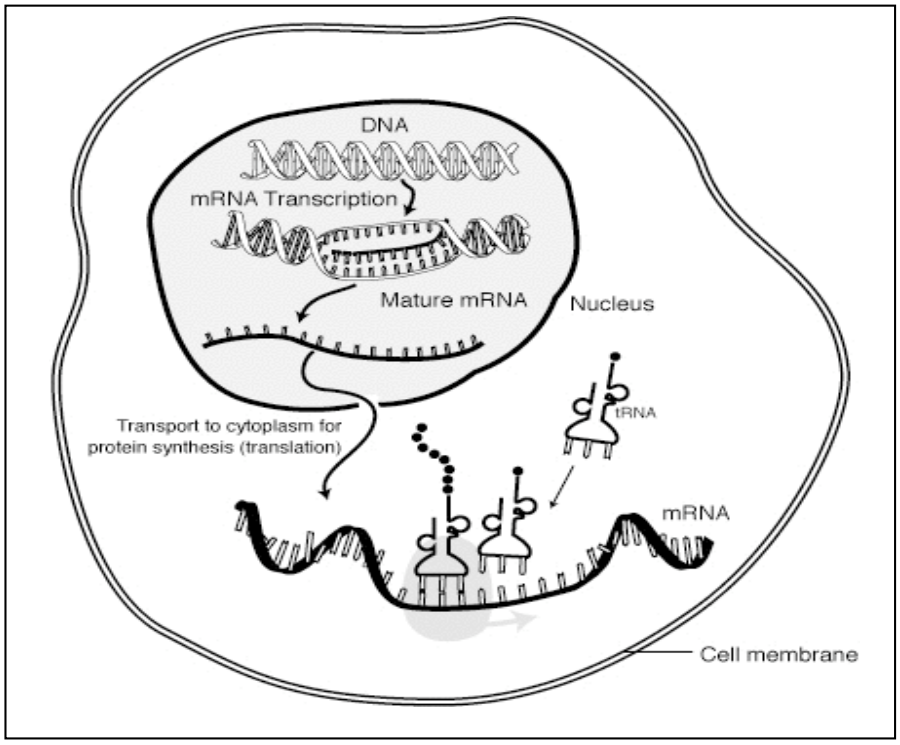


Figure 1: Protein biosynthesis

(Source: Wikipedia)

Structure of Protein

Generally, proteins contain from a range of 50 to 1000 amino acid residues per polypeptide chain. A peptide bond is an amide bond formed by the reaction of a α-amino group (NH_2) of one amino acid with the carboxyl group (COOH) of another, as shown below in Figure 2 [11].

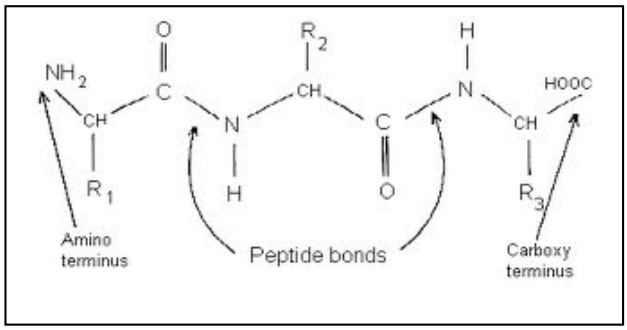


Figure 2: Peptide bond

(Source: Birkbeck College 1995)

Proteins are not an entirely rigid structure [14]. The shape into which a protein naturally folds is known as its native conformation [11]. Many proteins can fold unassisted; simply through the chemical properties of their amino acids, others require the aid of molecular chaperones to fold into their native states [27]. Proteins may shift between several related structures while they perform their biological function, these functional rearrangements, these tertiary and quaternary structures are referred to as conformations and transition between them are called conformational changes [7, 14, 28]. The structure of proteins can be divided into four parts. They are primary, secondary, tertiary and quaternary structures [11, 29-30]. The primary structure is responsible for the function of a protein [31]. This structure is composed of various amino acids held together by peptide bonds. Proteins may have one or more polypeptide chains. Each polypeptide in a protein has amino acids linked with each other in a specific sequence and it is this sequence of amino acids that is said to be the primary structure of that protein [32]. The repeating amino acid sequences stabilized by hydrogen bonds results in so-called secondary structures [31]. Most common e.g. are alpha helix, beta sheets and turns. Proteins also undergo extensive folding into complex three-dimensional geometry

called as tertiary structure that is maintained by hydrogen bonds, disulfide bonds, ionic bonds and hydrophobic interactions [32]. It coils and folds in such a way that the hydrophobic side chains are held interior and the hydrophilic groups are held outside. This arrangement gives stability to the molecule [33]. When these different protein subunits joined together they form quaternary structure known as protein complex [32-33]. Different protein structure has been shown below in figure 3.

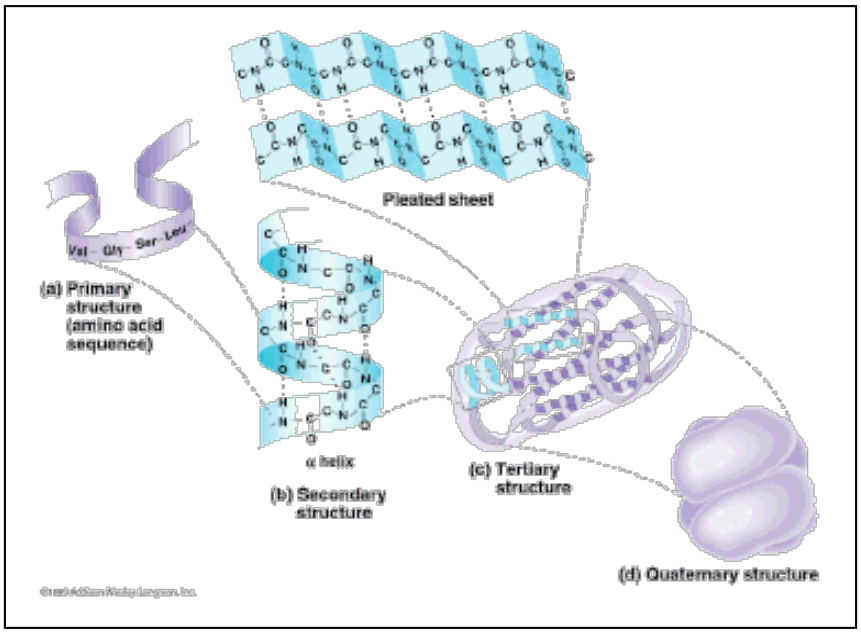


Figure 3: Structure of protein

(Source: Campbell, N. 5th edition)

Protein molecules have been crystallized and examined by X-ray diffraction crystallography, a technique that allows the visualization of the precise three-dimensional positioning of atoms in relation to each other in a crystal [32-34]. Nuclear magnetic resonance (NMR) technique can also be used to determine the structure of protein, only 9% of protein structure has been known by using NMR [7, 34]. Secondary structure composition of a protein can be determined by circular dichroism [35]. Cryo-electron

microscopy has recently been used to determine the protein structure to high resolution [36].

Biopharmaceuticals: The New Era

The roots of biotechnology can be traced back into antiquity when brewing, cheese making and baking were discovered. An important step for the humanity and science of biology was the invention of the microscope in 1670s. Antonie Van Leeuwenhoek was the first person to see the microbes with his invention [37-38]. Edward Jenner invented the smallpox vaccination and another scientist Louis Pasteur created a process of controlled heating for milk, beer, etc, without affecting flavors [39-41]. The advances in the science of biology in the 18^{th} and 19^{th} centuries allowed the development of microbial bases for the brewing and baking process [42]. The post second world war period saw the application of pharmaceutical biotechnology and increased the knowledge of the molecular basis of biological systems [42]. This accumulation of knowledge led to the development of new techniques with industrial applications.

Since the discovery that the human body is composed of cells and proteins that are susceptible to but can also fight off pathogenic microbes has challenged our imagination to develop biopharmaceuticals. The term "biopharmaceutical" was first used in 1980 and was used to describe a class of therapeutic drugs produced by modern biotechnological techniques, specifically via genetic engineering (1970) or by hybridoma technology (1975) (in case of monoclonal antibodies) [43]. These mainly include proteins (including antibodies), nucleic acids (DNA, RNA or antisense oligonucleotides) used for therapeutic or diagnostic purposes [43-45].

The first biopharmaceutical substance approved for therapeutic use was biosynthetic 'human' insulin made via recombinant DNA technology. Also “referred” to as rHI, under the trade name Humulin [43, 45]. Since then, different biopharmaceuticals products have been developed like blood factors used for treatment of haemophilia, thrombolytic agents used to treat thromboembolism, hematopoietic growth factors used in the treatment of blood related diseases, interferons used in the treatment of leukemia and multiple sclerosis, growth hormone, colony stimulating factors, growth factors like heparin binding proteins, interleukin based products etc. [46-55].

While most biopharmaceuticals approved are intended for human use, a number of products developed are used for veterinary application, e.g., recombinant bovine growth hormone used to increase milk yields from dairy cattle [56]. Till mid 2002, 120 biopharmaceutical products has been approved for marketing in USA and/or European Union which represents global biopharmaceutical market of $15 billion [43]. Since then the impact of biopharmaceuticals on global healthcare and the economy is substantial and growing rapidly. 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 [57-58]. The biopharmaceutical industry has invested $ 65.2 billion in research and development of new medicines in the year 2008 [59]. Figure 4 gives the rise in biopharmaceutical market.

