Purification of Protein

Dissertation Submitted in partial fulfillment for the degree of

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DECLARATION

I hereby declare that the dissertation entitled "***Purification Of Protein*** " submitted by me, for the degree of Master of Science to KIIT University is a record of bonafide work carried by me under the supervision of, ***Mr. Satish Kale*** *Assistant Manager of M J Biotech Pvt. Ltd., Pune, Maharashtra*

Date: 05/05/2018

Place: Pune, Maharashtra

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Abstract:

Insulin is a polypeptide hormone secreted from the beta cells of islets of langerhans gland in pancreas. This protein has two chains A and B chain, A chain carry 21 amino acid and B chain carry 30 amino acid. It helps to store glucose as glycogen in hepatic cell, muscle and it also help lipolysis by which the prevention of ketone body formation can done. But if there any auto immune reaction occur in beta cell the production of insulin can no longer be synthesized by and the result is Type-1 diabetes mellitus, so to prevent this doctor advice to take insulin injection. In the past century recombinant DNA technology was just an imagination but now this is the one and only the best way to produce protein like insulin. Recombinant human insulin has a faster onset of action and show lower immunogenicity than the conventional insulin which was produce from pig. The glucose concentrations in the blood become less when their therapy is switched from animal-source insulin to human insulin but the dosage should be as per the physician.

Recombinant Insulin can be produced in the form of Inclusion bodies or in the soluble form. Due to lower yield in case of soluble protein, the method of expressing the heterologous protein in the form of Inclusion bodies are preferred. This require the expressed protein denaturation and renaturation steps followed by its purification using different chromatography techniques.

In the current project, purification of recombinant human insulin was carried out by two type of anion exchange chromatography i.e., initial capturing the protein of interest using weak anion exchange chromatography followed by intermediate purification step wherein strong anion exchange chromatography is applied. Using these techniques, the purity of the insulin reaches> 90%.

For final polishing, high pressure chromatography (RP-HPLC) is used, wherein purity of protein of interest is further improved and reaches a value of > 99% purity. Along with these chromatographic techniques, various other steps are followed for Downstream processing such as enzymatic digestion using Carboy Peptidase B enzyme, which convert the pro-insulin into the active insulin by splicing the lysine arginine amino acid which join the N terminal of the A chain and C terminal of the B chain and other tangential flow filtration techniques for concentration and buffer exchange.

The main agenda of the process is to produce API conforming to national and international standards as per established GMP procedures.

➢ Abbreviations:

- CIP- Cleaning In Place
- SIP: Sterilization In Place
- WFI: Water For Injection
- cWFI: Cooled Water For Injection
- PuW: Purified Water
- RPHPLC: Reverse Phase
- c-GMP: Current Good Manufacturing Practice
- GDP: Good Documentation Practice
- TFF: Transient Flow Filtration
- HMWP: High Molecular Weight Protein
- OD: Optical Density
- TA: Total Absorbance
- NaCl: Sodium Chloride
- NaOH: Sodium Hydroxide
- NA: Not Applicable
- RM: Raw Material
- API: Active Pharmaceutical Ingredient

1.Introduction:

1.1 Background and Context:

Proteins square measure a unit big biomolecules, or macromolecules that which might be exist in nature as polymer, dimer, and monomer and consisting of 1 or lots of long chains of compound (amino acid residue). a massive vary of operate is performed among organs by macromolecule, furthermore as catalyzing metabolic reactions, deoxyribonucleic acid replication, responding to stimuli, and transporting molecules from one location to a distinct among the body. Proteins differs from each other primarily in their specific sequence of amino acids, that is distinguish by the nucleotide sequence of their genes, and that typically leads to organic process into a selected three-dimensional structure or tertiary structure that determines it's activity. To perform any quite quite in-vitro analysis, a macromolecule should to be pure far from various cellular elements. This methodology generally begins with cell lysis by a homogenizer or sonicator, throughout that a cell's membrane get disrupted and its internal contents get free into a solution referred to as a crude lysate, and the certain mixture will be purified by ultracentrifugation and other technique, that fractionates the varied cellular elements into fractions containing soluble proteins; membrane lipids and proteins, cellular organelles, and nucleic acids. Precipitation by a way known as salting out and salting in and it will concentrate the proteins from this lysate. varieties of activity (i.e RPHPLC, Size exclusion action, gel filtration action e.t.c.,) square measure then wont to isolate the proteins of interest supported it's properties like relative molecular mass, net charge and binding affinity e.t.c. If the relative molecular mass and iso-electric points of the specified protein's are far-famed then the extent of purification will be monitored by a versatile gel electrophoresis (i.e SDS-PAGE), and by spectroscopy analysis however in this case the protein should has identical spectroscopical features, or by an enzyme assays however in this case the protein ought to should has the enzymatic activity. in addition, by the treatment of electro focusing proteins are often isolated in keeping with their charge. For natural proteins, a series of purification steps should be required to get the knowledge that's the protein sufficiently pure for the laboratory applications. To oversimplify this method, recombinant DNA technology is commonly wont to add chemical options to proteins that build them easier to purify while not poignant their specific structures or activity. currently a days a spread of assorted tags have in addition been developed to help researchers to purify the particular proteins from advanced mixtures. one in all this sort of technique could be a "tag" that consists of a particular amino acid sequence, usually a series of essential amino acid residues (a "His-tag"), that is connected to at least one terminus of the supermolecule either C-terminus or the N-terminus. As a result, once there's state of affairs involves pass the lysate over a column that contained by nickel

metal, the histidine residue helps to lygate the nickel and fix to the column whereas the untagged parts of the lysate clear.

