

## Evaluation of Recombinant Fusion Protein Purification Using Mixed Mode Chromatography (MMC)

Bairavabalakumar N, Amol M, Pravinkumar D, Bhargav Prasad K, Sathyabalan M, Bharath R Shenoy, Sunit M and Vivek H\*

Zumutor Biologics, Bangalore, India

### Article Information

Received date: Mar 20, 2017

Accepted date: Apr 15, 2017

Published date: Apr 20, 2017

### \*Corresponding author

Vivek H, Zumutor Biologics Private Limited, Bangalore, India, Email: vivek.halan@zumutor.com

**Distributed under** Creative Commons CC-BY 4.0

**Keywords** Mixed mode chromatography; Affinity chromatography; Monoclonal antibody; Fusion protein; Resins

**Abbreviations** RPM: Revolutions Per Minute; OD: Optical Density; CHO: Chinese Hamster Ovary; UPLC: Ultra Pressure Liquid Chromatography; RP-LC: Reverse Phase Liquid Chromatography; PMSF: Phenyl Methyl Sulphonyl Fluoride

### Abstract

Peptides are most widely used in treating various life threatening diseases and peptide therapeutics are emerging in a fast pace in recent years. Recombinant peptides are in the sweet spot between small molecule drugs and large biotherapeutic proteins. Exenatide, a therapeutic synthetic peptide used in the treatment of Type 2 diabetes was originally developed by Amylin Pharmaceuticals and approved in April 2005, marketed in the name of "Byetta". It is a 4.18 kDa peptide with an iso-electric point (pI) of 4.70, which was expressed in microbial system as a fusion protein with a molecular weight of 21.1 kDa. The fusion protein contains thioredoxin as a solubility tag in order to express in soluble form and His-tag as an affinity tag to ease in the purification process and also to reduce proteolytic effects. The recurrent problem faced with ion exchange chromatography was that the recombinant fusion protein typically eluted as an aggregate. Hence, an attempt was carried out to screen various mixed mode chromatography resins by taking advantage of its ability to bind through more than one type of interaction. This study outlines the purification of recombinant fusion protein using different mixed mode resins and also compares the purification efficiency in terms of purity with that of ion exchange and affinity chromatography. The main aim of the study was to find an alternative to affinity chromatography capture step. The current screening study revealed that it is feasible to purify recombinant protein from *E.coli* using mixed mode chromatography as a comparable capture step. Mixed mode chromatography can be a powerful alternative to affinity chromatography in downstream processing of biotherapeutics.

### Introduction

The market of recombinant protein production is increasing exponentially in the last five years. Last three decades, many platforms and technologies were developed in protein purification, yet challenges remain especially in the purification of recombinant proteins, peptides, biologics and biosimilars from both mammalian as well as microbial cells. In order to attain higher purity and recovery most of the proteins particularly peptides pose extreme challenges in purification. Recombinant peptide covers around 25 percent of recombinant protein market. They are difficult to express in *E.coli* due to less solubility and are also highly prone to protease degradation. Using fusion tags in expression vector helps in increasing the solubility of peptides and thus, in turn increases the yield and eases the purification processes.

Fusion partners can promote different effects when located at the N-terminus or C-terminus of the target protein. Generally N-terminal tags are advantageous than C-terminal fusion tags due to the following reasons: (a) Efficient translation initiation sites on the tag and (b) fusion tags can be removed without leaving any additional residues that may hinder the activity of recombinant protein. There are number of fusion tags available to increase the solubility such as Maltose Binding Protein (MBP), Thioredoxin (TRX), Nitrogen Utilizing Substrate A (NusA), Small Ubiquitin Modified (SUMO), Glutathione-S-Transferase (GST), IgG domain B1 of Protein G (GB1) Solubility enhancing Ubiquitous Tag (SNUT), Seventeen kilodalton protein (Snp), IgG repeat domain ZZ of Protein A (ZZ) and other [1]. MBP is a relatively cysteine less protein with a MW of ~ 40.6 kDa and it is thermodynamically moderately stable, used mainly to enhance the solubility of target protein and can be purified easily by affinity chromatography [2]. TRX is a 12-kDa intracellular thermostable protein of *E. coli* that is highly soluble, and most employed fusion tag than other fusion tags. GST is a 26 kDa protein from *Schistosoma japonicum* used for expression and fusion proteins can be purified using a single step affinity chromatography. In addition to solubility tags, tags such as histidine tag can also be used for the ease of purification process. These types of affinity tags are highly specific where protein of interest can be purified with highest purity and recovery also will be better than other types of chromatography.

In recent years, fermentation processes have been improved proportionally to reach higher cell densities with high product yield. There is a greater demand now on purification to keep up with the booming biopharmaceutical industry with additional challenges. Development of efficient purification processes for biotherapeutic proteins has been highlighted as one of the

major challenge in biotechnology industries. There are various types of chromatography techniques available and selection of most appropriate chromatographic technique is the key to successful and efficient protein purification platform. By optimizing the best conditions to suit the requirements, combining them in a rational way to increase yield and minimize the number of steps is very essential. Hence, chromatography becomes an important tool in every laboratory where protein purification is carried out.

Various types of chromatography being used in protein or peptide purification which includes Affinity Chromatography, Ion exchange Chromatography, Hydrophobic Interaction Chromatography and Size Exclusion Chromatography (Gel filtration Chromatography). Affinity chromatography separate proteins based on the reversible interaction (affinity) between a protein or group of proteins and a specific ligand coupled to a chromatography matrix. The technique gives high selectivity, high resolution and usually high binding capacity for the proteins of interest. There are various types of affinity resins used for purification of wide range of proteins such as Ni-NTA resins for purification of proteins containing Histidine tag (Immobilized Metal Affinity Chromatography -IMAC), Protein A and Protein G resins for purification of Monoclonal or Polyclonal antibodies. Lectin family of proteins called Concanavalin A (Con A) employed for the separation of glycoproteins, polysaccharides and glycolipids. Ion exchange chromatography is used for the purification of proteins based on the charge and there are two types namely Anion Exchange Chromatography and Cation Exchange Chromatography, where proteins are separated based on isoelectric point (pI). Hydrophobic Interaction chromatography, where proteins are separated based on hydrophobicity. Size exclusion chromatography, in which protein separation occurs based on the size of the protein. Few other chromatography types used commonly for protein purification includes Dye based chromatography such as Reactive yellow, Reactive blue agarose resin generally being used for purification of endotoxins, Interferons, blood factors, kinases and other heparin binding proteins. In addition to all, recently mixed mode chromatography has attracted the attention of biopharmaceutical industry due to its different type of binding and elution properties. In recent years, mixed mode chromatography is widely being used for the purification of monoclonal antibodies in addition to recombinant fusion proteins.

Here, we made an attempt to employ mixed mode chromatography as a capture step to purify a recombinant fusion protein expressed in *E.coli*. The chromatography conditions used here are based on previous experience with same fusion protein with same pI of the target protein. The target protein contains exendin-4 peptide fused with Thioredoxin fusion tag to increase the solubility during expression and histidine tag to ease the purification process.

### Mixed Mode Chromatography

Mixed Mode Chromatography (MMC) is a type of chromatography where more than one type of interaction occurs between analyte and resin. MMC widely used for purification of recombinant proteins, peptides, oligonucleotides and Ribonucleic Acids (RNAs); oligosaccharides. Table 1 shows the list of mixed mode resins and possible mode of interaction with analyte or protein. In addition to the list mentioned in Table 1, Avantor has both mixed mode and multimode resins for purification of various types of