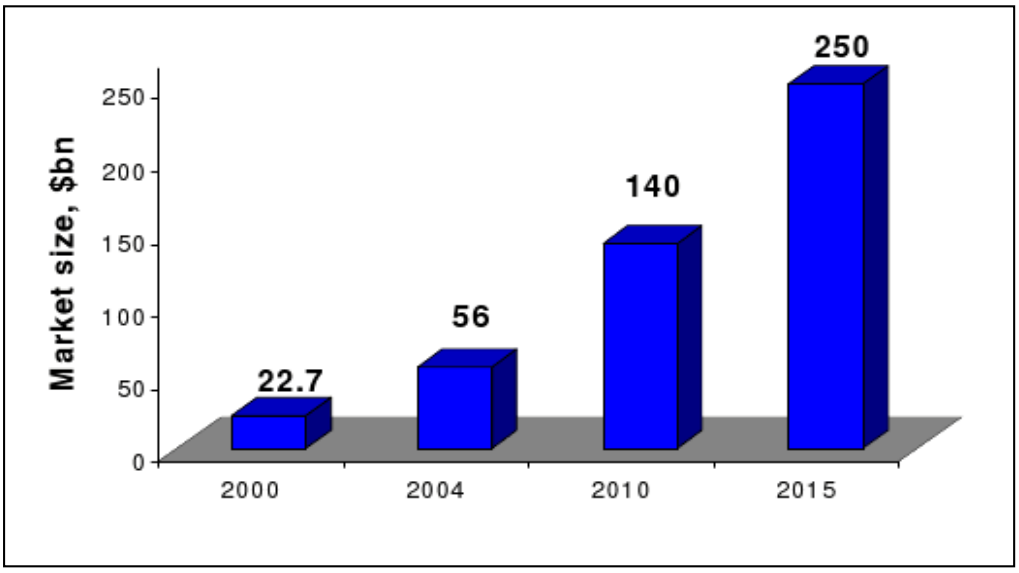


Figure 4: Biopharmaceutical market overview

(Source: Business Insight 2006)

The total number of patents granted for biopharmaceuticals has risen significantly since the 1970s. In 1978 the total patents granted was 30, which then climbed to 15,600 in 1995, and by 2001 there were 34,527 patent applications [60].

Heparin Binding Proteins

One important class of protein biopharmaceuticals are heparin binding proteins which consist of a wide range of protein families including epidermal growth factors (EGF's), fibroblast growth factors (FGF's), connective tissue growth factors (CTGF), midkines, etc. [61]. Heparin (highly sulfated glycosoaminoglycans) and heparan sulfate glycosaminoglycans (HSGAGs) (present on the surface of all the adherent cells) mediate a wide variety of complex biological processes by binding to this specific class of more

than 200 extracellular proteins [62]. In many instances these interactions have been demonstrated to require distinct HS chemical characteristics. As one extreme example, the binding of antithrombin III to heparin and HS requires a specific pentasaccharide sequence containing a unique 3-O sulfate on the central glucosamine residue [62]. HSGAGs are complex acidic polysaccharides that are characterized by a disaccharide repeating unit of α-d-glucosamine (1 → 4) linked to uronic (α-l-iduronic/β-d-glucuronic) acid showed in figure 5 [61, 63]. This repeating disaccharide subunit provides the structural heterogeneity within the HSGAG polysaccharide. The sulfates groups or sites for sulfation present on the HSGAG make it one of the most acidic biopolymer. Similar to DNA and fibrous proteins like collagen, HSGAGs adopt a helical structure. The conformational flexibility due to iduronic acid enhances the specificity of HSGAG oligosaccharides binding to proteins [61].

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 [61-64]. While it has been well accepted that interactions with HSPGs play critical roles in mediating growth factor signaling to cells, it is not clear what defines the specificity of growth factor-HS interactions or how unique and exclusive those interactions are [63].

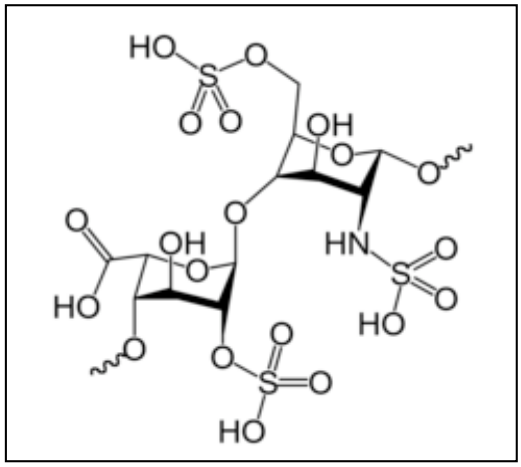


Figure 5: Heparin sulfate

(Source: Glycosan Biosystems)

Fibroblast Growth Factors and its Receptor

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 [65-66]. FGFs are also used as biopharmaceutical drugs to improve wound healing caused by metabolic diseases such as obesity, diabetes, infection, chronic liver failure, malnutrition, and second-degree burns [65-66]. FGFs exert their biological activity by binding to tyrosine kinase receptors (FGFRs) on the cell surface in the presence of HSPGs [67]. The signaling involves ligand-induced receptor dimerization and autophosphorylation, followed by a downstream transfer of the signal showed in figure 6 [68]. However, in the absence of cell surface proteoglycan, heparan sulphate results in a dramatic decrease in FGFR activity for proliferation, mitogenesis, wound healing, and neuro-vascularization [67-69]. FGFRs contain an extracellular

domain, a short transmembrane domain, and a cytoplasmic tyrosine kinase domain. The FGFR consists of extracellular immunoglobulin-type domain having three structural subdomains named D1, D2 [Fig 7], and D3. The D2 subdomain contributes to the ligand (FGF) binding [67-69].

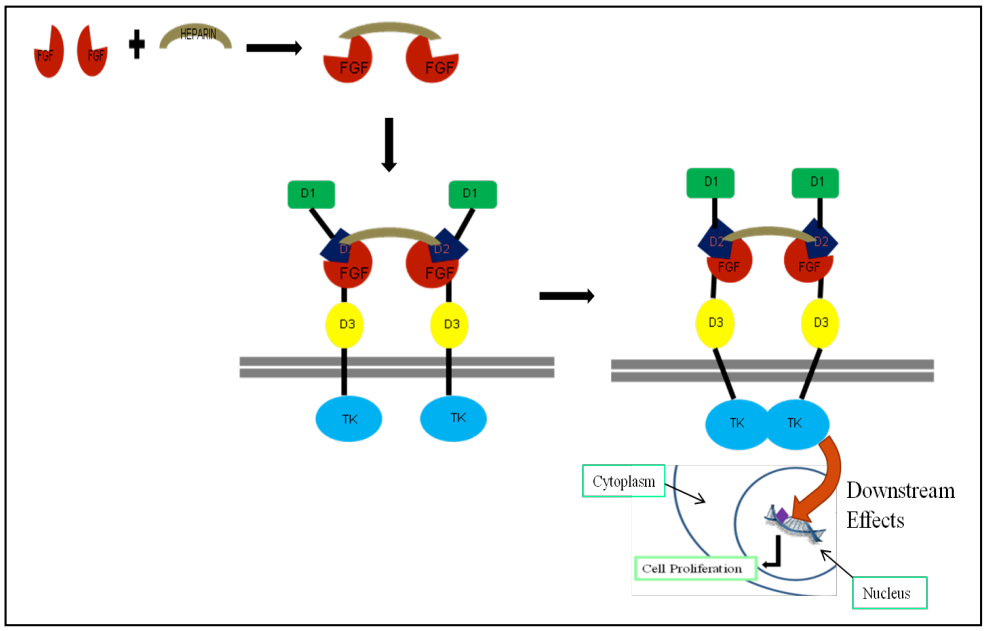
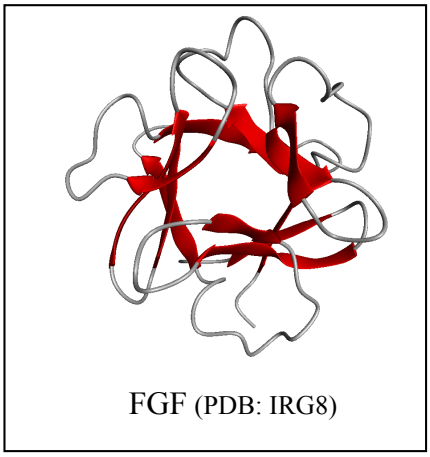


Figure 6: Signaling mechanism of FGF

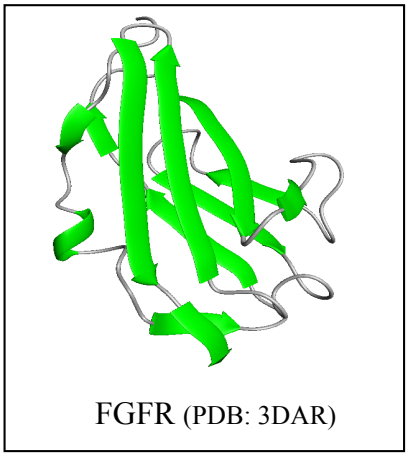
Fibroblast growth factor was first found in pituitary extracts by Armelin and then was also found in a cow brain extract [Fig. 7] [70-71]. On further fractionation two slightly different forms were extracted using acidic and basic pH and isolated that were named "acidic fibroblast growth factor" (FGF1) and "basic fibroblast growth factor" (FGF2). These proteins had a high degree of amino acid identity but were determined to be distinct mitogens [72].