Insulin is a ploypeptide internal secretion created by beta cells of the pancreatic gland islets, and it's thought of to be the most anabolic internal secretion of the body. It regulates the metabolism of carbohydrates, proteins and fats by promoting the absorption of, especially, the aldohexose from the human blood into liver, fat and skeletal muscle cells. During this case the tissues absorbe aldohexose that get regenerate into either glycogen via glycogenesis or fats (triglycerides) via lipogenesis, or, among the case of the liver, it regenerate into each. Production and secretion of aldohexose by the liver is powerfully inhibited by high concentrations of hypoglycemic agent internal secretion molecule among hypoglycaemic agent insulin macromolecule within the blood. Hypoglycaemic agent whereas gift in blood it jointly affects the synthesis of proteins in a very large choice of tissues. For this reason it's known as an anabolic peptide hormone, which promoting the conversion of tiny molecules within the blood into giant molecules within the cells. However low hypoglycaemic agent levels within the blood have the alternative impact by promoting widespread organic process, particularly of reserve body fat. hypoglycaemic agent has 2 variety of peptide chain. The A-chains and B- chains, each chain coupled along by disulfide bond (S-S). it's but initial synthesized as one peptide chain known as preproinsulin in beta cells. The preproinsulin contains a 24-residue signal peptide chain that directs the emerging peptide chain to the rough endoplasmic reticulum (RER). The signal peptide chain is cleaved because the peptide is translocated into lumen of the endoplasmic reticulam, by that proinsulin get fashioned. With in the rough endoplasmic reticulam the proinsulin folds into the right conformation wherever three disulfide bonds get fashioned. During the time of assembly in rough endoplasmic reticulam to make the immature granules the proinsulin is transported from rough endoplasmic reticulum to the trans-Golgi network (TGN) within 5-10 minutes. Transport to the TGN it should take regarding half-hour. The maturation of activeinsulin from pro-insulin is done through the action of cellular endopeptidases enzyme that is known as prohormone convertases, in addition because the exoprotease carboxypeptidase B. The endopeptidases cleave at a pair of positions of proinsulin and, cathartic a fraction that termed because the C-peptide, and deed a pair of amide chains, the B- and A-chains, coupled by a pair of disulfide bonds (S-S). The cleavage sites are situated with in a selected combine of a basic residues. When cleavage of the C-peptide, these a pair of pairs of basic residues are removed by the carboxypeptidase B it itself is a protein. In case of proinsulin the C peptide is the main portion, and therefore the primary sequence of proinsulin goes within the order "B-C-A" (the B and A chains were known on the premise of mass and therefore the C peptide discovered) By that the

guaranteeing mature hypolycaemic agent get prepackaged at intervals mature granules anticipating metabolic signals (such as essential organic compound, arginine, essential amino acid and mannose) and vegal nerve stimulation to be exocytosed from the cell into the circulation. Beta cells at intervals the islets of langerhans unhitch hypoglycemic agent in a very two section. The first-phase unharness is apace triggered in response to raised glucose levels, and it lasts for concerning ten minutes. The second section could be a sustained, slow unharness of recently fashioned vesicles triggered severally of sugar, peaking with-in a pair of to three hours. Reduced first-phase hypoglycemic agent unharness is also the earliest detectable cell defect predicting onset of kind a pair of polygenic disorder. The first phase unharness and hypogyceemic agent sensitivity is freelance predictors of polygenic disorder or diabetes which is a herediatery disease

The description of first phase release is as follows:

- Glucose enters into the β-cells through the glucose transporters, GLUT2. These glucose transporters (GLUT2) has a relatively low affinity for glucose, ensuring that the rate of glucose entry into the β-cells is proportional to the extracellular glucose concentration (within the physiological range). During low blood sugar levels very little glucose enters into the β-cell and at high blood glucose concentrations large quantities of glucose enter into these cells.
- The glucose that enters into the β-cell is get phosphorylated to glucose-6-phosphate (G-6-P) by glucokinase (hexokinase IV) which is not inhibited by glucose-6-phosphate in the way that the hexokinases which is present in other tissues (hexokinase I – III) are affected by this product. This means that the intracellular glucose-6-posphate concentration remains proportional to the blood sugar concentration.
- Glucose-6-phosphate(G-6-P) enters into the glycolytic pathway and after that via the pyruvate dehydrogenase reaction,it get enters into the Krebs cycle, where multiple, high-energy ATP molecules are get produced by the oxidation of acetyl CoAenzyme (the Krebs cycle substrate), leading to a rise in the ATP and ADP ratio within the cell.
- An increased intracellular ATP and ADP ratio closes the ATP-sensitive potassium channel. This potassium ions (K^+) is very useful because this there is no facilitated diffusion occur in the cell, andit also help to buildup of intracellular potassium ions. As a result, the within of the cell becomes less negative with relevancy the skin, leading to the depolarization of the cell surface membrane.
- Based upon depolarization, the voltage-gated calcium ion (Ca^{2+}) channels get open, and allowing the calcium ions to move into the cell by facilitated diffusion.