proteins. BRX-aXM Type I & BRX-aXM Type II resins used in the screening are from Bio-Rad and both are in development stage. Maria *et al* employed two different MMC resins namely Capto MMC and HEA Hypercel followed by anion exchange membrane adsorbers in the purification of recombinant monoclonal antibody and results obtained was comparable with conventional method, where Protein A was used as a Capture step [3]. 4'-(1H-imidazol-1-yl) aniline Sepharose CL 6B, a novel glutathione based mixed mode resin was developed and studied for dynamic binding capacity (DBC), adsorption isotherm. The resin performance was evaluated in the purification of IgG from a mixture of IgG and Bovine Serum Albumin [4]. Zhang *et al* developed a pyridine-based, mixed mode resin and the ligand is 4'-terpyridinylsulfanylethylamine (40-TerPSEA) and compared this resin with Protein A resin in the purification of humanized monoclonal antibody from CHO cell culture. The result revealed that the performance of this mixed mode ligand was comparable between two different chromatography [5]. Prepacked mixed mode resins (HEA, PPA and MEP HyperCel) in small columns employed for fast purification of proteins. All three resins showed higher capacity than conventional HIC resins with unique selectivity respectively [6]. Recombinant GST protein was expressed in *E.coli* and purified using mixed mode resins namely HEA HyperCel and PPA HyperCel. The recovery of GST in HEA HyperCel or PPA HyperCel is 93% and 96% respectively [7]. Four mixed mode resins namely PPA HyperCel, HEA HyperCel, MEP HyperCel and Capto adhere were evaluated for the capture of monoclonal antibodies from CHO cell culture fluid. Based on DoE, high throughput screening in microplate format was performed to find the best conditions [8]. TOYOPEARL MX-Trp-650M resin was evaluated to separate aggregates of two humanized monoclonal antibodies using dual salt mixtures, where sodium chloride was employed with three different salts such as citrate, glutamate and acetate [9]. Zhang *et al* developed a novel mixed mode resin using a pyridine-based compound, 40-terpyridinyl sulfanyl ethylamine (40-TerPSEA) as a ligand. The resin was studied in the purification of humanized IgG from CHO cell culture fluid. The performance of this mixed mode resin with respect to binding capacity, selectivity and reusability is comparable with Protein A based resins [10]. Shi *et al* developed a mixed mode resin by immobilizing a new ligand 4-(1H-imidazol-1-yl) aniline on Sepharose CL-6B and evaluated in the purification of IgG from bovine serum [11]. Hirano *et al* demonstrated the separation of monomeric BSA from the oligomeric forms and purification of monoclonal antibody against interleukin-8 using arginine as eluent [12]. Wang *et al* developed a novel mixed mode membrane adsorber and compared the binding capacity, productivity, residence time and ligand density with other three commercially available mixed mode resins such as MEP Hypercel, Capto MMC, Capto MMC Imp Res. This adsorber was employed in bind and elute mode. The result revealed that new mixed mode membrane adsorber showed high binding capacities and high load productivities [13]. Mixed mode chromatography step was employed in a mAb purification process and studied aggregate removal in comparison with other two ion exchange resins namely Poros HQ50™, Poros HS50™ and one Hydrophobic interaction chromatography resin (Phenyl Sepharose HP™). Mixed mode chromatography step was incorporated in mAb manufacturing process [14]. Kaleas *et al* evaluated Capto MMC as a Capture step to purify mAbs directly from CHO cell culture fluid and roughly twelve mAb feed stocks were examined under different elution conditions

and compared with conventional Protein A chromatography step [15]. Mixed mode chromatography is also employed for the purification of bispecific antibodies such as IgG4 bispecific antibodies using SepaxZenix SEC-300 column [16].

In addition to recombinant proteins, plasmid DNA also purified using mixed mode resins. Capto adhere resin was employed for plasmid DNA purification from *E.coli* crude extract [17]. Matos *et al* employed strong mixed mode anionic resin to separate small deoxyribonucleic acid fragments, where binding and elution behavior of ss- and dsDNA molecules in the size range 19-33 mers were evaluated using Capto adhere mixed mode resin. The result showed efficient separation of DNA fragments [18]. Mixed mode chromatography is also used for separation of Chito Oligosaccharides (COS), where hydrophilic interaction/weak cation-exchange (HILIC/WCX) mixed-mode chromatography resin was developed to efficiently separate COS in mixed mode operation [19].

encoding exenatide peptide was cloned in pET32a vector. Thioredoxin fusion tag and histidine tag were used to enhance the solubility, to ease the purification process and enzyme cleavage site between fusion tag and protein of interest to cleave the fusion tag respectively. Briefly, glycerol stock was inoculated into Luria Bertani broth (LB) media containing 100 µg/ml ampicillin and grown overnight at 37°C (250 RPM). The overnight culture was used as an inoculum and inoculated into Chemically Defined Media (CDM) in a 5 Liter scale BioStat BDCU-II fermenter at 4.5 Liter scale in fed batch mode. Cells were induced at 10 OD (600 nm) by IPTG at 1 mM final concentration and after induction, culture was continued for 4 more hrs for protein production. Cells were harvested and bacterial culture was divided into twelve equal parts for screening different mixed mode resins. Cell culture was centrifuged at 9000 RPM for 1 hr at 2-8°C to separate cells from media.

**Cell lysis and Clarification:** Since the loading conditions such as pH and salt concentration were different for various mixed mode

**Table 1:** List of mixed mode resins.

Sr.no.	Resin	Type of interaction during binding	Elution technique
1	Capto MMC™ (20)	Hydrophobic and ion exchange	High salt and pH gradient
2	Capto™ Adhere (21)	Hydrophobic and ion exchange	High salt gradient
3	CHT™ Ceramicfluorapatite (22)/ CFT™ Ceramicfluorapatite(23)	Affinity and ion exchange	Phosphate/sulphate based gradient elution
4	HEA HyperCel™ (24) PPA HyperCel™ (24)	Hydrophobic and electrostatic repulsion	Changing the pH <pKa of the ligand
5	MEP HyperCel™ (25)	Hydrophobic and electrostatic repulsion	Changing the pH <pKa of the ligand
6	CMM Hypercel™ (26)	Hydrophobic and electrostatic repulsion	Changing the pH <pKa of the ligand
7	CaptoCore™ 700 (27)	Hydrophobic and ion exchange	Product of interest in flow-through
8	Toyopearl MX-Trp-650M (28)	Hydrophobic and ion exchange	High salt gradient
9	EshmunoHCX (29)	Hydrophobic and ion exchange	High salt gradient
10	Nuvia cPrime (30)	Hydrophobic and ion exchange	High salt gradient and pH gradient
11	BRX-aXM Type I	Hydrophobic and ion exchange	High salt gradient and pH gradient
12	BRX-aXM Type II	Hydrophobic and ion exchange	High salt gradient and pH gradient

## Materials & Methods

### Materials

*Escherichia coli* BL21 (DE3) strain was used for the expression of recombinant fusion protein, pET32a as a vector, AKTA Purifier 100 and C10/10 column from GE Healthcare. Different resins used were: (a) MEP Hypercel from Pall life sciences (b) Capto adhere, Capto MMC from GE Healthcare (c) Nuvia cPrime, BRX-aXM Type I, BRX-aXM Type II from Bio-Rad. In addition to that, anion exchange resin Q Sepharose and Ni Sepharose from GE Healthcare were also evaluated. AEX mixed mode resins from Bio-Rad are in development stage (BRX-aXM Type I in alpha testing stage, BRX-aXM Type II in beta testing stage).

Waters Acquity H Class with PDA (Photo Diode Array) Detector UPLC System and RP C18 2.1X 50 mm with 1.8 µm from Agilent for fusion protein analysis. All the solutions and buffers prepared were at desired pH and conductivity. The buffers and cell lysate supernatants were filtered through 0.45 µm filter before loading onto chromatography column.

### Methods

**Plasmid construction and protein expression:** The sequence

chromatography, different lysis buffers were used during cell lysis in each mixed mode chromatography respectively. In the present study, 72 grams of cell pellet was re-suspended in 360 ml of respective lysis buffer. Table 2 shows different lysis buffer used in cell lysis. The first step in purification of the recombinant fusion proteins was cell lysis, where pre-chilled lysis buffer was added to the cell pellet in 1: 5 ratio and cells were lysed using High Pressure Homogenizer in two passages after adding DNase and PMSF to the cell suspension at 0.5mg/ml and 1 mM final concentration respectively. The whole cell lysate was clarified by centrifugation at 9000 RPM for 1 hr at 4°C for loading onto mixed mode column. In each MMC, only ~50 ml of clarified supernatant was loaded to maintain uniform loading conditions.

### Chromatography purification

**MEP hypercel:** Clarified supernatant (~50ml) was loaded onto pre-equilibrated MEP Hypercel C10/10 column in Equilibration buffer. Flow through was collected separately. Afterwards, a wash step was applied using equilibration buffer to remove weakly bound proteins followed by second wash step using Wash buffer (pH 6.0). Bound proteins eluted from the column by Elution buffer-1 in pH gradient from pH 6.0 to pH 4.0 over ten column volume length followed by Elution buffer-2 in single step and collected in fractions.

**Citation:** Bairavabalakumar N, Amol M, Pravinkumar D, Prasad BK, Sathyabalan M, Bharath RS, et al. Evaluation of Recombinant Fusion Protein Purification Using Mixed Mode Chromatography (MMC). Ann Chromatogr Sep Tech. 2017; 3(1): 1030.