The involvement of these proteins in several biological processes stated above showed their importance for biopharmaceutical and research purposes (i.e., to understand

the structural- functional relationship of these proteins) and encouraged biochemists in devising newer techniques for purification of these biomolecules.



FGF (PDB: IRG8)



FGFR (PDB: 3DAR)

Figure 7: Three dimensional structure of FGF-1 and D2 domain of FGFR

Protein Purification

To enable functional and structural studies it is required to obtain the protein in highly pure form because presence of even minute quantities of contaminants can severely disturb the structure determination or otherwise can destabilize the sample. In general protein purification varies from simple one step precipitation procedure to a series of complex processes that are intended to isolate a single type of protein from a complex mixture [7, 73]. In most of the protein purification protocols the starting material is usually a biological tissue or a microbial culture which are induced to overexpress desired protein of interest. [30, 74]

The various steps in the purification process allows the protein to get freed from the matrix and then gradually as the purification goes on separate the protein and non-protein parts of the mixture, and finally separate the desired protein from all other

proteins [7]. Separation of one protein from all others is typically the most laborious aspect of protein purification. Pure proteins are then analyzed by different techniques for homogeneity and biological activity. Proteins are separated on the basis of differences in their size, physico-chemical properties, binding affinity and biological activity [30, 73-74]. Purpose of protein purification is to produce a relatively large quantity of purified proteins for various uses. Examples include the preparation of commercial products such as enzymes (e.g. lysozyme), nutritional proteins, and certain biopharmaceuticals (e.g. insulin) [30, 75-76].

Today scientists are using recombinant DNA technology to produce large quantities of desired protein if present in very less quantity in natural source. Recombinant expression also allows the protein to be tagged, e.g. by a His-tag, to facilitate and reduce the steps involved in purification [30, 61, 77].

Different proteins can be purified by different ways like precipitation, ultracentrifugation, chromatographic method based on size, charge, hydrophobicity, affinity, metal binding, HPLC [30, 73-74, 78]. Here we are going to discuss about two important heparin binding protein human FGF-1 and D2 domain of FGFR which are traditionally purified by using affinity chromatographic methods [Fig. 8].

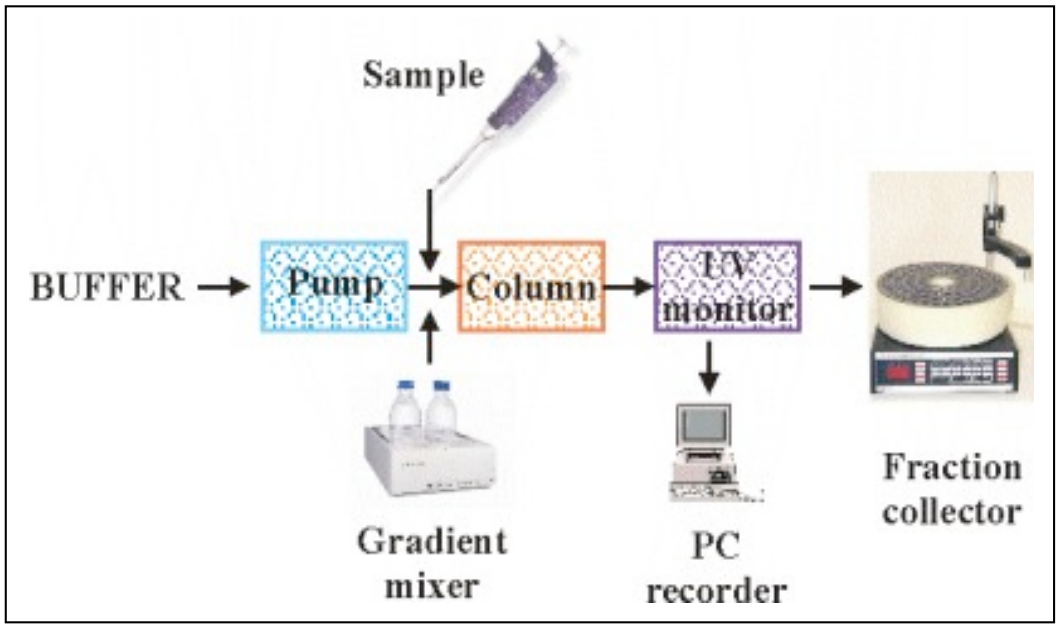


Figure 8: Affinity chromatography

(Source: Proteincrystallography.org)

Objective of Study

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 [79-81]. Proteins are a novel type of compound in comparison to traditional small molecules. They present new and significant challenges to the realization of their full potential as therapeutic agents [79]. FGF-1 and D2 domain of FGFR are those proteins which, due to their intrinsic property to adopt alternative conformations during protein expression, folding, unfolding and aggregation during isolation presents numerous challenges for their isolation/separation, as the separated proteins become essentially irrelevant from a

biochemical perspective, which in turn leads to the use of sophisticated methodologies with multiple steps. These challenges result in higher cost for production of these recombinant proteins, which currently limits their effective application [80-81].

The objective of this study was to devise a novel simple, cost effective and scalable method for large scale recombinant protein purification. Low cost protein purification methods are in high demand for mass production of low selling price biopharmaceuticals that play an important role in the upcoming bio-economy.

This new technique is based on ion exchange chromatography where inexpensive amberlite weak cation exchange resin has been used in place of a heparin/Ni-NTA resins for purification of fibroblast growth factor and D2 domain of fibroblast growth factor to achieve pure protein in biologically active conformation. Ion exchange chromatography has been known to be an efficient technique for separation of proteins [82]. Ion exchange chromatography is based on the principle of interaction between oppositely charged protein molecules (FGF-1 and D2 domain) and solid phase molecules (IRC 50) [82-83].

MATERIALS AND METHODS

Materials

E. 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. Lysozyme was purchased from Sigma-Aldrich. All other chemicals used in this study were of American Chemical Society reagent grade.

Methods

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

Human FGF-1 residues 14-154 (GenBank # X59065) and D2 domain of human FGFR, residues 145-259 (GenBank # AK026508) from full length FGFR, cDNAs were made by procedures essentially as previously described and cloned into a pJExpress414 vector (DNA2.0) and transformed into BL21(DE3) E. coli cells for the overexpression [84]. The gene sequences were optimized using DNA2.0 codon bias algorithm to maximize the yield of the proteins, prior to cloning [85]. *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 hours 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.

Purification of FGF-1 using on-column heparin affinity chromatography

The protein was expressed as soluble form and the purification was achieved by heparin sepharose affinity column [Fig. 9]. The soluble protein was loaded onto heparin sepharose affinity column. FGF bound to heparin sepharose column was washed with 4 bed volumes each (~100 mL) of two different elution buffers 20 mM phosphate buffer (pH 7.2) and 20 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 [87-90].

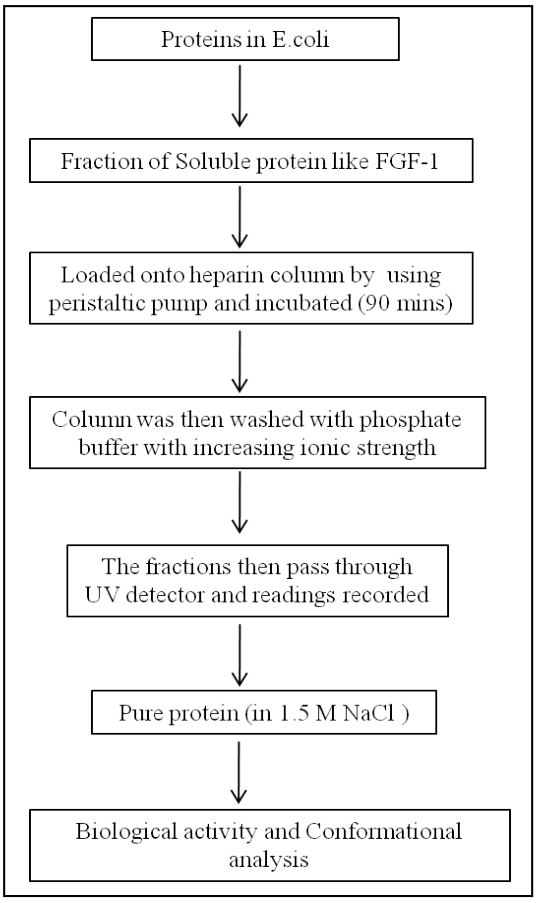


Figure 9: Schematic representation of FGF purification by affinity chromatography

Purification of D2 domain using on-colum heparin and Ni-NTA affinity chromatography

The proteins expressed in the inclusion bodies were dissolved and extracted in 20 mM phosphate (pH 7.5), containing 300 mM NaCl and 8 M urea at room temperature. Protein purification was achieved in two steps [Fig. 10]. Inclusion bodies dissolved in 20 mL of 8 M urea were loaded onto Ni-NTA nickel affinity column. D2 domain bound to the column was washed with 10 bed volumes (~100 mL) of the elution buffer [20 mM phosphate buffer (pH 7.5) containing 300 mM NaCl, 20 mM Imidazole, and 50 mM ammonium sulfate]. Finally, the refolded protein was eluted with an elution buffer containing 500 mM imidazole. Further purification was achieved using heparin sepharose affinity column. The partially pure protein obtained from Ni-NTA column was loaded on a heparin sepharose column and washed with a 20 mM phosphate buffer (pH 7.2) to remove the contaminating proteins. D2 domain bound to heparin sepharose was eluted with 1.5 M NaCl [91-92].