- The cytosolic calcium ion concentration can also get increased by the release of calcium ion from intracellular stores via activation of ryanodine receptors.
- The calcium ion concentration in the cytosol of the beta (β) cells can also, or additionally, get increased through the activation of phospholipase C which results the binding of an extracellular ligand (hormone or neurotransmitter) to a G protein-coupled membrane receptor (GPCR). Phospholipase-C cleaves the lipid membranes of phospholipid, phosphatidyl inositol 4,5-bisphosphate,which get converted into inositol 1,4,5-trisphosphate and diacylglycerol. Inositol 1,4,5-trisphosphate (IP3) then binds to the receptor proteins in the plasma membrane of the endoplasmic reticulum (ER). This allows the discharge of Calcium ions from the endoplasmic reticulam via the IP3-gated channels, that raises the cytosolic concentration of Ca ions (Ca^{2+}) severally of the results of a high blood sugar concentration. During this manner the parasympathetic stimulation of the pancreatic islets operates via this pathway to extend insulin secretion into the blood.
- The considerably increased quantity of metallic element ions (Ca2+) within the living substance of the cells causes the discharge into the blood of antecedently synthesized internal secretion (insulin), that has been keep in living thing secretory vesicle. Other substances proverbial to stimulate internal secretation (insulin) unleash embody the amino acids like essential amino acids like arginine threonine e.t.c, the parasympathetic unleash of neurotransmitter(acetylcholine , sulfonylurea, cholecystokinin) , antidiabetic, cholecystokinin (CCK, conjointly via phospholipase C), and therefore the gastrointestinally derived incretins, like glucagon-like peptide-1(GLP-1) and glucose dependent insulinotropic peptide (GIP). catecholamine (noradrenaline) will powerfully inhibit the discharge of internal secretation, By that the glucose level get increased throughout stress condition. It seems that the unleash discharge of catecholamines by the sympathetic system an ervosum has conflicting influences on insulin release by beta cells, as a result of unleash of insulin will solely inhibited by α2-adrenergic receptors and stirred up by β2-adrenergic receptors. Cyberspace result of catecholamine from sympathetic nerves and insulin from adrenal glands on internal secretion unleash is inhibition thanks to dominance of the α-adrenergic receptors.

When the aldohexose level comes all the way down to it's usual physical worth, endocrine (insulin) unleash from the β-cells additionally slows or stops. If the blood sugar level drops not up to this, particularly into perilously low levels, unleash of hyperglycemic hormones (most conspicuously glucagon from islets of Langerhans alpha cells) forces unleash of aldohexose into the blood from the liver glucose stores, supplemented by gluconeogenesis if the glucose stores become depleted. By increasing blood sugar, the hyperglycemic hormones stop or currect severe symptom condition of an individual's being. Evidence of the impaired first-phase insulin unleash will be seen by the aldohexose tolerance check, incontestable by a well elevated blood sugar level

at half hour when the body process of a glucose load(75 or one hundred g of glucose), followed by a slow drop over successive one hundred minutes, to stay on top of one hundred twenty mg/100 metric capacity unit when 2 hours when the beginning of the check. In an exceedingly traditional person the blood sugar level is corrected by the tip of the check. Well this is often the natural manner for synthesis and unleash of insulin in human body.

But as we know that when the level of insulin in blood become low as it should present in the blood then doctors advice us to intake insulin injection.

Production of insulin in-vivo we have to undergo recombinant DNA technologies. Where we over expressed the insulin and after that we rupture the cell and release the protein into the outside but in this way we can not get the insulin or any protein in pure form there must be other proteins and inclusion bodies are also present normally we have called this impurities or unwanted proteins. So to remove the impurities and to get the pure form of insulin we have to do protein purification. The process to synthesis the protein in a live cell and to rupture the cell for releasing the protein is undergoes the Upstream process and the purification steps is defined as Downstream process. There are lots of way to purify the protein but here we have done the ion exchange chromatography, HPLC, Size exclusion chromatography, e.t..c In ion exchange chromatography we have undergoes anion exchange chromatography. Here two type of anion exchange chromatography is use to purify the insulin protein. The first one is chromatography-1 and the second one is chromatography-2 and after getting the crude insulin a HPLC is perform to finally purify the product and the next step is to covert the product into a powdered form via lyophilization.

1.2 Scope and Objectives:

Downstream processing includes all steps needed to purify a biological product from cell culture broth to final purified product. It involves multiple steps to capture the target macromolecule like protein and to get rid of host cell connected impurities (e.g., host cell proteins, DNA, etc.), method connected impurities (e.g., buffers, leached ligands, antifoam, etc.) and product connected impurities (e.g., aggregates, fragments, clipped species, etc.). Every purification step is capable of removing one or additional categories of impurities. Downstream process sometime encompasses 3 main stages, namely

(i) Initial recovery (extraction or isolation),

(ii) Purification (removal of most contaminants)

(iii) Polishing (removal of specified contaminants and unwanted forms of the target protein insulin that may have formed during isolation and purification).

Initial recovery involves the separation between cell and supernatant (broth clarification). For this purpose, the main operations employed are centrifugation, filtration, sedimentation, and flotation. Insulin is produce extracellularly, the clarified broth is submitted to concentration (e.g., ultrafiltration) followed by purification. Secreted and soluble insulin in the culture media can be directly recovered by centrifugation. Samples then be concentrated and the target protein purified from the supernatant by processes such as precipitation, and chromatography. For intracellular protein, the cells harvested must be submitted to lysis (e.g., high-pressure homogenizer, sonication, passing through mills, etc.) followed by clarification to remove cell debris. The target protein is purified from the clarified cell homogenate (usually by precipitation and/or chromatography). In cases where proteins are expressed as inclusion bodies (as some recombinants produced by *E. coli*), an extra step of protein refolding (buffer exchange) is required. These additional steps significantly contribute to increases in production time and costs for intracellular protein insulin.