**Table 2:** Buffers used in cell lysis and Chromatography.

Sr.No	Resin Name	Equilibration buffer	Wash buffer	Elution buffer
1	MEP Hypercel	30mM Tris, 500mM NaCl, pH 7.55	30mM Sodium Phosphate , 500mM NaCl, pH 6.0	Elution buffer-1: 30mM Sodium acetate, 50mM NaCl, pH 4.0 Elution buffer-2: 30mM Sodium acetate, pH 4.0
2	Capto adhere I	30mM Tris, 25mM NaCl, pH 7.56	30mM Sodium Phosphate , 25mM NaCl, pH 6.0	Elution buffer-1: 30mM Sodium acetate, 100mM NaCl, pH 4.0 Elution buffer-2: 30mM Sodium acetate, 1M NaCl, pH 4.0
3	Capto adhere II	30mM Tris, 25mM NaCl, pH 7.56	30mM Sodium acetate, 50mM NaCl, pH 4.05	30mM Tris, 1M NaCl, pH 7.5
4	Capto adhere III	30mM Tris, 25mM NaCl, pH 7.56	30mM Sodium acetate, 50mM NaCl, pH 4.05	30mM Tris, 1M NaCl, pH 7.5
5	Capto MMC	30mM Tris, 25mM NaCl, pH 7.56	No specific Wash buffer was used	30mM Tris, 1M NaCl, pH 7.5
6	Nuvia cPrime	30mM Sodium acetate, 150mM NaCl, pH 4.0		Elution buffer-1: 30mM Tris, pH 7.5 Elution buffer-2: 30mM Sodium acetate, 1M NaCl, pH 4.0
7	BRX-aXM Type I	30mM Tris, 25mM NaCl, pH 7.56		30mM Tris, 1M NaCl, pH 7.5
8	BRX-aXM Type II (Run I)	30mM Tris, 25mM NaCl, pH 7.56		30mM Tris, 1M NaCl, pH 7.5
9	BRX-aXM Type II (Run II)	30mM Tris, 25mM NaCl, pH 7.56	30mM Tris, 25mM NaCl, pH 6.5	30 mM Tris, 1M NaCl, pH 8.55
10	Q-Sepharose	30 mM Tris, 25 mM NaCl, pH 8.56	30mM Tris, 25mM NaCl, pH 7.50	30 mM Tris, 1M NaCl, pH 7.50
11	Ni Sepharose (IMAC)	30mM Tris, 500mM NaCl, 20 mM Imidazole, pH 8.02	30mM Tris, 200mM NaCl, 35 mM Imidazole pH 8.02	30mM Tris, 200mM NaCl, 500 mM Imidazole pH 8.02

**Capto adhere (Run-I):** Clarified supernatant (~50ml) was loaded onto pre-equilibrated Capto Adhere C10/10 column in Equilibration buffer (30mM Tris, 25mM NaCl, pH 7.56). Collected flow through separately and then started wash step with equilibration buffer to remove loosely bound proteins. Once the absorbance ( $A_{280}$ ) reached close to baseline, second wash step was initiated by Wash buffer at pH 6. Bound proteins elution from the column by elution buffer I was accomplished in pH gradient from pH 6.0 to pH 4.0 over 10 column volume length where the pH 4.0 buffer contains 30mM Acetate, 100mM NaCl. Fractions were collected and stored. After pH gradient elution, second elution was started with high salt (30mM Sodium acetate, 1M NaCl pH 4.0), where elution peak started rising and fractions were collected until the peak reached close to baseline.

**Capto adhere (Run-II):** Clarified supernatant (~50ml) with high salt (500mM NaCl used in lysis buffer) was loaded onto pre-equilibrated Capto Adhere C10/10 column in Equilibration buffer (30mM Tris, 25mM NaCl, pH 7.56). Collected flow through separately. Equilibration buffer was applied to remove weakly bound proteins and other impurities. Once the absorbance reached close to baseline, wash step was initiated using Wash buffer containing 50 mM NaCl (pH 4.05) followed by purified water wash to elute proteins bound by hydrophobic interaction. Re-equilibrated the column in equilibration buffer and elution was initiated by elution buffer containing 1 M NaCl in single step and fractions were collected.

**Capto adhere (Run-III):** Clarified supernatant (~50ml) was loaded onto pre-equilibrated Capto Adhere C10/10 column in Equilibration buffer (30mM Tris, 25mM NaCl, pH 7.50). Collected flow through separately. Wash step using Wash buffer pH 4.05 was initiated after passing the equilibration buffer. Passed purified water to remove hydrophobic proteins and then re-equilibrated with equilibration buffer to bring down the conductivity to binding conditions. Bound

proteins eluted from the column by elution buffer in salt gradient over 10 column volume and collected in fractions.

**Capto MMC:** Clarified supernatant (~50ml) was loaded onto pre-equilibrated Capto MMC C10/10 column in Equilibration buffer (30mM Tris, 25mM NaCl, pH 7.56). Collected flow through separately. After loading the sample, wash step with Equilibration buffer was applied to remove weakly bound proteins. Bound proteins eluted from the column by elution buffer (25 mM NaCl to 1 M NaCl) in salt gradient over 10 column volume. Eluents were collected in fractions.

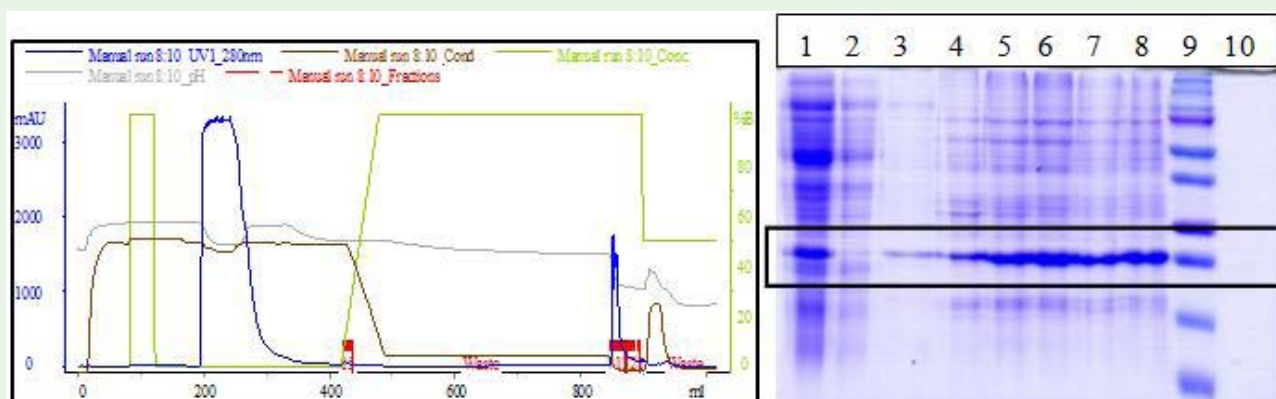
**Nuvia cPrime:** Clarified supernatant (~50ml) was loaded onto pre-equilibrated Nuvia cPrime C10/10 column in Equilibration buffer (30mM Sodium acetate, 150mM NaCl, pH 4.00). Collected Flow through separately. Wash step was applied by equilibration buffer. Protein elution was initiated by elution buffer in pH gradient from pH 4.0 to pH 7.50 over 10 column volume. Fractions were collected. After pH gradient elution, second elution was started with high salt in a single step.

**BRX-aXM Type I:** Clarified supernatant (~50 ml) was loaded on to pre-equilibrated BRX-aXM Type I resin C10/10 Column in equilibration buffer (30mM Tris, 25mM NaCl, pH 7.56) Collected flow through and removed loosely bound proteins from the column using equilibration buffer. After passing equilibration buffer,  $A_{280}$  was reached close to baseline. Elution was carried out by elution buffer (30 mM Tris, 1M NaCl at pH 7.50) in salt gradient and fractions collected.

**BRX-aXM Type II(Run-I):** The same procedure followed in BRX-aXM Type I was followed for BRX-aXM Type II mixed mode resin. But, the purity was low.

**BRX-aXM Type II(Run-II):** Clarified supernatant (~50 ml) was





**Figure 1:** Chromatogram for MEP Hypercel and SDS-PAGE Analysis (10%).

Lane details: Lane 1: Load, Lane 2: FT, Lane 3: Fraction #1-2, Lane 4: Fraction #7-8, Lane 5: Fraction #9, Lane 6: Fraction #10, Lane 7: Fraction #11-15, Lane 8: Fraction #16-19, Lane 9: Bio-Rad Pre-stained Broad range Protein Marker (Cat # 1610374), Lane 10: Empty.

precipitated at various concentration of ammonium sulphate, precipitation at acidic pH to remove impurities, where low pH precipitation does not help. But, ammonium sulphate at 0.4M concentration showed efficient removal of impurities (data not shown). In this Chromatography, clarified supernatant pH was adjusted to 8.50 and then precipitated at 0.4M ammonium sulphate. The clarified sample was then loaded onto pre-equilibrated BRX-aXM Type II resin C10/10 Column in equilibration buffer (30 mM Tris, 25 mM NaCl, pH 8.55). Collected Flow through separately and loosely bound proteins were removed from the column using equilibration buffer. After passing equilibration buffer, A280 was reached close to baseline. Elution was carried out by elution buffer (30 mM Tris, 1M NaCl at pH 8.55) in salt gradient and fractions collected.