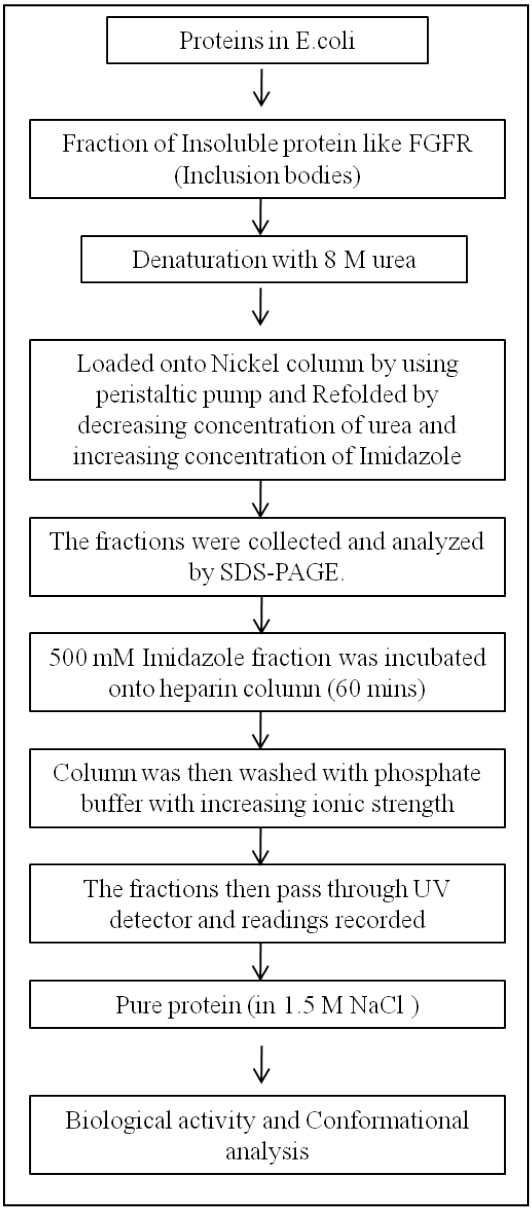


Figure 10: Schematic representation of FGFR purification by affinity chromatography

Purification of recombinant FGF-1 using Amberlite weak cation exchange resin

Purification of FGF-1 was achieved by applying clear soluble fraction of bacterial cell lysate onto an IRC column packed in 15 mL centrifuge tubes and allowed to stand for 45 minutes, centrifuged at 12000 rpm for 10 minutes and the supernatant 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 12000 rpm for 10 minutes the supernatant fractions were then collected separately. FGF-1 was further eluted from 8 the resin by washings with 10 mM phosphate buffer (pH 7.2) containing 1.5 M NaCl, incubated for 30 minutes, centrifuged at 12000 rpm for 10 minutes, and collected as separate fractions. Throughout the purification, the temperature was maintained at 4 °C. The protein was further desalted and concentrated by ultrafitration 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 (Σ280=17420 M-1 cm-1) calculated from the amino acid sequence of FGF-1 [86]. The homogeneity and authenticity of the protein was assessed by using SDS-PAGE, MALDI-TOF mass spectrum analysis, heparin binding affinity and steady state fluorescence.

Purification of recombinant D2 domain of FGFR using Amberlite weak cation exchange resin

Purification of the D2 domain was achieved by denaturation of the inclusion bodies (insoluble fraction of bacterial cell lysate) using 5 mL of denaturation buffer (8 M urea, 10 mM Tris buffer) to a final concentration of 2 mg/mL protein. The suspension was then

centrifuged for 10 minutes at 12,000 rpm and the supernatant was loaded onto the IRC column packed in a 15 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 method from 8 to 0 M over a 2 hours period. After refolding, the protein was allowed to stand for 60 minutes, centrifuged at 12,000 rpm for 5 minutes and collected. The resin was then washed thrice with 10 mL of 10 mM phosphate (pH 7.4), centrifuged, and fractions were collected separately. Further elution was done by washing the resin twice with 10 mM phosphate (pH 7.2) containing 1.5 M NaCl, incubated for 40 minutes, centrifuged and collected as separate fractions. The protein was further 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 (Σ280=24075 M-1 cm-1) 9 calculated from the amino acid sequence of the D2 domain [86]. Purified D2 domain was assessed for homogeneity and authenticity by using SDS-PAGE, MALDI-TOF mass spectrum analysis, heparin binding affinity and steady state fluorescence.

Heparin affinity Chromatography

The purified protein (FGF-1 or D2 domain) was loaded onto heparin sepharose column and incubated for 90 mins. The column was then washed with 10 mM phosphate buffer (pH 7.2). The column was further washed 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. Throughout the purification the temperature was maintained at 4 °C. The authenticity of the protein was checked by SDS-PAGE.

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.

RESULTS AND DISCUSSIONS

Low cost protein purification methods are in high demand for production of low cost biopharmaceuticals which shows the importance of biomolecules in therapeutics. The driving force for devising new inexpensive purification and characterization techniques for biopharmaceuticals is because of their potential to cure number of diseases with fewer side effects because of their specificity. In therapeutics biopharmaceuticals drugs structurally mimics a number of compounds found within the body for example, cytokines, enzymes, hormones, clotting factors, vaccines, monoclonal antibodies, antisense drugs, and peptides which require to produce these compounds in pure form for research purposes, i.e to understand the structural-functional relationship of these compounds which will be helpful in fabrication or synthesis of newer drugs which help to cure diseases caused by inactivity or hyperactivity of these compounds [87].

Overexpression of FGF-1 and D2 domain

The FGF-1 construct used in the present study is 140 amino acids long from the full length human FGF-1. SDS-PAGE of the bacterial cells induced by IPTG showed that most of the protein was expressed in soluble form [Fig. 11A]. The D2 domain construct used in the present study is 114 amino acids long from the full length human FGFR. SDS-PAGE of the bacterial cells induced by IPTG showed that most of the expressed protein is trapped as inclusion bodies [Fig. 11B].

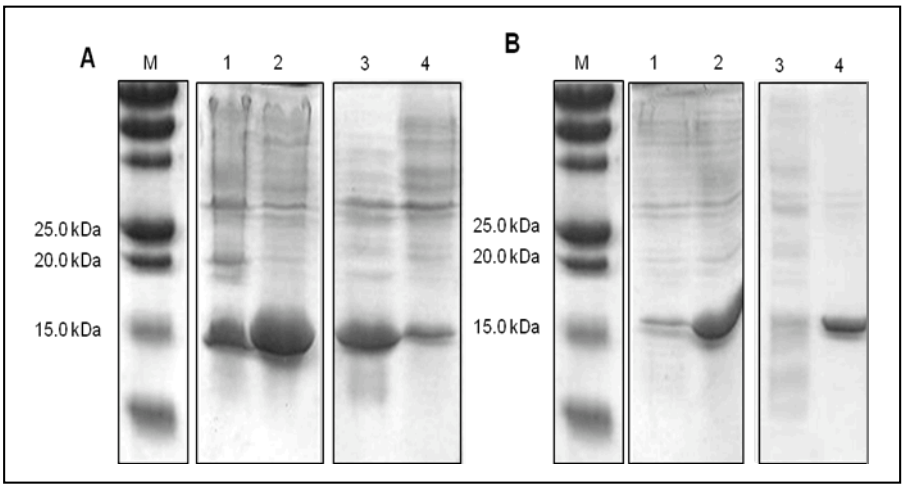


Figure 11: **A-** SDS-PAGE depicting the expression and purification of FGF-1 from *E. coli.* Lane M represents the molecular weight marker; lane 1, uninduced sample; lane 2, induced sample; lane 3, lysate of induced sample soluble fraction; lane 4, lysate of induced sample insoluble fraction. **B-** SDS-PAGE 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.

Purification of the FGF-1 using on-column heparin affinity chromatography

The purification of the recombinant FGF-1 was facilitated by using heparin affinity resin. FGF-1 was bound to heparin quite strongly and the unbound contaminating protein was eliminated by washing the resin with 100 mL of wash buffer [10 mM phosphate buffer (pH 7.2)]. Other impurities which nonspecifically bind to heparin resin were further removed by washing the column with 10 mM phosphate buffer (pH 7.2) with 0.75 mM NaCl. FGF-1 was eluted with 10 mL phosphate buffer (pH 7.2) containing 1.5 M sodium chloride. SDS-PAGE of the purified FGF-1 sample yielded an intense single band corresponding to a molecular mass of ~16 kDa with about 98% purity [Fig. 12].