Efficient recovery and purification of insulin have been referred as a critical part of the production process. Purification process must be robust, reliable, easily scaled-up, and capable of removing both processes- and product-related impurities to ensure product safety. The achieved purity, the speed of process development, overall recovery yield, and throughput are some of the main key parameters that must be taken into consideration during downstream process development. To reach the stringency of purity required in the industry, exceeding 95%, chromatography steps are usually required. Chromatography allows for high resolution and has traditionally been the workhorse for protein purification and polishing. However, chromatography has also been the major cost center in purification processes, mainly due to media cost and relatively long cycle times. In addition, the industry still faces practical limitations in terms of throughput and scalability.

The main objective of downstream process should be to deliver the highest yield of the purest product at the shortest time and cost. However, traditional processes and quality control does not bring the efficiency needed to keep pace with current upstream production. To address current issues, some general trends emerge as most relevant including single use modules, continuous production, process analytical technology, and quality by design. The disposable units are compatible with continuous mode and bring faster routine operation because no cleaning or cleaning/validation has to be performed. Continuous processes generally result in higher productivity, less buffer consumption, and smaller footprint. A general end-to-end continuous process can be accomplished by perfusion cell reactors coupled with a continuous capture step, integrated with some of the downstream technology.

1.3 Achievements:

For the last six month I have done my project in MJ Biotech Pvt.Ltd and during this session I got a good exposure to learn a lot of things, also to handle many technique. The main product of this industry is manufacturing of GMP compliant insulin, which has been marketed in the form of API (Active Pharmaceutical Ingredient). So for the last six month ,I got a chance to work in DSP department where in the purification of the insulin is carried out. So to this a lots of instruments are used like anion exchange chromatography, RPHPLC, TFF, Nutsche filter, e.t.c, to purify the protein. Personnel involved in the manufacturing activity has to adhere to the standard operating procedures for process operation and equipment cleaning along with various sterilization steps, and to maintain their documents as per the guidelines mentioned in ICHQ7.

1.4 Overview of Dissertation:

After getting the refolded enzymatic treated protein solution, anion exchange chromatography (chromatography-1) is performed to start the purification of the product. For doing the chromatography we need five types of buffer and each buffer has a significant role which help to purify the protein of interest (insulin). The product thus eluted is treated with 1M HCl to bring down the pH and kept it for 12-14 our incubation. Then after 14 hour, a filtration is performed to filter the product.

Then the next step of purification is zinc precipitation and after that centrifugation is carried out. This step is followed by another type of anion exchange chromatography (Chromatography 2). Then after that CPB digestion is carried out in order to convert the pro-insulin to insulin, and again a HPLC system run to purify the product and then finally the main active and purified insulin is converted to powdered form. The main agenda of the downstream department is to concentrate as well as to purify the product, and to reach the purity level $\geq$ 99.95%.

The process of conversion of IBs to active and purified form of insulin has described in the bellow. For doing the downstream process of insulin purification here different kind of technique, solution or buffer is use and as well as different kind of instrument.

- Chromatography:

Different strategies based on sequences of classical chromatography have been described for nucleic acids, peptides, and proteins purification. In fact, chromatography is a very effective purification technique with a wide range of industrial applications and currently represents the favorite choice due to its high resolution capacity. The separation principle in chromatography is based on the differences in the affinity of the species carried by a fluid mobile phase toward a solid stationary phase. When a sample is introduced and transported by the eluent along the column, some of its components will have more

powerful interactions with the stationary phase than others, generating concentration profiles that will percolate the chromatographic column at different speeds. The less retained species will elute earlier from the column than the most retained ones, eventually allowing the collection of the products of interest with a high purity degree. Based on the interaction between the solid stationary phase and protein, chromatographic techniques can be summarized into five classes: (i) affinity, (ii) ion-exchange, (iii) hydrophobic interactions, (iv) size exclusion, and (v) mixed-mode chromatography. Traditional choices in chromatographic set ups include particle-based resins, batch mode operation, and packed columns. In the pharmaceutical industry some alternatives are done based on the above parameters, especially the chromatographic separations based on simulated moving bed (SMB), expanded bed adsorption (EBA), and single block monolith columns.

To purify the merchandise here the ion exchange natural action has used. By an anion-exchange chromatography the protein could be separated according to their charges victimization associate ion-exchange organic resin compound containing positively charged teams, such as diethyl-amino ethyl groups (chromatography-1). In a solution, the resin is always covered with the positively charged counter-ions or cations. To purify proteins, amino acids, sugars and other acidic substances with a negative charge at higher pH scale levels the anion exchange chromatography is very much preferred. The tightness of the binding between the substance and therefore resin is predicated on the strength of the negative charge of the substance and also the positive charge of the protein molecule. The mobile phase is generally a low to medium conductivity or low medium salt concentration in a solution to help the binding of all charged molecule. The adsorption of the molecules to the solid support is driven by the ionic interaction between the oppositely charged ionic teams within the sample molecule and within the purposeful substance on the support. The strength of the interaction is decided by the amount and therefore the location of the charges on the molecule and on the functional group. By increasing the salt concentration the molecules that has the weakest ionic interactions begin to elute from the column initial. The molecules which is bind with resin by strong ionic interaction it needed the a higher salt concentration and the elution of that molecule also occur later by the help of gradient flow.. The binding capacities of ion exchange resins are typically quite high. This has the key importance in process scale chromatography, however it is not essential for analytical scale separations.