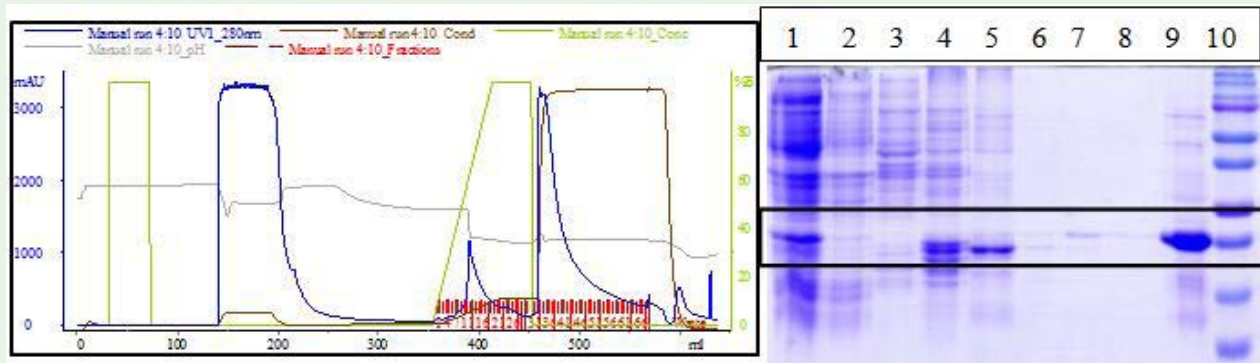
**Q-Sepharose:** Q Sepharose C10/10 column (Packed with GE's Q-Sepharose AEX resin) was equilibrated in equilibration buffer (30mM Tris, 25mM NaCl, pH 7.56). Clarified cell lysate after centrifugation (~50ml) was loaded onto the column. Flow through collected separately. Loading was followed by low pH wash buffer (30mM Tris, 25mM NaCl, pH 6.50). Initiated the elution by elution buffer (30mM Tris, 1M NaCl pH 7.50) in gradient mode and fractions were collected.

**Ni-Sepharose:** Ni Sepharose C10/10 column was equilibrated in equilibration buffer (30mM Tris, 500mM NaCl, 20 mM Imidazole pH 8.02). Clarified cell lysate after centrifugation (~50ml) was loaded onto the column. Flow through collected separately. Loading was followed by wash with equilibration buffer and then by wash buffer. Initiated the elution by elution buffer containing 500 mM Imidazole.

## Result and Discussion

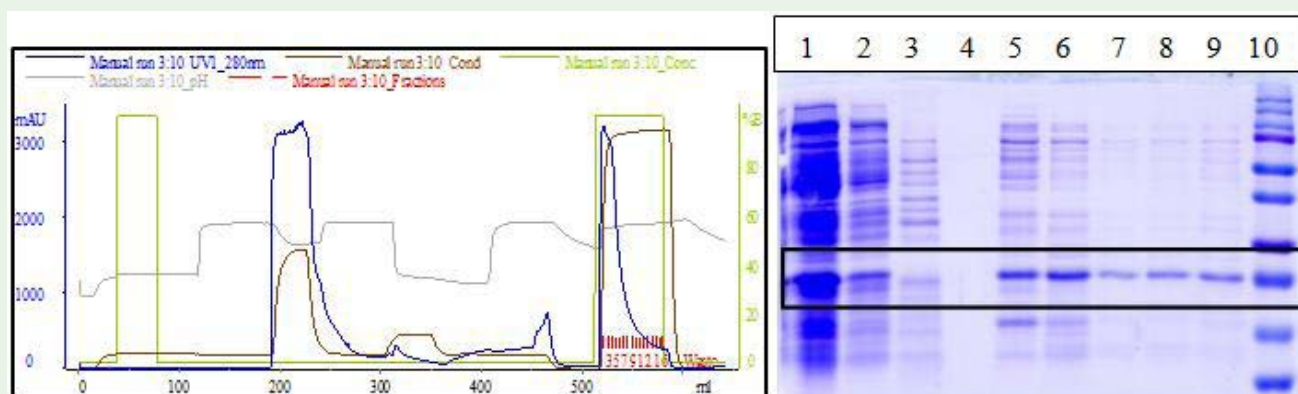
Different MMC resins were evaluated for its recovery and purity. The result from each mixed mode chromatography showed that multimodal resins has different mode of interaction. The purity and separation of the fusion protein varied in each mixed mode chromatography.

In MEP Hypercel mixed mode chromatography (Figure 1), two sharp elution peaks were observed. SDS-PAGE analysis (Figure 1) result revealed that fusion proteins eluted along with some impurities and slight level of fusion proteins observed in flow through. MEP Hypercel eluted sample can be further purified using either anion exchange chromatography or metal affinity chromatography as an intermediate step and this yields product with better purity.



**Figure 2:** Chromatogram for Capto Adhere (Run I) and SDS-PAGE Analysis (10%).

Lane details: Lane 1: Load, Lane 2: FT, Lane 3: Fraction #2-4, Lane 4: Fraction #5-10, Lane 5: Fraction #11-20, Lane 6: Fraction #21-24, Lane 7: Fraction #25-28, Lane 8: Fraction #29-32, Lane 9: Fraction #33-55, Lane 10: Bio-Rad Pre-stained Broad range Protein Marker (Cat # 1610374).



**Figure 3:** Chromatogram for Capto Adhere (Run II) and SDS-PAGE Analysis (10%).

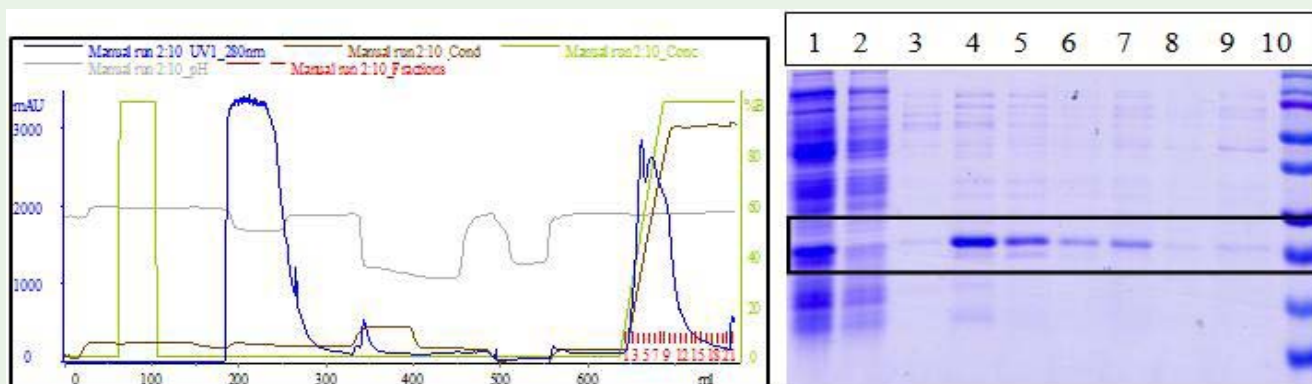
Lane details: Lane 1: Load, Lane 2: FT, Lane 3: Low pH wash, Lane 4: Purified water wash, Lane 5: Fraction #1, Lane 6: Fraction #2-3, Lane 7: Fraction #4, Lane 8: Fraction #5-6, Lane 9: Fraction #7-12, Lane 10: Bio-Rad Pre-stained Broad range Protein Marker (Cat # 1610374).

By optimizing the loading conditions such as loading at high pH, high salt concentration and in presence of non-ionic detergent, the level of target protein binding onto the resin can also be increased. HCIC showed binding of fusion protein and impurities distributed throughout the fractions, when product elution was performed at acidic pH. The elution from MEP Hypercel can be directly loaded on to either anion exchange chromatography or anionic mixed mode chromatography to purify further. RP-LC analysis showed, MEP Hypercel chromatography produced target protein with 56% purity (Figure 11). The elution of bound proteins in Capto adhere mixed mode chromatography -Run-I (Figure 12) was observed in both pH gradient from pH 6.0 to 4.0 (decreasing pH) as well as in high salt elution buffer. The corresponding SDS-PAGE (Figure 2) showed the co-elution of high level of impurities along with protein of interest in pH gradient when compared to high salt elution condition. This condition yielded around 70% of target protein purity (Figure 13).

In case of Capto adhere chromatography Run-II (Figure 3) cells were lysed in high salt concentration and loaded the clarified sample at high salt to create binding of proteins onto resin by hydrophobic interaction instead of ionic interaction. In this chromatography run,

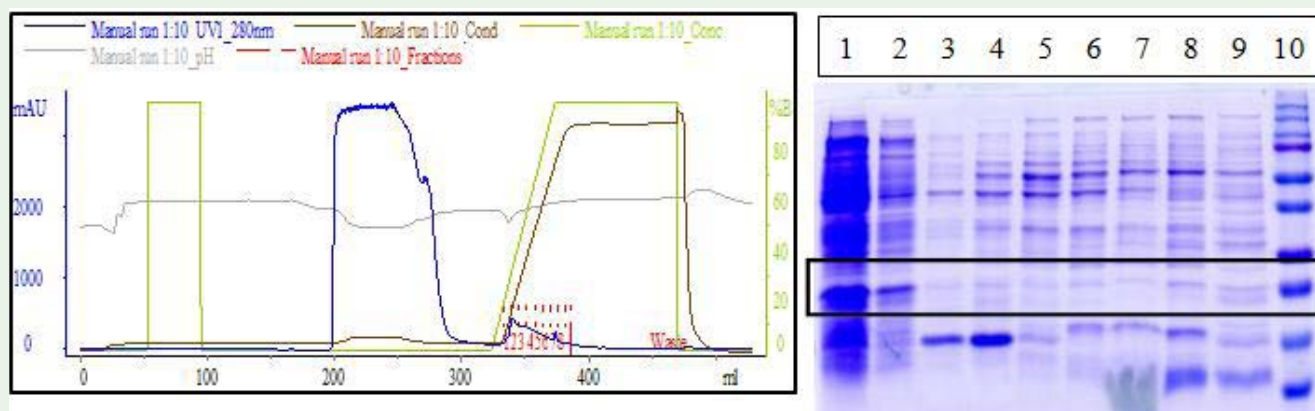
half of the target protein was observed in FT and the reason may be due to dominance in ionic interaction than hydrophobic interaction. Removal of impurities was observed in low pH wash and target protein was eluted during re-equilibration of the column with equilibration buffer. Purified water was used in the elution to elute hydrophobically bound proteins also. Since most of the proteins eluted completely in high salt elution condition, optimizing the loading pH and salt concentration in elution may help in reducing the impurities. Based on RP-LC analysis of elution pool, observed purity was around 42% (Figure 14).