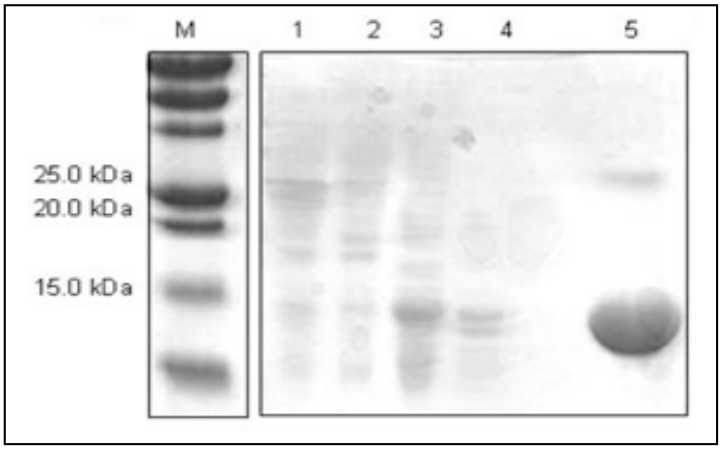


Figure 12: SDS-PAGE depicting the purification of FGF-1 using heparin column. Lane M, represents the molecular weight marker; lane 1, depicts protein bands contained in fraction collected as flow through; lane 2 and 3, depicts protein bands contained in fractions eluted in 10 mM phosphate buffer; lane 4, depicts protein bands contained in fractions eluted in 10 mM phosphate buffer containing 0.75 M NaCl; and lane 5, depicts protein bands contained in fraction eluted in 10 mM phosphate buffer containing 1.5 M NaCl.

Purification of the D2 domain using on-column Ni-NTA and heparin affinity chromatography

The inclusion bodies were denatured using 8 M urea. Urea basically alters the water structure and dynamics and thereby diminishes the hydrophobic effects by encouraging solvation of hydrophobic groups. In addition, urea also weakens the water structure which then competes for intraprotein electrostatic interactions. Ureal also interacts directly with polar residue and peptide backbone, thereby stabilizes the nonnative conformation. In this ways urea denatures the protein both directly and indirectly as well [88]. The refolding of the recombinant D2 domain was facilitated by incubating the denatured protein fraction with Ni-NTA affinity resin and refolded using a linear gradient (8 M to ~ 0 M) by washing with 20 mM phosphate buffer (pH 7.5) containing 20 mM, 50 mM, 50 mM, 100 mM imidazole concentration and finally the protein was eluted in 500 mM imidazole concentration. Increasing concentration of imidazole has been used for two main purposes; firstly, to reduce the concentration of urea to get the protein in native conformation and secondly, to elute the protein by competing for positively charged sites on Ni-NTA column. Further purification was facilitated by incubating 500 mM imidazole fraction onto heparin affinity resin. The impurities were eluted in 10 mM phosphate buffer (pH 7.2) and 10 mM phosphate buffer (pH 7.2) containing 0.75 mM NaCl. The D2 domain was eluted with 10 mL phosphate buffer (pH 7.2) containing 1.5 M sodium chloride and yielded an intense single band (purity ~ 98 %) on SDS-PAGE corresponding to a molecular mass of ~ 13 kDa [Fig. 13].

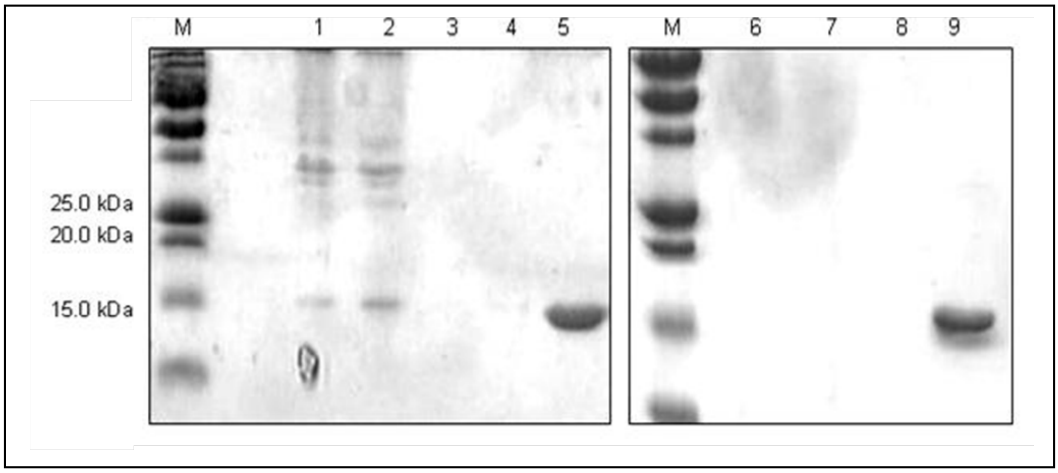


Figure 13: SDS-PAGE depicting the purification of D2 domain using nickel (lanes 1-5) and heparin affinity (lanes 6-9) columns. Lane M, represents the molecular weight marker; lane 1-5, depict protein bands contained in fractions eluted in 20 mM, 50 mM, 50 mM, 100 mM, 500 mM Imidazole concentration, respectively from the nickel column. Lane 6-9, depicts fractions collected from heparin column. Lane 6, depicts protein bands contained in fraction collected as flow through; lane 7, depicts protein bands contained in fraction eluted in 10 mM phosphate buffer; lane 8, depicts protein bands contained in fraction eluted in 10 mM phosphate buffer containing 0.75 M NaCl concentration; and lane 9, depicts protein bands contained in fraction eluted in 10 mM phosphate buffer containing 1.5 M NaCl.

Traditional synthetic heparin affinity and Ni-NTA affinity chromatography methods are effective with a high yield of 32 mg and 20 mg per litre of FGF-1 and D2 domain respectively, they also exhibit several disadvantages including, time-consuming experimental procedures for purification, and regeneration, a requirement of expensive affinity resins and difficulties in maintenance of the resins as bacterial growth causes it to degrade [89-95]. These disadvantages associated with previously reported procedures results in high cost of commercially available purified proteins which have many biopharmaceuticals applications.

In order to overcome the disadvantages of affinity chromatography and to reduce the cost of the purified protein, in the present study we have devised an inexpensive and efficient off-column purification method to be used as an alternative to conventional affinity chromatography methods using Amberlite cation exchange resin.

Cation exchange chromatography is based on principle of adsorption of the molecules to the solid phase which is driven by the ionic interaction between positively charged protein molecules and negatively charged solid phase particles [Fig. 14]. The strength of the interaction is determined by the number and location of the charges on the molecule and on the functional group. The protein molecules are then eluted by varying the pH close to pI of the protein or increasing the salt concentration [82-83]. By increasing the salt concentration and varying the pH the molecules with the weakest ionic interactions start to elute from the column first. Molecules that have a stronger ionic interaction require a higher salt concentration and elute later in the gradient [82-83]. The interaction between the protein and the solid phase depends on several factors such as net charge, charge distribution, ionic strength, pH of solvent, nature of ions, etc [83].

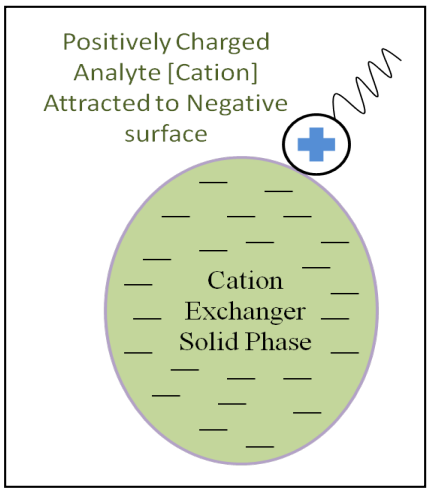


Figure 14: Cation exchange chromatography

Amberlite ion exchange resins for protein purification were first used for the removal of pectinmethylesterase from a preparation of pectinpolygalactonurase using an amberlite IR 100, a polystyrene cation exchanger (sulfonic acid resin) [74]. Since then amberlite cation exchange resin IRC 50 has been used for successful chromatographies of a number of proteins, such as growth hormones, cytochrome c, lysozymes, proteases and other hormones and proteins etc. [96-108].

Purification of the FGF-1 using IRC 50 resin

The purification of the recombinant FGF-1 was facilitated by using an amberlite weak cation exchange resin IRC [Fig. 15]. 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 nonspecifically bind to IRC resin were further removed by washing the column with 10

mM Tris buffer (pH 7.4) with 50 mM NaCl. FGF-1 was eluted with 10 mL phosphate buffer (pH 7.2) containing 1.5 M sodium chloride. SDS-PAGE of the purified FGF-1 sample yielded an intense single band corresponding to a molecular mass of ~ 16 kDa with ~ 98% purity [Fig. 16].