Beside this one more strong anion exchange chromatography is use to increase the purity of the insulin, which is Chromatography-2. Four protein can bind in one resin particle in case of Chromatography 2 that is the reason it's called strong anion exchange chromatography. Normally the strong or the weakness of a resin depends on the pH, the weak chromatography work upon very low range of pH where as the strong anion exchange chromatography works on a very vast range of

pH. Weak anion exchanger function poorly above a pH of 9. When working with ion exchange resin such as diethyl aminoethyl (chromatography-2) resin, it is important to work within the supplier-provided working pH range. Strong ion exchangers are most well linked resins for several pH applications because their performance is unaffected by pH. However, weak ion exchangers can be powerful separation tools in cases where strong ion exchangers fail as a result of selectivities of weak and roubst strong ion exchanger often differ.

After running the Chromatography 2 still the protein can not give proper purity so to remove the remaining impurities and to get the proper yield and purity a one more chromatography is use which is RPHPLC (Reverse Phase High Performance Liquid Chromatography). Reversed-phase HPLC (RP-HPLC) is one of most important techniques for protein separations. RP-HPLC has been applied on the analytical scale, and has also been scaled up for preparative purification. In addition, preparative RP-HPLC is often used for large-scale purification of proteins, in industrial scale. Because of its compatibility with mass spectrometry, In proteomic research RP-HPLC is an indispensable tool. Reversed-phase high-performance liquid chromatography separate the molecules on the basis of their hydrophobicity. The separation depends on the hydrophobic binding between the protein and the resin where the protein is present in mobile phase and the resin is present in stationary phase.. RP-HPLC is a very powerful and important technique for the analysis of peptides and proteins because of a number off actors that include:

(1) Under a wide range of chromatographic conditions for very closely related molecules as well as structurally quite distinct molecules the excellent resolution technique can be achieved.

(2) By changing the characteristics of mobile phase the chromatographic selectivity can be manipulated

(3) The generally high recoveries and, hence, high productivity.

(4) The stability of the sorbent materials under a wide range of mobile phase conditions is caused by the excellent reproducibility of repetitive separations which takes a long time.

For the isolation of peptides and proteins from a wide variety of synthetic or biological sources RPHPLC is a very important technique and is used for preparative applications. RP-HPLC is generally employed for the final large-scale purification.

- Resin:

The resin which is used for chromatography-1 is Sepharose fast flow. Sepharose fast flow resin is weak resin for chromatography. Sepharose is a crosslinked, beaded-form of agarose, a polysaccharide polymer material extracted from seaweed. Its brand name is derived from Separation-Pharmacia-Agarose.

There are lots of positively charged resin is present for anion exchange chromatography one of those is DEAE resin. It is used for the separation and purification of proteins and nucleic acids. This positively charged gel based resin help to strongly bind the negatively charged proteins or nucleic acids into the resin. When the salt concentration of a buffer increase it get dissociate into the column and the negative ion replace the protein (Which is already bind into column) from the resin and by this way the protein get eluted from the column. Cromatography-1 is a weak anion exchanger. This exchanger is utilized to separate proteins that have extremely different charges. Like all anion exchangers, the resin carries a positive charge that interacts favorably with negative charges. The positive charge of chromatography-1 cellulose resin is due to a protonated amine group. The binding of resin and protein is depends on the charge. This resin is a weak exchanger because only two amino group are attached with it and it get ionized very fast besides that in weak anion exchange resin only two protein molecule can bind and the other thing is tis kind of resin can not act very well with high pH range . For elute out the protein from the column NaCl or KCl are typically used because the chloride anions will attach to the resin and replace the protein, and then the protein will come out from the column. But here is one more thing is from thr ccolumn the proteins are the only thing which is come out during the process the and the resin which is cellulose, dextran, agarose based are remain unaffected because they compose inert matrices, though it is strong or weak cation and anion exchange chromatography.

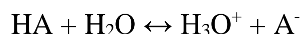
Beside this one more strong anion exchange chromatography is use to increase the purity of the insulin, which is Chromatography-2. Four protein can bind in one resin particle in case of Chromatography-2 that is the reason it’s called strong anion exchange chromatography. Normally the strong or the weakness of a resin depends on the pH, the weak chromatography work upon very low range of pH where as the strong anion exchange chromatography works on a very vast range of pH. During the acid dissociation of the resin’s functional group if the pH of the buffer no can’t match the pH of resin, then this will this be a reason resin’s capacity loss. Strong anion exchanger function a vast range of pH. When working with ion exchange resin such as Q sepharose fast flow resin, it is important to work within the supplier-provided working pH range. Strong ion exchangers are often preferred resins for many pH applications because their performance is unaffected by pH. Now a days for industrial chromatography purpose the Q Sepharose fast flow is very much popular, because of its higher chemical stability, well proven CIP and sanitization protocol. It is composed of cross linked 6% agarose beads, with quaternary ammonium (NH_3) strong anion exchange groups. For scale-up with Q sepharose fast flow resin it is available in a range of pre-packed process development tools.

For the analysis of proteins RP-HPLC is the most commonly employed experimental procedure, it generally involves the use of a C18-based sorbent and a mobile phase. The microscopic porous silica are used as a packing material for chromatography and it also allows the use of high linear flow velocities resulting in favorable mass transfer properties and rapid analysis times. Bearing an n-alkyl hydrophobic ligand in silica it get chemically modified by a derivatized silane. The most commonly used ligand is C18. The process of chemical immobilization of the silica surface results in approx half of the surface of silanol group being modified. To manipulate the selectivity of peptide and protein separations and to influences the retention between the peptides and proteins a type of n-alkyl ligand are used. Protein samplesis totally depends on the choice of ligand to influence the protein recovery and conformational integrity. Short less hydrophobic n-butyl ligands the recovery of the protein get higher.