Some impurities were removed in low pH wash step and there was no effect of purified water in removing impurities in Capto adhere MMC Chromatography Run III (Figure 4). It was observed in SDS-PAGE (Figure 4) that elution by salt gradient led to two peak, where fractions from first peak containing high level of target protein with impurities when compared to second elution peak. Half of the target protein did not bind to the resin and eluted in flow through. The product purity was better, when eluted using salt gradient. This chromatography conditions yielded to approximately 52 % purity (Figure 15).



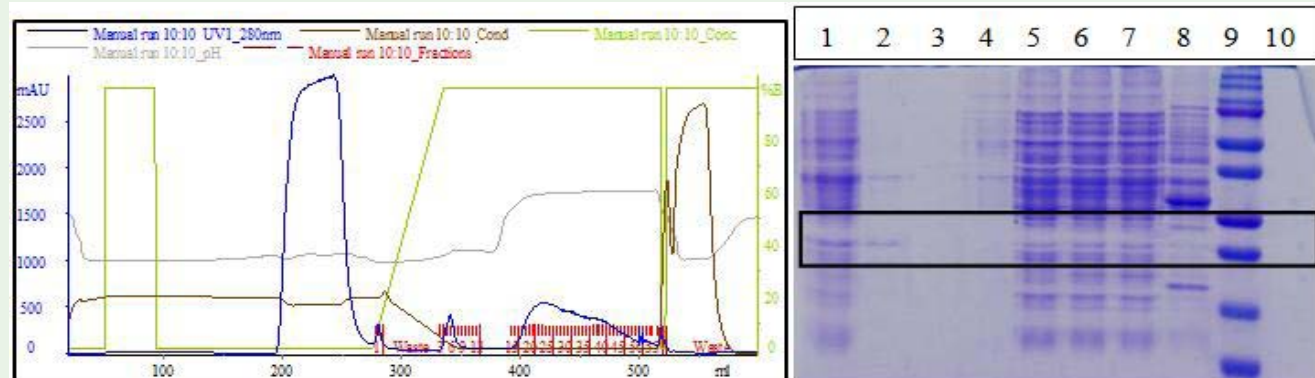
**Figure 4:** Chromatogram for Capto Adhere (Run III) and SDS-PAGE Analysis (10%).

Lane details: Lane 1: Load, Lane 2: FT, Lane 3: Fraction #1, Lane 4: Fraction #2-4, Lane 5: Fraction #5, Lane 6: Fraction #6, Lane 7: Fraction #7-8, Lane 8: Fraction #9-11, Lane 9: Fraction #12-20, Lane 10: Bio-Rad Pre-stained Broad range Protein Marker (Cat # 1610374).



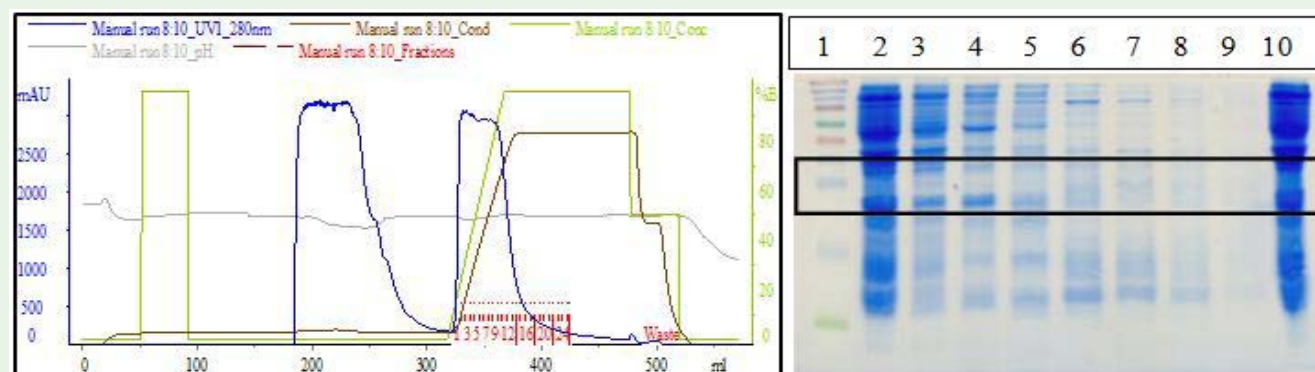
**Figure 5:** Chromatogram for Capto MMC and SDS-PAGE Analysis (10%).

Lane details: Lane1: Load, Lane 2: FT, Lane 3: Fraction #1, Lane 4: Fraction#2, Lane 5: Fraction#3, Lane 6: Fraction #4, Lane 7: Fraction#5, Lane 8: Fraction #6, Lane 9: Fraction#7-9, Lane 10: Bio-Rad Pre-stained Broad range Protein Marker (Cat # 1610374).



**Figure 6:** Chromatogram for Nuvia cPrime and SDS-PAGE Analysis (10%).

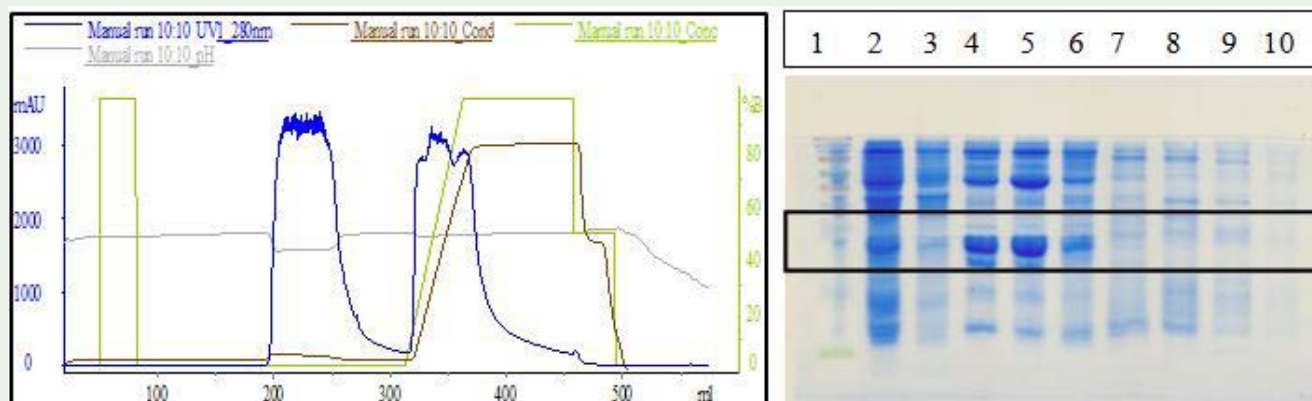
Lane details: Lane 1: Load, Lane 2: FT, Lane 3: Fraction #1-2, Lane 4: Fraction # 3-7, Lane 5: Fraction #16-29, Lane 6: Fraction #30-36, Lane 7: Fraction #37-50, Lane 8: Fraction #56-57, Lane 9: Bio-Rad Pre-stained Broad range Protein Marker (Cat # 1610374), Lane 10: Empty.



**Figure 7:** Chromatogram for BRX-aXM Type I and SDS-PAGE Analysis (10%).

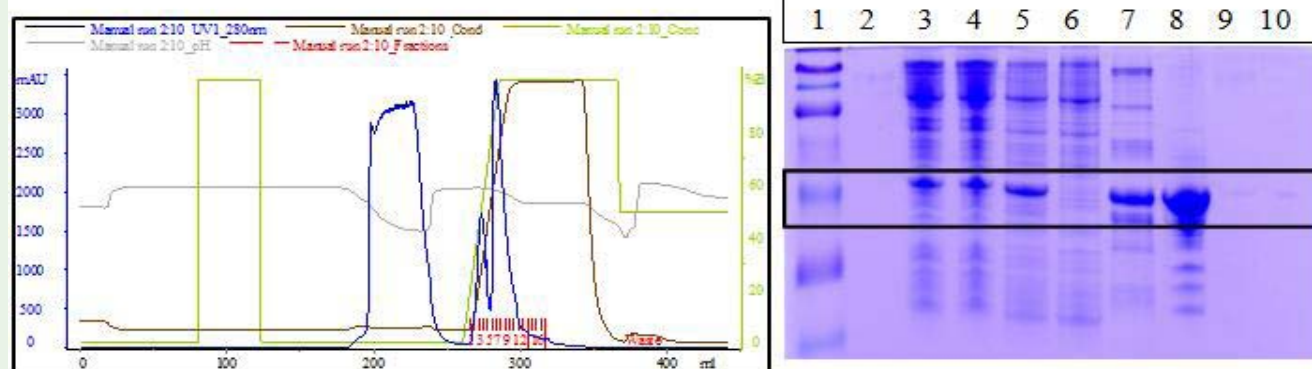
Lane Details: Lane 1: ThermoFisher Scientific Protein Marker (Cat # 26634), Lane 2: Load, Lane 3: FT, Lane 4: Fraction #2, Lane 5: Fraction #4, Lane 6: Fraction #7, Lane 7: Fraction #9, Lane 8: Fraction #11, Lane 9: Fraction #14, Lane 10: Cell Lysate.