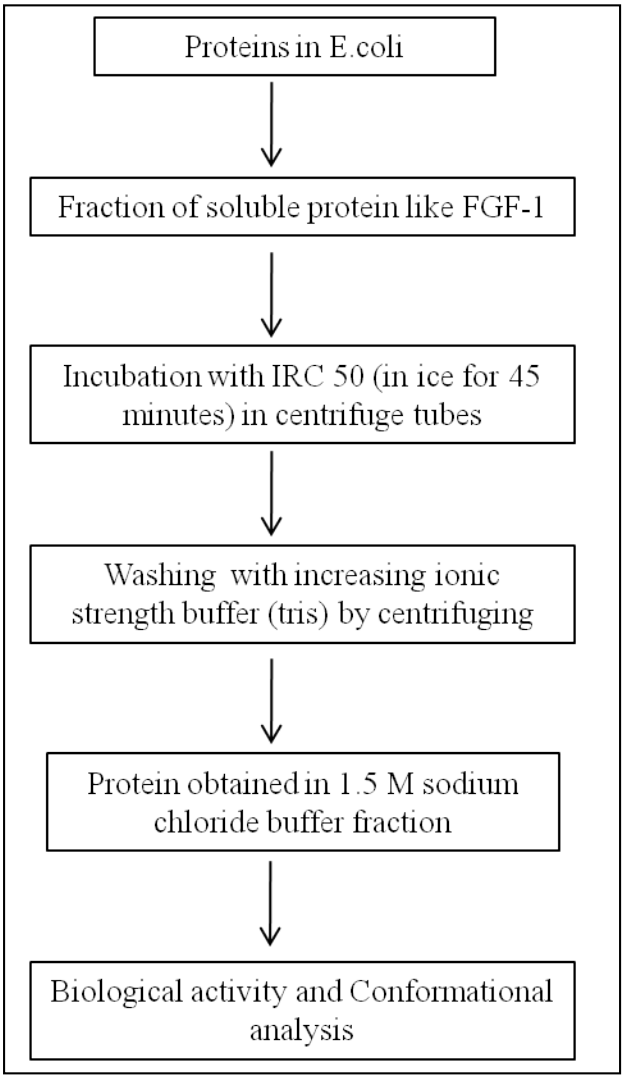


Figure 15: Schematic representation of FGF purification by IRC resin

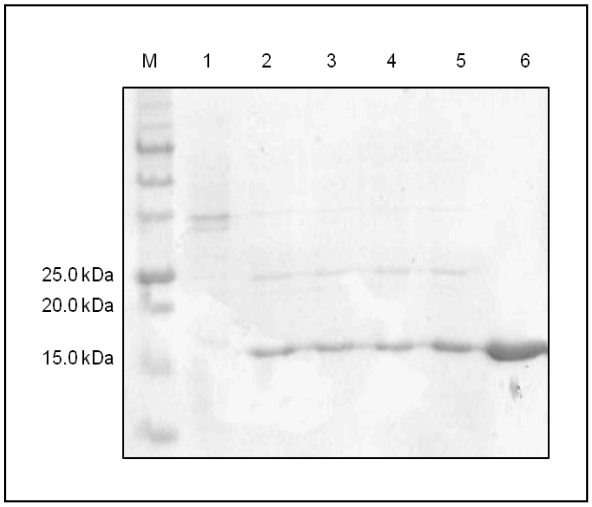


Figure 16: SDS-PAGE of fractions collected with buffer containing varying concentration of sodium chloride. Lane M represents molecular weight marker; lane 1, represents flow through; lane 2, depicts protein bands contained in fraction eluted in 10 mM Tris buffer; lanes 3-5, depicts protein bands contained 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.

Purification of the D2 domain of FGFR using resin

The refolding of the recombinant D2 domain was facilitated by incubating the denatured protein fraction with affinity IRC resin and refolded using a linear gradient (8 M to ~ 0 M) by 10 mM Tris buffer (pH 7.4) containing 50 mM NaCl [Fig. 17]. 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. Repeated washing of the column with the wash buffer also served as an effective protocol to remove any residual denaturant. The D2 domain was eluted with 10 mL phosphate buffer (pH 7.2) containing 1.5 M sodium chloride and yielded an intense single band (purity ~ 98 %) on SDS-PAGE corresponding to a molecular mass of ~ 13 kDa [Fig. 18].

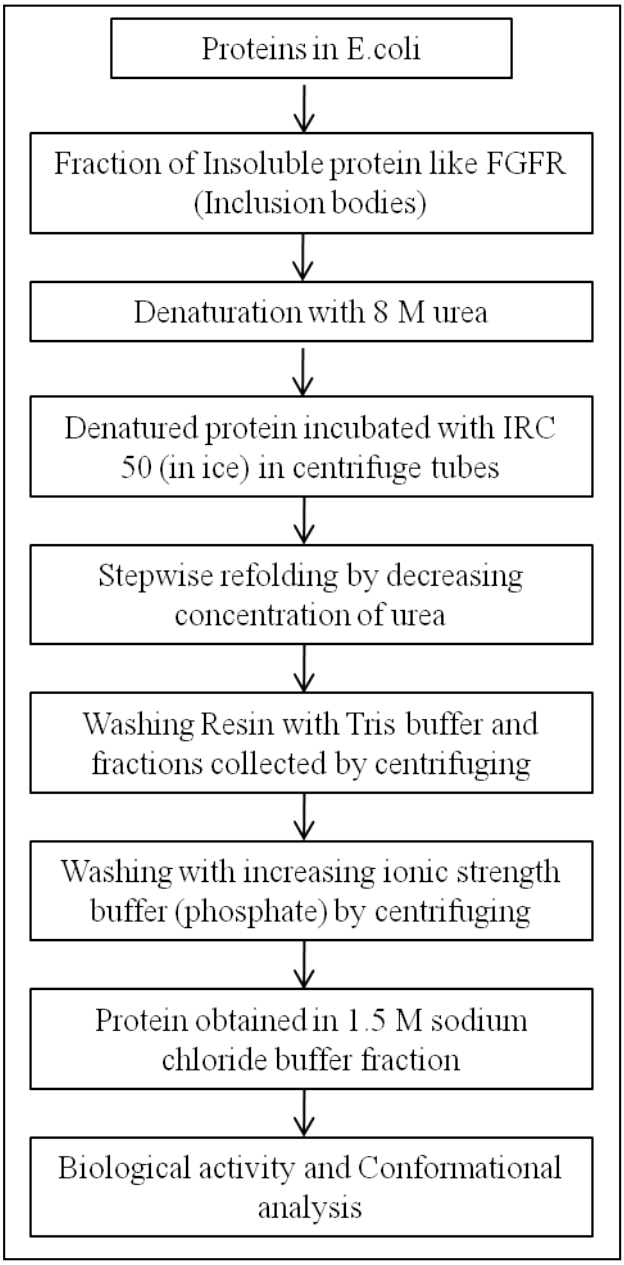


Figure 17: Schematic representation of D2 domain purification by IRC resin

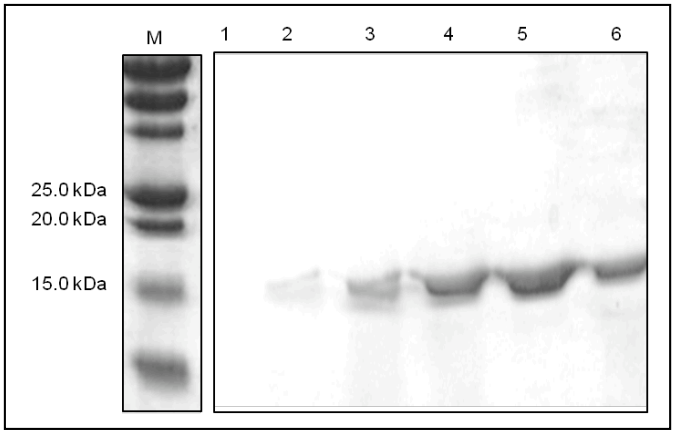


Figure 18: SDS-PAGE of fractions eluted in buffer containing varying concentration of sodium chloride. Lane 1-3, depicts protein bands contained in fractions eluted at 10 mM phosphate buffer; and lane 4-6, depicts protein bands contained in fractions eluted in 10 mM phosphate buffer containing 1.5 M NaCl.

Assessment of purity and homogeneity of the proteins purified using IRC 50 resin

SDS-PAGE of the purified protein samples yielded an intense single band per lane corresponding to a molecular mass of ~13 kDa and ~16 kDa for D2 domain and FGF-1 respectively, with ~ 98% purity when compared with the purity of the 98% pure lysozyme obtained from Sigma-Aldrich [Fig. 19A]. MALDI-TOF mass spectrum analysis of the purified recombinant FGF-1 and D2 domain showed an expected molecular mass of ~16 kDa and ~13 kDa respectively [Fig. 19B and 19C].