- Buffer:

It keeps a control over the variables in an experiment or an analysis on an important element to make sure that the analysis is accurate and repeatable. There are many variables that might have to consider, both chemical and physical, during the analysis. For example, a variable like temperature can be controlled by using an oven or water bath. When adding small amounts of a sample to a solvent, there are chemical changes observed such as a change in pH. This can lead to further changes such as changes in solubility and sample precipitation and we can control by a variable like pH. A buffer is simply a solution that resists changes and a buffer is a kind of solution that also resists changes to a solution's pH when small quantities of acid or alkali are added. So in chromatography, if adding a sample to a mobile phase can shift the pH, a buffer can be added to the mobile phase to resist the pH changes giving the analyst control over the pH. There are lots of ions in solution that can cause a change in pH such as hydrogen ions (H^+) or hydroxide ions (OH^-). Addition of hydrogen ion increases the concentration of hydrogen ions, so reducing the pH,o hydrxyl ion combine with hydrogen in solution forming water, by removing hydrogen ions, and thus increase the pH. A buffer solution has to resist these changes. There are two basic forms of buffer solution, acidic buffer and alkali buffer. Although there are different kind buffers which work in slightly different ways, the process is very similar. An acidic buffer produced by a weak acid (HA) and its conjugate base (A^-), and in case of any alkali buffer the thing istotally opposite.

In solution the two species are in equilibrium giving:



$$HA + H_2O \leftrightarrow H_3O^+ + A^-$$

If an acid is added to the solution, the equilibrium helps to get rid of an additional hydrogen (H^+) ions. In case of an alkali then the opposite thing will occur. In each cases the added reagent is effectively removed because the equilibrium changes and also the pH changes very little. This is an example of Le Chatelier's principle in action. Once a buffer contains the incorrect concentration or ion, it can even prevent binding of the protein of interest to the column resin. The charged species of a buffers and a resin for ion exchange chromatography should be always same. In case of any buffer preparation the appropriate pH and conductivity of that specific buffer should always maintained because if it will differ then in that case the chromatography will not give appropriate result. Definition of a Conductivity is a condition where the salt can increase the electricity of a solution. Electricity can flow easily through a material with has high conductivity. Conductivity is measured in siemens per centimeter. Factors such as temperature have a higher effects on conductivity. Conductivity is reciprocal from resistivity so if any solution has high conductivity which means it has lower resistivity, and if a solution has higher resistance then the conductor must be poor. pH stands for "power of hydrogen", by which the amount of hydrogen ions in a solution can be detected, actually the acidity or the alkalinity of the solution can be detected by this. Mathematically the pH is equal to the negative logarithm of hydrogen ion in a particular solution. If a solution shows the pH grater than 7 that means the solution is basic and if the solution shows the pH range less than 7 that means the solution is acidic in nature, and the 7 pH is the neutral pH normally the purified water has the neutral pH.

For anion exchange chromatography different kind of buffer are used but here for protein purification a selective buffer are used. For chromatograpy-1 five selective buffer are used these are the followings:

- Equilibration-1 Buffer: This is prepared by tris solution, whose molecular weight is 121.14,basically this is used to equilibrate the column. After passing the purified water the tris is transferred which help to bring the resin in a proper condition that the resin can bind the negative protein molecule. This buffer solution works best in pH 7-8 range, and conductivity range is 10-26 ms/cm. Normally the pH of tris is bellow the desire range but to bring the pH in the desired range generally 1M HCL is used.
- Equilibration-2 Buffer: This is also prepared by the tris solution and sodium chloride. Basically tris is used as buffering agent and Nacl maintain the conductivity. It's pH range is 7-8 and the conductivity range is grater than 10ms/cm. During passing the Nacl it get dissociate into Na^+ and Cl^- ions and the Cl^- ion remove the strong impurities which does not come out with the Purified wash as well as the Eq-1 solution. Cl^- ion is negative and the protein is also negative so protein get dissociate from the resin it comes out so it can also say that it help to do the CIP of the resin before loading the protein.

- Wash-1 Buffer: After loading the sample wash is very important because in the sample not only the protein is present there are other proteins ,SOD complex and other impurities also present and to remove this at-first wash-1 buffer is normally transferred from the chromatography. It has the composition of tris and NaCl, and it works properly in 9-16 ms/cm range of conductivity and 7-8 range of pH. The working principle of NaCl is to remove the impurities from the resin and the tris work as a buffering agent.
- Wash-2 Buffer: Due to presence of different kind impurities which get attach with the resin very tightly and loosely only one time wash is not sufficient so a second time was has given to sample with the composition of tris, NaCl, and IPA(Iso Propyl Alchohol). Here NaCl again do the same work releasing the impurities by adding the Cl^- ion the resin but only with NaCl and tris the sample does not become pure there are lots of impurities which can not get remove by NaCl so to remove this an organic solvent is needed and in this case here IPA is the better option. The impurities get attach with the protein by hydrophobic bond, and NaCl can not break the hydrophobic bond, so IPA is use to break the hydrophobic bond. This buffer gives its best activity in 7-8 pH and bellow 1 ms/cm conductivity range.
- Elution Buffer: This buffer is main and very important buffer it helps to dissociate the protein of interest from the resin. Elution buffer is composed by tris, NaCl and IPA. Salt help to dissociate the protein to release the resin or to lose the bind between resin and protein and so that the purified protein come out from the column and further process can done, IPA the organic solvent help to break the hydrophobic bond and tris is used as the buffering agent of the solution. The pH range of the solution is 7-8 and the conductivity range is again bellow 1 ms/cm. Generally the conductivity range is high in this solution so that the protein can come out side.
- Regeneration-1 Buffer: Usually different resin has different time span it is not use for one time so because it is more costly and there is no need to use it for one time but to reuse the resin it should be regenerated with different kind of salt and alkali solution, so that it get its activity back. NaCl is used as a regeneration buffer. It help to remove the other protein or impurities from the column after the elution has come. It get dissociate into Na^+ and Cl^- ion and the Cl^- ion remove the negative charge impurities and bind with the column, in other ward it help to clean the column.
- Regeneration-2 Buffer: In the load there are lots of acidic impurities are also present which can not remove by salt so to remove the impurities sodium acetate is used.
- Regeneration-3 Buffer: Only salt is not enough to clean the column or to remove all the impurities so to clean the column and for making it again active to bind with the protein NaOH is also used it basically degrade the unwanted protein impurities and make the column junk free.