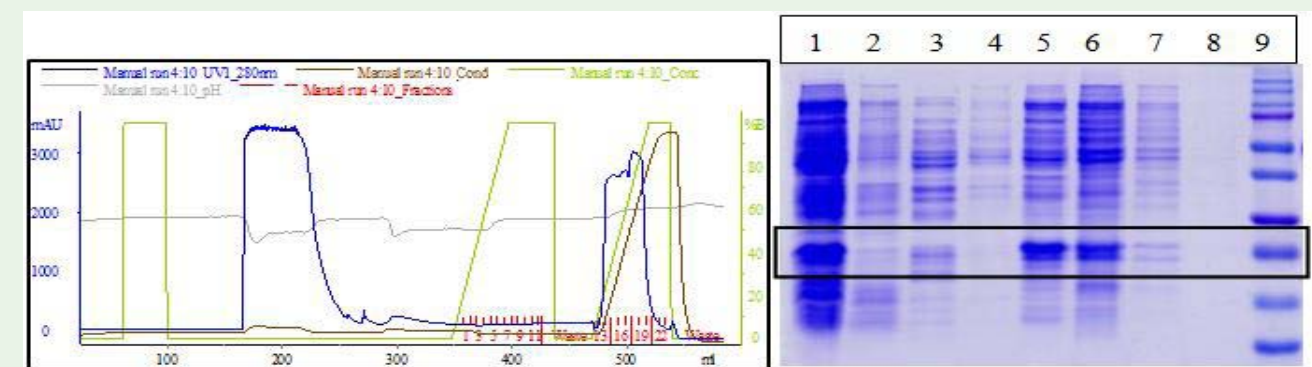
**Figure 8:** Chromatogram for BRX-aXM Type II (Run-I) and SDS-PAGE Analysis (10%).

Lane Details: Lane 1: ThermoFisher Scientific Protein Marker (Cat # 26634), Lane 2: Load, Lane 3: FT, Lane 4: Fraction #2, Lane 5: Fraction #3, Lane 6: Fraction #5, Lane 7: Fraction#8, Lane 8: Fraction#10, Lane 9: Fraction #13, Lane 10: Fraction #17.



**Figure 9:** Chromatogram for BRX-aXM Type II (Run-II) and SDS-PAGE Analysis (10%).

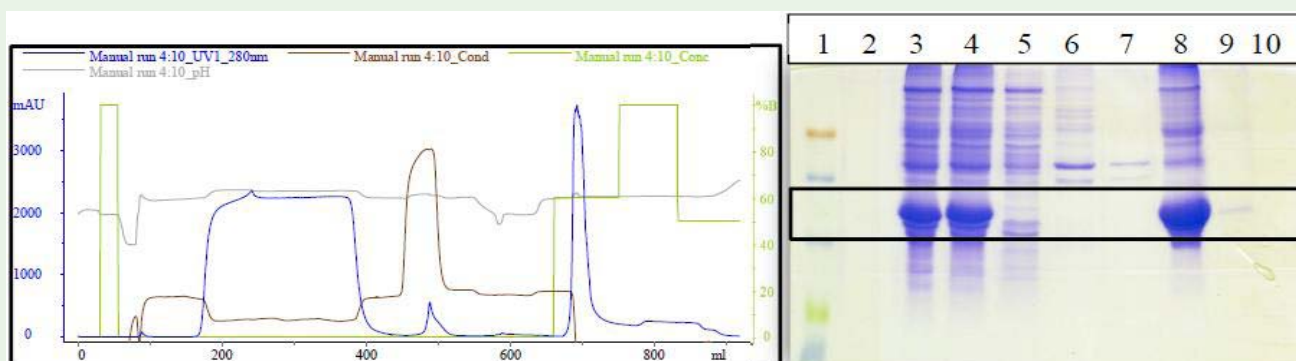
Lane Details: Lane 1: Colorburst Sigma Protein Marker (Cat # C1992), Lane 2: Empty Lane 3: Passage #1, Lane 4: Passage #2, Lane 5: Load (after ammonium sulphate precipitation), Lane 6: Flow Through, Lane 7: Fractions # 1 to 4, Lane 8: Fractions # 6 to 13, Lane 9: Fraction#14, Lane 10: Fraction #16.



**Figure 10:** Chromatogram for Q-Sepharose 6FF and SDS-PAGE Analysis (10%).

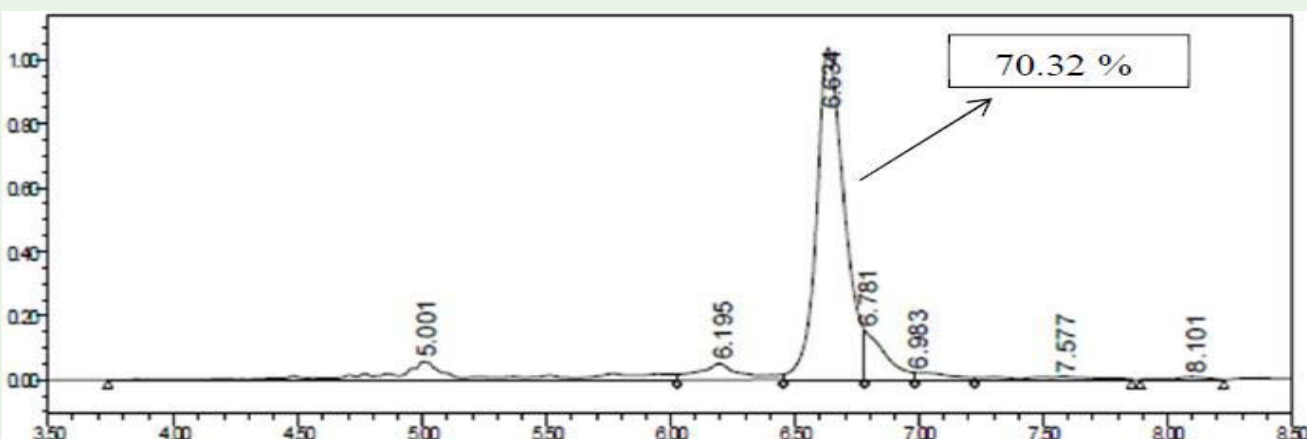
Lane details: Lane 1: Load, Lane 2: FT, Lane 3: pH 6.5 wash, Lane 4: Fraction#13, Lane 5: Fraction#14-16, Lane 6: Fraction #17, Lane 7: Fraction #18-19, Lane 8: Fraction #20-22, Lane 9: Bio-Rad Pre-stained Broad range Protein Marker (Cat # 1610374).



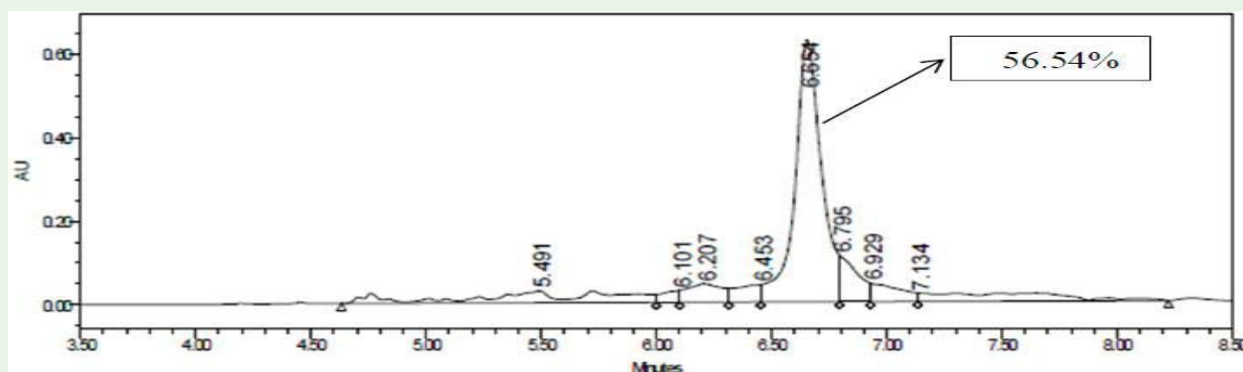


**Figure 11:** Chromatogram for IMAC Nickel Sepharose FF and SDS-PAGE Analysis (10%).

Lane details: Lane 1: Molecular weight Marker (Thermo Fisher Spectra Multi color Low range, Cat # 26628), Lane 2: Empty, Lane 3 and 4: Load, Lane 5: FT, Lane 6: Wash 01 (High salt Wash), Lane 7: Wash 02 (40 mM Imidazole Wash), Lane 8: IMAC Elution 01, Lane 9: IMAC Elution 02, Lane 10: Empty.