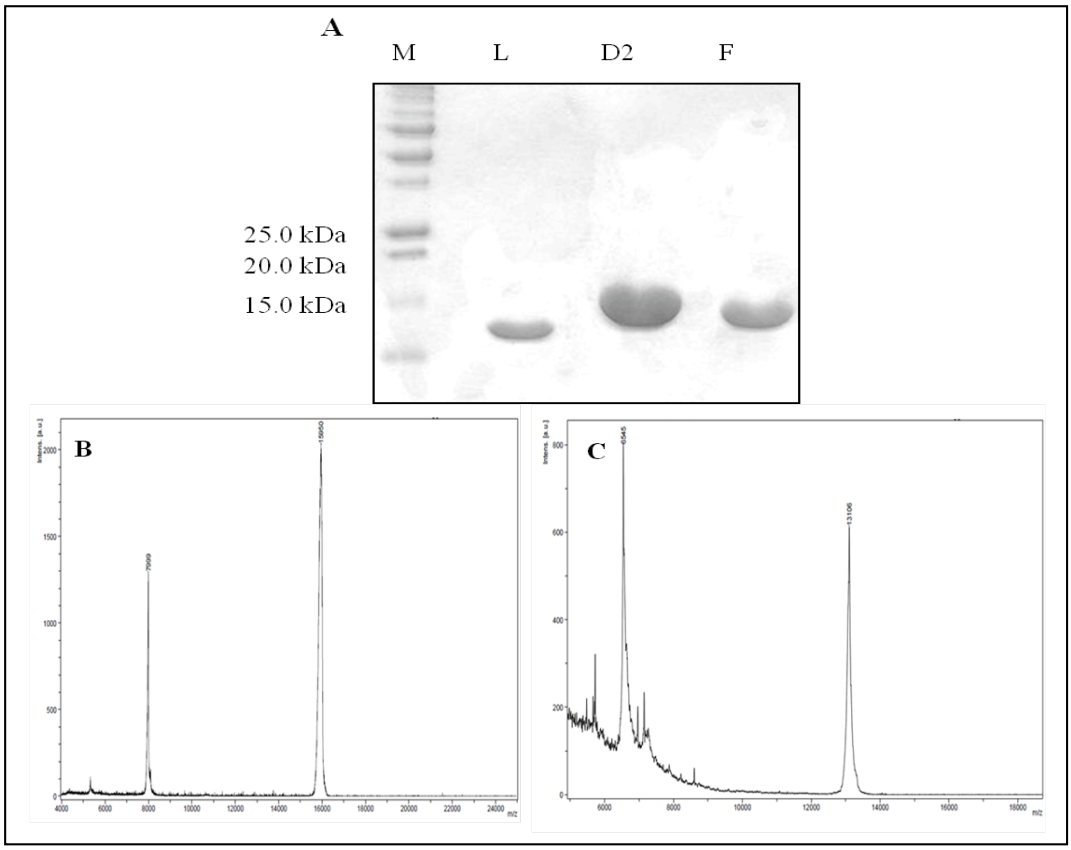


Figure 19: **A** - SDS-PAGE depicting the homogeneity and purity of purified FGF-1 and D2 domain. Lane M, represents the molecular weight marker; lane 1, represents 98% pure lysozyme; lane 2, represents purified D2 domain of FGFR; lane 3 represents purified FGF-1. **B-** MALDI-TOF mass spectrum of the purified FGF-1 sample. **C-** MALDI-TOF mass spectrum of the purified D2 domain sample.

Amberlite weak cation exchange resin IRC 50 resin is composed of copolymerized methacrylic acid and divinylbenzene. The resin was found to be more successful because the methacrylic acid part alone gives 11.6 mmol of carboxylic acid per gram which is very high in order of binding of resin with protein. The remaining groups in smaller amounts are 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. The hydrophobic matrices do not bind irreversibly to the molecules and release them while washing with buffer (suitable pH) [74]. The pH range varies from 0 to 14. IRC 50 has a high pKa of 6.5 suited for basic proteins [74].

The efficiency of this new method was based on the less time required to perform the whole purification, which is approximately 1/3rd of conventional heparin chromatography (in case of proteins expressed in soluble form) also noteworthy is the lower cost of the resin as compared to other affinity sepharose resins.

The IRC 50 resin has also been proven useful in cases where proteins are expressed in inclusion bodies e.g. D2 domain of FGFR. In this case, the time required for refolding the protein is reduced by 1/4th and refolding occurs on the same column which again shows that the technique is cost effective as it does not require another column. The maintenance and regeneration of this resin is very convenient, and could be achieved by simply incubating the resin in 0.1 M HCl and washing it several times with distilled water. Overall, this off-column purification strategy using IRC resin is economical, efficient and easy to maintain.

Biological Activity of Recombinant FGF-1 and D2 Domain

It is important to verify whether the recombinant protein purified by the off-column method is in its biologically active conformation after the 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 [89-91, 94]. Both FGF-1 and D2 domain were loaded onto the heparin column [previously washed with 10mM phosphate buffer (pH 7.2)] separately. No proteins were eluted in the washings with both 10 mM phosphate buffer (pH 7.2) as well as with 10 mM phosphate buffer (pH 7.2) containing 0.75 M NaCl. The proteins were eluted on separate SDS-PAGE as a single peak in 10 mM phosphate buffer (pH 7.2) containing 1.5 M sodium chloride and yielded an intense single band corresponding to a molecular mass of about 16 kDa and 13 kDa, respectively. 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 and could bind strongly with heparin [Fig. 20A & 20B].

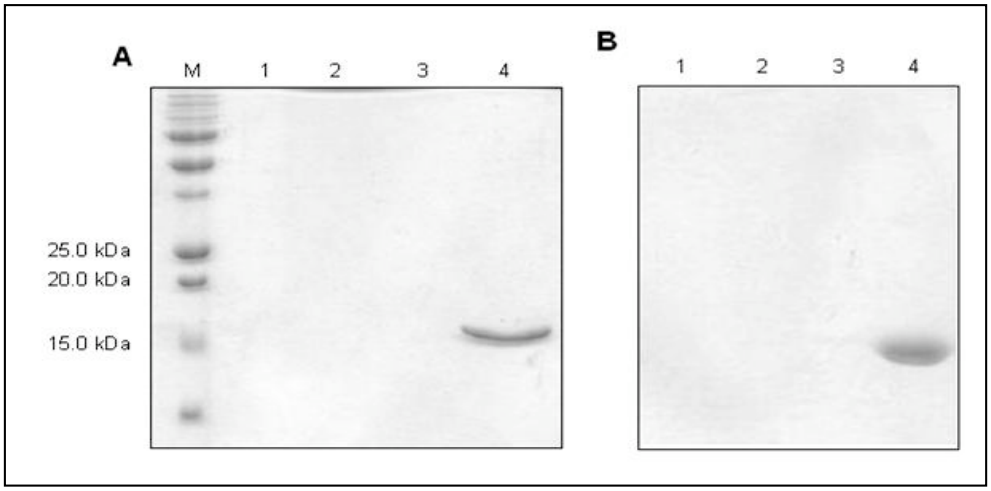


Figure 20: A- SDS-PAGE depicting heparin affinity chromatography of FGF-1. Lane M, represents molecular weight marker; lane 1, represents flow through; lane 2, depicts protein bands contained in fraction eluted in 10 mM phosphate buffer; lane 3, depicts protein bands contained in fraction eluted in 10 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.

Biophysical Characterization of Recombinant FGF-1 and D2 domain

The main objective of biophysical characterization of proteins in the context of structural studies is to evaluate the suitability of the sample after the purification for structure determination, i.e. if the protein is in a folded, partially folded or unfolded state, the homogeneity or potentially aggregated state of the protein, the purity and the stability of the protein.

We 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) [109-110]. Tryptophan, tyrosine and phenylalanine are the three amino acid residues responsible for inherent fluorescence properties of proteins [Fig. 21]. These residues have different absorption and emission wavelength in different conformations which helps in tracking the tertiary structural changes in a protein molecule.

The emission spectrum of FGF-1 at ~ 308 nm in its native state is dominated by a tyrosine emission peak. But in the completely unfolded state FGF-1 exhibits an emission spectrum dominated at ~ 350 nm which is because of florescence of single well-conserved tryptophan at position 121 in the full form of the primary sequence [Fig. 22A] [109-110]. These spectral features are ideal to monitor the denaturant induced unfolding of the protein. The conformational stability assessed by equilibrium thermal denaturation showed that 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) which suggests that thermodynamic stability of the protein is enhanced upon binding to the SOS [Fig. 22B] [92].

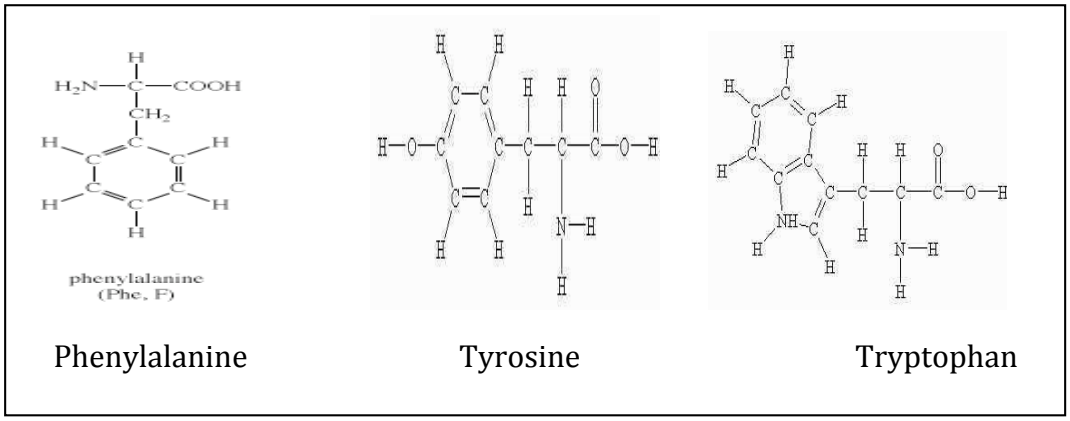


Figure 21: Chemical structure of tyrosine and tryptophan

(Source: Lehninger, 5th ed)

These results clearly indicate that the recombinant FGF-1 is in a stable, folded and in biologically active conformation.