After chromatography-1 Chromatography-2 is perform to increase the purity level because there lots of impurities are present in the sample so only chromatography-1 is not enough to bring that much purity that it can be further proceed. It is a strong anion exchanger and it can

work in vast range of pH. In Chromatography-2, Q sepharose fast flow resin are normally use here and in Chromatography-2 four protein molecule can bind to the one resin molecule and that is one reason it is called strong anion exchanger. For Chromatography-2 there are little bit changes in case of conductivity and pH, and the details of the buffer are given bellow:

- Equlibration-1 Buffer: The main function of this buffer is to equilibrate the column, in other ward to make resin that much proper condition that it can bind the protein. The composition of that buffer is 0.5M tris but the pH of the buffer is 8-9 and conductivity is below 10ms/cm. To equilibrate the column it need to run this buffer two times of the column volume.
- Equilibration-2 Buffer: This is also help to equilibrate the column and also increase the ion level in the column so that if there are any impurities are present it can comes out or dissociate from the resin and column become clean. This solution works best in the pH range 8-9 and not more than 2 ms/cm conductivity range. NaCl is the salt which is used to maintain the conductivity of the buffer.
- Wash Buffer: Before Chromatography 2 there are lots of stage is present to purify the product or protein so only one time wash of the column is enough after loading the sample into the column. This buffer play the main function of to remove the junk from the column after loading the sample into the column. Here in 8-9 pH and less than 2 ms/cm conductivity range the tris and NaCl works work properly to loose the unwanted protein from the column and IPA is also the main component of this buffer composition, which help to create the hydrophobic environment by which other protein can comes through outlet valve.
- Elution Buffer:After loading the whole sample into the column and washed with wash buffer to remove allunwanted proteins from the column, then during this time this buffer helps to elute the bound proteins from the column. Most frequently, by increasing the salt concentration the bounded proteins get elutedor occasionally, by changing the pH of the buffer. As ionic strength increases the salt get dissociate and the ions typically the $Na+$ or Cl^-compete with the bound proteins for charges on the surface of the medium and one or more of the bound species begin to elute and move down the column. The proteins with the lowest charge at the selected pH will first eluted from the column as the ionic strength get increases. Similarly, the proteins with the highest charge at a certain pH will be most strongly retained and it will elute last. The higher net charge of the protein or the higher ionic strength that is needed for elution. By controlling changes in ionic concentration using different percentage of gradient, proteins are eluted differently in a purified form or the concentrated form. Here tris and NaCl is normally used and to loose the protein which is attached with the column by hydrophobic interaction IPA is use.
- Regeneration Buffer: A final wash with high ionic strength buffer regenerates the column and removes any impurities which is still get bound with the column. This ensures that the column is ready for the next run. The column is then re-equilibrated withequilibration-1 buffer before starting the next run. Normally in three steps the column is regenerated.

- NaCl: By transferring it generate high salt concentration in the buffer so that the impurities can comes out normally it transfer three times of the column volume.
- Sodium acetate: To remove the acidic impurities sodium acetate is run in the column two times of the column volume.
- NaOH: NaOH is act as a protein denaturant. It is transferred three times of the column volume, by this three stage the column become reactive to run the next cycle.

For final polishing and to get the purity level above 97% one more chromatography is also performed which RPHPLC. Here the solute mixture is initially applied to the sorbent in the presence of aqueous buffers, and then solutes are eluted by the addition of organic solvent into the mobile phase. Elution depends on either by isocratic conditions where the organic solvent concentration is constant, or by gradient elution where the amount of organic solvent is increased gradually over a period of time. By using any of this two conditionsthe solutes get, eluted in order of increasing molecular hydrophobicity. Preparation of aqueous mobile phase is the most difficult step in reversed-phase chromatography (RPC) method development for the ionic analytes. The most important thing in this case is the consideration affects of pH on analyte retention, and the type of buffer for use, and mostly its concentration, solubility in the organic modifier and its affect on detection, among other considerations. In reversed phase chromatography if the choice of the buffers get wrong in terms of the buffering species, ionic strength and pH, it will give a poor and irreproducible retention and tailing of the product. Buffers are solutions of a weak acid and its conjugate base, or a weak base and its conjugate acid. They mitigate the influence of hydrogen and hydroxide ions and subsequently reduce the pH fluctuations, in case of dilution also. The identical pH range for reversed-phase on a silica-based packing is about pH 2 to 8. Choice of buffer is totally depends on the desired pH range. It is very important that the buffer should has a pK_a which is close to the desired pH because buffers control the pH best at their pK_a. According to the thumb rule the choice of a buffer depends it's pKa value <2 units of the desired mobile phase pH. Organic acid like Phosphoric acid and its salts like sodium or potassium salts are the most common type of buffer systems for RPHPLC. Phosphate's has two pK_a values, such as 2.1 and 7.1, and UV transparency make it ideal for most HPLC separations.