**Figure 12:** RP-LC analysis of Fraction #11-15 from MEP Hypercel Chromatography.



**Figure 13:** RP-LC analysis of Fraction #33-55 pool from Capto Adhere Run I Chromatography.

**Citation:** Bairavabalakumar N, Amol M, Pravinkumar D, Prasad BK, Sathyabalan M, Bharath RS, et al. Evaluation of Recombinant Fusion Protein Purification Using Mixed Mode Chromatography (MMC). Ann Chromatogr Sep Tech. 2017; 3(1): 1030.

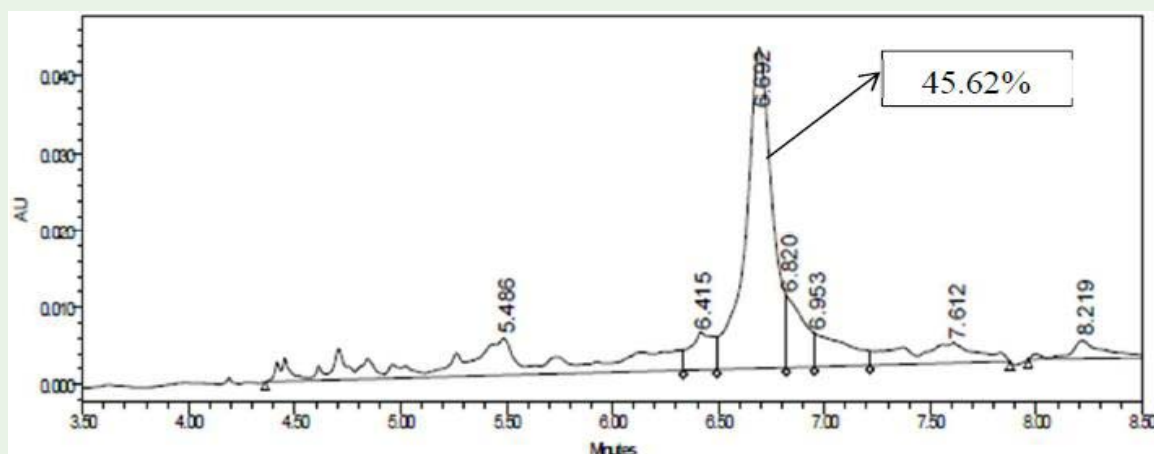


Figure 14: RP-LC analysis of Fraction #04-12 pool from Capto Adhere Run II Chromatography.

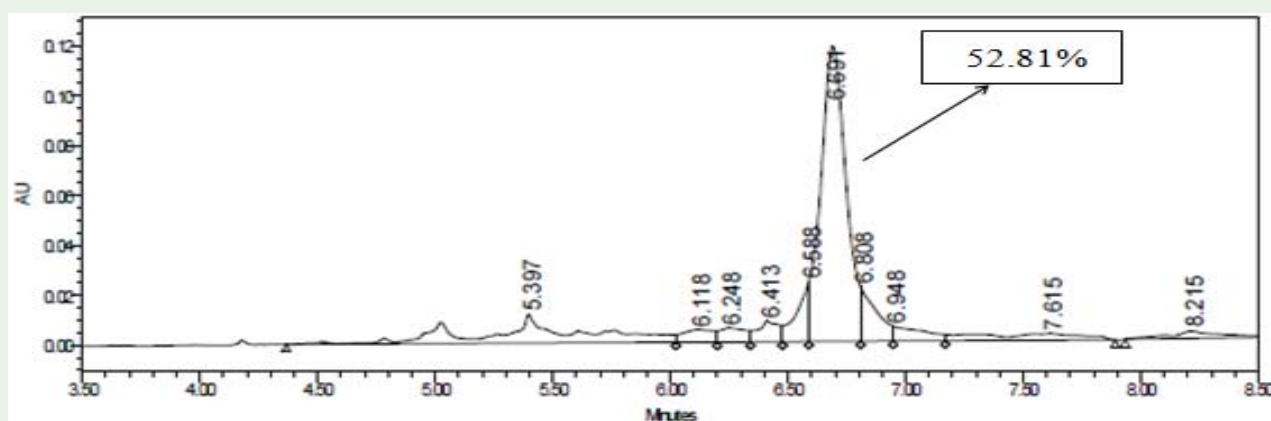


Figure 15: RP-LC analysis of Fraction #05-11 pool from Capto Adhere Run III Chromatography.

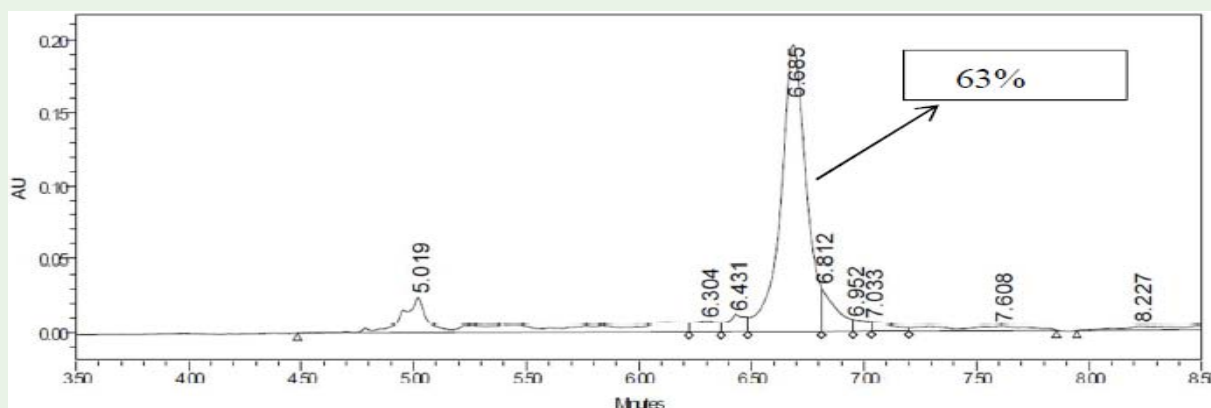
Capto MMC was employed as a negative binding mode chromatography (Figure 5) where sample was loaded in acidic pH (pH 4.0). Chromatography profile showed a small elution peak, when eluted in salt gradient mode. SDS-PAGE analysis (Figure 5) revealed the presence of fusion protein in FT, but mainly with high level of host cell proteins. Only host cell proteins with traces of fusion proteins bound to the column. Here, an attempt was made to check the purity by operating in negative binding mode (Flow through mode) as a capture stage. In general, chromatography in FT mode as a capture step will not be a viable step unless good purity and highest recovery is achieved.

Nuvia cPrime, a cationic mixed mode resin used as a Capture step, where cell lysis was performed at pH 4.0 since the pI of protein is ~ 5.20. The solubility of fusion protein is less at low pH (data not shown). In this chromatography (Figure 6), fusion protein was observed in both flows through as well as in elution. But, the purity of the fusion protein was better in flow through. The elution contained target protein, but was co-eluted with majority of host cell proteins.

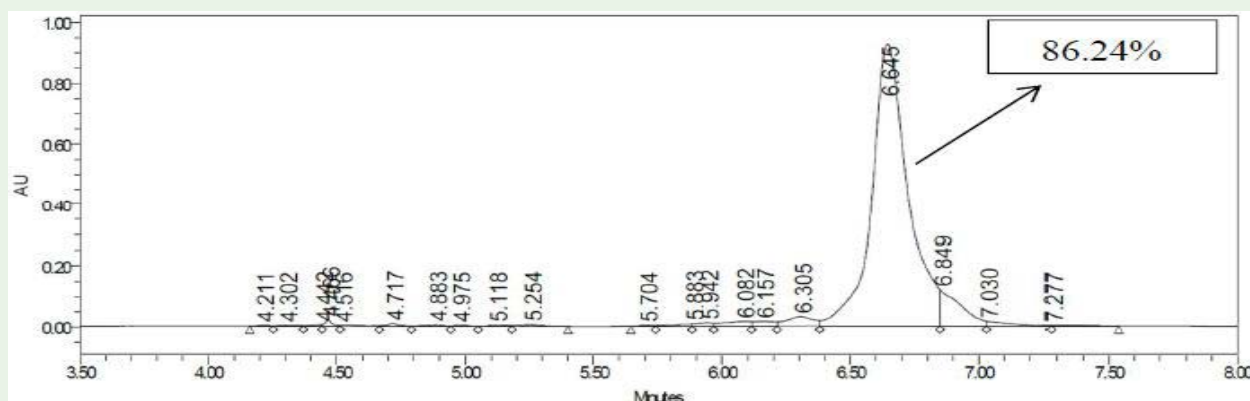
In the case of anion exchange mixed mode chromatography,

where two types of resins employed in purification, BRX-aXM Type I (Figure 7) and BRX-aXM Type II Run I (Figure 8), fusion protein was observed in flow through and some amount of fusion protein eluted along with impurities. In both types of resin, fusion protein eluted with lots of host cell proteins. But, the profile was different between two chromatography. In BRX-aXM Type I mixed mode chromatography, higher amount of impurities were eluted along with very less fusion protein and half of the fusion protein even did not bind to the resin and eluted in flow through. But, in BRX-aXM Type II Run-II (Figure 9), fusion protein eluted in flow through was less when compared to BRX-aXM Type I MMC but the impurities level was higher as like in BRX-aXM Type I. Evaluated ammonium sulphate and acidic pH based precipitation to reduce the impurities before loading on the capture column. Ammonium sulphate at 0.4 M concentration efficiently removed (data not shown) the impurities. The purity of target protein is better in BRX-aXM Type II Chromatography Run-II. Based on RP-LC analysis, the purity of fractions pool was approximately 63% (Figure 16), and purity can be further increased by changing chromatography conditions.