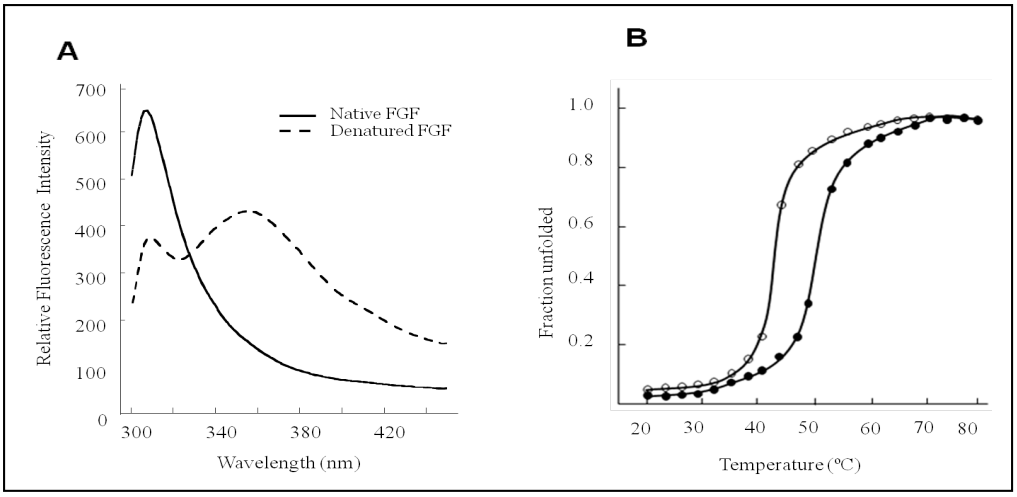


Figure 22: **A-** The figure shows the emission spectra of FGF-1 in its native state (continuous line) and in the denatured state(s) state (broken line). **B-** Thermal induced unfolding of FGF-1 in the presence (closed circle) and absence (open circle) of SOS.

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 full form of the primary sequence [93]. 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 D2 domain at ~ 338 nm in its native state suggests that the tryptophan residues are buried in the interior of the well organized tertiary structure of the protein. However, in the completely unfolded state D2 domain exhibits an emission spectrum dominated by tryptophan fluorescence at ~ 350 nm [Fig. 23A]. The conformational stability assessed by equilibrium thermal denaturation showed that 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) which suggests that thermodynamic stability of the protein is enhanced upon binding to the SOS. These results clearly indicate that the recombinant FGF-1 is in a stable, folded and in biologically active conformation [Fig. 23B].

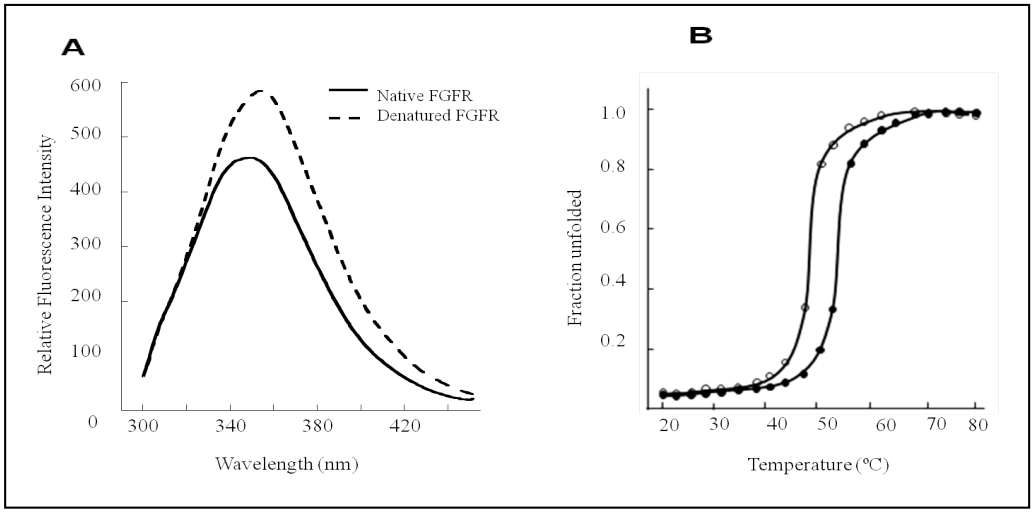


Figure 23: **A-** The figure shows the emission spectra of D2 domain of FGFR in its native state (continuous line) and in the denatured state(s) state (broken line). **B-** Thermal induced unfolding of D2 domain of FGFR in the presence (closed circle) and absence (open circle) of SOS.

We have found the off column purification method to be efficient yielding 30 mg and 24 mg per litre of FGF-1 and D2 domain respectively in the native biologically active conformation compared to column affinity chromatography which yielded 32 mg and 20 mg per litre of FGF-1 and D2 domain respectively. It also does not involve the disadvantages associated with conventional heparin/Ni-NTA sepharose column chromatography [Table 1].

	Amberlite IRC 50	Heparin/Ni-NTA
Time Require (Unit Operation)	~ 3-4 hrs	~ 8-9 hrs
Cost of Resin	~ $60 (250 gm)	~ $ 500 (50 ml)
Regeneration Time Require	~ 1 hr	~ 6-8 hrs
Regeneration Efficiency	Efficiency not decreased	Efficiency decreased after few regenerations
Disintegration	Not prone to bacterial disintegration	Prone to bacterial disintegration after a period of time
Scale up process	Possible Doesn't require high-tech technique	Possible Require high-tech equipments
Cost of Protein	~ 5-6 times lesser then traditional heparin purification	~ 1mg FGF $ 1250 ~ 1mg of D2 $ 2500
Resource requirements	Centrifuge, Centrifuge tubes, SDS-PAGE, Dialysis bag or Membrane, Nitrogen Gas	Centrifuge, Column, Peristaltic pump, UV detector, Recorder, SDS-PAGE, Dialysis bag or Membrane, Nitrogen gas, FPLC (sometimes),

Table 1: Comparison of amberlite IRC 50 and column affinity chromatography

Implementation of this new protocol will in turn reduces the cost of commercially available purified protein, when compared to pure protein obtained by conventional method by eliminating the need for sophisticated instruments.

CONCLUSION

This is the first report in which amberlite cation exchange resin have been used for purification of heparin binding proteins. 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 IRC resin and thereby devised an efficient and economical method for the purification of this biologically important class of proteins. 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.

FUTURE STUDIES

Natural sources are often rare and expensive because of which most of the protein biopharmaceuticals are produced recombinantly which results in higher cost of this therapeutics. The development of biopharmaceuticals which are economical and in their biologically active form is increasing day-by-day. Execution of this efficient and inexpensive protocol for the purification of other heparin binding proteins or other class of proteins having similar properties can result into isolation of proteins which are of high importance to mankind. Fig. 24 represents the schematics of Off-Column purification.

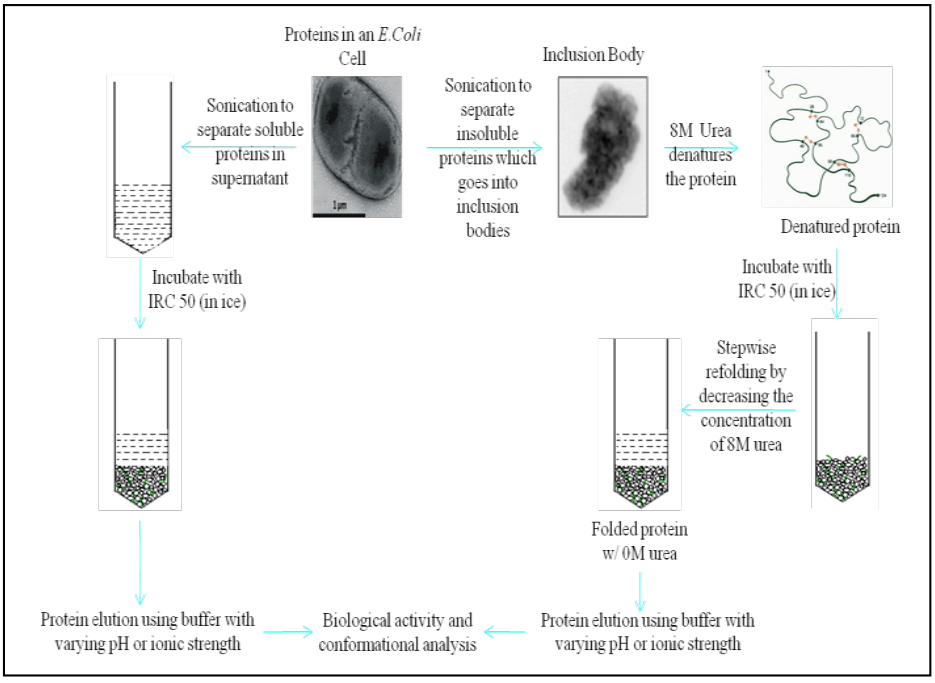


Figure 24: Schematic representation of off-column purification

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