**Citation:** Bairavabalakumar N, Amol M, Pravinkumar D, Prasad BK, Sathyabalan M, Bharath RS, et al. Evaluation of Recombinant Fusion Protein Purification Using Mixed Mode Chromatography (MMC). Ann Chromatogr Sep Tech. 2017; 3(1): 1030.



**Figure 16:** RP-LC analysis of Fractions # 6 to 13 BRX-aXM Type II (Run-II) Chromatography.



**Figure 17:** RP-LC analysis of single fraction from IMAC Nickel Sepharose FF Chromatography.

MMC was compared with Anion exchange chromatography to check the purity and recovery. In anion exchange chromatography, target protein was partially bound to column and observed high level of impurities in the elution when eluted in linear salt gradient. Additionally, the solubility of target protein was also low due to lysis at low salt concentration. Cabanne *et al.* carried similar kind of studies, where affinity chromatography was compared with mixed mode chromatography in the purification of a recombinant Maltose-Binding Protein (MPB). Yield of purified protein was higher with mixed-mode resins than affinity resins, and purity was comparable to affinity chromatography [31].

Due to higher specificity, IMAC yielded higher recovery and purity. Based on RP-LC results, purity achieved in affinity chromatography was ~ 86 % (Figure 17) and it is possible to achieve either similar or higher purity using MMC by optimizing various binding and elution conditions.

## Conclusion

Downstream processing of therapeutic proteins comprises upto 80 percentage of entire production cost. In recent years, many resins are being developed to reduce the cost involved in downstream

processing and the focus is high on improving the downstream process. Especially, affinity chromatography in the purification of biotherapeutic proteins add more cost the production. Here, we describe the screening of various mixed mode resins in the purification of recombinant protein in comparison with affinity chromatography and ion exchange chromatography with limited chromatography conditions. Many other conditions can be evaluated to increase the purity and recovery in MMC. Most of the host contaminants were removed in mixed mode Chromatography step which is similar to affinity chromatography in terms of purity. MMC can be a good alternative to affinity chromatography, where mixed mode chromatography shows good selectivity in capture step. Therefore, mixed mode chromatography can be a good choice to employ in the purification of recombinant fusion proteins.

## References

1. Sofia Costa, André Almeida, António Castro and Lucília Domingues. Fusion tags for protein solubility, purification, and immunogenicity in *Escherichia coli*: the novel Fh8 system. *Frontiers in Microbiology*. 2014; 5: 1-20.
2. Ajamaluddin Malik. Protein fusion tags for efficient expression and purification of recombinant proteins in the periplasmic space of *E. coli*. *3 Biotech*. 2016; 44: 1-7.

**Citation:** Bairavabalakumar N, Amol M, Pravinkumar D, Prasad BK, Sathyabalan M, Bharath RS, et al. Evaluation of Recombinant Fusion Protein Purification Using Mixed Mode Chromatography (MMC). *Ann Chromatogr Sep Tech*. 2017; 3(1): 1030.



3. Sophie Maria, Gilles Joucla, Bertrand Garbay, Wilfrid Dieryck, Anne-Marie Lomenech, et al. Purification process of recombinant monoclonal antibodies with mixed mode chromatography. *Journal of Chromatography*. 2015; A1393: 57-64.
4. Qing-Hong Shi, Zheng Cheng, Yan Sun. 4-(1H-imidazol-1-yl) aniline: a new ligand of mixed-mode chromatography for antibody purification. *Journal of Chromatography A*. 2009; 33: 6081-6087.
5. Chunfang Zhang, Dale P Fredericks, Eva M Campi, Milton TW Hearn. Application of 4'-terpyridinylsulfanylethylamine resins for the purification of monoclonal antibodies by mixed-mode Chromatography. *Protein Expression and Purification*. 2014; 104: 34-40.
6. Virginie Brenac Brochier, Anthony Schapman, Patrick Santambien, Lothar Britsch. Fast purification process optimization using mixed-mode chromatography sorbents in pre-packed mini-columns. *Journal of Chromatography A*. 2008; 1177: 226-233.
7. M Sivapragasam, A Norhafizah. Purification of Glutathione S-transferase (GST) using Mixed Mode Chromatography. *International Journal of Engineering Research & Technology (IJERT)*. 2014; 3: 1292-1299.
8. Jerome Pezzini, Gilles Joucla, René Gantier, Magali Toueille, Anne-Marie Lomenech, Caroline Le Sénéchal, et al. Antibody capture by mixed-mode chromatography: a comprehensive study from determination of optimal purification conditions to identification of contaminating host cell proteins. *Journal of Chromatography A*. 2011; 1218: 8197- 8208.
9. Judith Vajda, Egbert Mueller and Eva Bahret. Dual salt mixtures in mixed mode chromatography with an immobilized tryptophan ligand influence the removal of aggregated monoclonal antibodies. *Biotechnology Journal*. 2014; 9: 555-565.
10. Chunfang Zhang, Dale P Fredericks, Eva M Campi, Milton TW Hearn. Application of 4'-terpyridinylsulfanylethylamine resins for the purification of monoclonal antibodies by mixed-mode chromatography. *Protein Expression and Purification*. 2014; 104: 34-40.
11. Qing-Hong Shi, Zheng Cheng, Yan Sun. 4-(1H-imidazol-1-yl) aniline: A new ligand of mixed-mode chromatography for antibody purification. *Journal of Chromatography A*. 2009; 1216: 6081-6087.
12. Atsushi Hirano, Tsutomu Arakawa, Tomoshi Kameda. Interaction of arginine with Capto MMC in multimodal chromatography. *Journal of Chromatography A*. 2014; 1338: 58-66.
13. Juan Wang, Eleanor W Jenkins, Julie R Robinson, Anastasia Wilson, Scott M Husson. A new multimodal membrane adsorber for monoclonal antibody purifications. *Journal of Membrane Science*. 2015; 492: 137- 146.
14. Rebecca A Chmielowski, Sandra Meissner, David Roush, Thomas O. Linden, Edward Glowacki, Janelle Konietzko, et al. Resolution of heterogeneous charged antibody aggregates via multimodal chromatography: A comparison to conventional approaches. *Biotechnology Progress*. 2014; 30: 636-645.
15. Kimberly A Kaleas, Margaret Tripodi, Scott Revelli, Vikas Sharma, Shelly A Pizarro. Evaluation of a multimodal resin for selective capture of CHO-derived monoclonal antibodies directly from harvested cell culture fluid. *Journal of Chromatography B*. 2014; 969: 256-263.
16. Xiaoyu Yang, Ying Zhang, Fengqiang Wang, Larry (Jin) Wang, Daisy Richardson, Mohammed Shameem, et al. Analysis and Purification of IgG4 Bispecific Antibodies by a Mixed-Mode Chromatography. *Analytical Biochemistry*. 2015; 484: 173-179.
17. Tiago Matos, João A. Queiroz and Leif Bülow. Plasmid DNA purification using a multimodal chromatography resin. *Journal of Molecular Recognition*. 2014; 27: 184-189.
18. Tiago Matos, João A. Queiroz, Leif Bülow. Binding and elution behavior of small deoxyribonucleic acid fragments on a strong anion-exchanger multimodal chromatography resin. *Journal of Chromatography A*. 2013; 1302: 40-44.
19. Xuefang Dong, Aijin Shen, Zhimou Gou, Deliang Chen, Xinmiao Liang. Hydrophilic interaction/weak cation-exchange mixed-mode chromatography for chitooligosaccharides separation. *Carbohydrate Research*. 2012; 361: 195-199.
20. GE-Healthcare product manual.
21. GE-healthcare product manual.
22. Bio-Rad product manual.
23. Bio-Rad product manual.
24. Pall Corporation product manual.
25. Pall Corporation product description.
26. Pall Corporation product description.
27. GE Healthcare product manual.
28. Tosoh Biosciences product manual.
29. Merck Millipore product manual.
30. Bio-Rad product manual.
31. Charlotte Cabanne, Jérôme Pezzini, Gilles Joucla, Agnès Hocquellet, Caroline Barbot, Bertrand Garbay, et al. Efficient purification of recombinant proteins fused to maltose-binding protein by mixed-mode chromatography. 2009; 1216: 4451- 4456.