- Buffer Concentration: A buffer which has higher concentration that enhance buffering capacity and it will also give more productive separation of the compounds partially ionized at the pH of the mobile phase, by reducing the local perturbations of pH of the moving peak of analyte.
- Buffer Solubility: When the separations is done based on gradient then this condition is especially important. Determination of solubility can become clear by mixing a given volume fractions of buffer and the organic solvent. Appearance of precipitates solution indicates solubility issues. A general rule is not

more than 50% organic should be used with a buffer. This will depend on the specific buffer and it's concentration.

- Effects on Detection: The choice of buffer is also dependent upon the sample also. During the measurement of absorbance by spectrophotometer, the buffer needs to be effectively transparent in this region, and especially it should be more critical for the sample when the separation is done by gradient.

So, for performing RPHPLC two types of buffer we need:

- Buffer A: This one is prepared by ammonium sulphate, and the pH and conductivity is adjusted y Ortho phosphoric acid. The pH range of this buffer is grater than 2.5 and the conductivity range is less than 7. Ammonium sulphate is normally used to increase the salt concentration of the buffer.
- Buffer B: This one is also prepared by same molarity of salt such as ammonium sulphate. But in this one is extra add which is IPA which is basically decrease the level of conductance of the buffer simultaneously increase the capacity of the buffer to break the hydrophobic bond the protein of interest and the resin, so that the protein of interest get eluted.
- Regeneration Buffer: For regenerate the RPHPLC column the buffer B is needed. Because in the buffer B IPA is present which is useful for removing the unwanted proteins from the column or in buffer B salt is also present which help to remove the impurities by replacing the charged group.
- Dilution buffer: This one is needed to dilute the elute and and to adjust the pH and conductivity of the elute for the next process which is TFF. This one is prepared by sodium citrate whose pH is between 7-8 and the conductivity is grater than 2 ms/cm.
- Crystallization Buffer: This one is prepared by three steps,
  - Stock solution: The stock solution is prepared by sodium citrate, CWFI, citric acid. First the sodium citrate get dissolved in autoclaved CWFI and the pH of the solution get adjusted with the citric acid to grater than 7.5.
  - Solution A: Solution A is prepared by gently adding the ethanol to final concentrate solution and mix well after the filtration is done.
  - Solution B: Solution B is prepared by sodium citrate, zinc acetate, citric acid, and CWFI. All these are mixed well and thefor adjusting the pH citric acid is use the pH range is above 5.8.

➢Procedure of the protein purification:

- 1st Step: After receiving the load at fast around 2ml sample has taken and after check the absorbance at 280 nm, the sample has sent to check the purity and concentration in QC department, and the chromatography-1 has started. First the purified water has passed through the column around 3 CV. This step is basically for neutralization of the column because the column is already stored in storage buffer. This buffer is prepared by NaOH, after doing the CIP of the column it is very important to store the column otherwise it become dry and the binding capacity of the resin will become very low.

  Then the start buffer or the equilibration-1 buffer has transferred to the column to equilibrate the column and give that condition that the resin can comes to the condition to bind the protein during loading, and the buffer transfer into the column until the suitable condition arise.

  The next step is to transfer the equilibration-2 buffer this one is also consider the start buffer. All the charged groups which are present in stationary phase are bound with replaceable counter ions after equilibrium get reached into the column, such as chloride or sodium. For binding the more amount specific protein the pH and the conductivity should be appropriate. This buffer is transferred into the column until the column get the conductivity and pH of that particular buffer.

  Now one of the final step in chromatography-1 which is loading the sample into the column. During this step the negative charged protein get bind with the positively charged protein, All other proteins simply pass through the column and are collected during this step.

  Now it is the time to release the unwanted protein from the column and to do that two time with two type of wash buffer is transferred into the column, the first one is wash-1 buffer which is prepared by tris and NaCl, the whole chromatography process is done based on the conductivity, so the salt get dissociate and help to release the loosely bound protein from the column but still there are lots of protein which get tightly attached with the resin by hydrophobic bond so to release this kind of impurities the second buffer is transferred from the column which is wash-2 and here IPA is extra and very important component which helps to break the hydrophobic bond and increase the purity level of the sample. Wash is transferred for grater than 1CV and wash-2 is transferred for at-least 2CV. During transferring the wash-2 buffer the flow rate of the column become decreasing cause it is prepared by IPA and density of IPA is higher than water so it gives a pressure during washing and the back pressure also become increased so we have to decrease the flow rate otherwise it will disturb our column bed.

  After washing the main step comes and that is elution, where the protein of interest comes out from the column. Most frequently, proteins are eluted by increasing the salt

concentration of the buffer. As ionic strength increases the salts are (the Na+ or Cl- ions) start to compete with the protein molecule for charges which is bound with the column and by which one or more of the bound proteins begin to elute and move down from the column. The proteins which has the lowest net charge at the specific pH will be the first ones eluted from the column as ion concentration get increases. On the other hand, the proteins which has the highest charge at a certain pH will be most strongly retained with the column and will be eluted last. The high net charge of the protein, the higher the ion concentration is needed for elution. Proteins are eluted differently in a purified or as a concentrated form from the column by controlling changes in ionic strength using different forms of gradient. During elution of the sample the flow rate of the column become less cause it is also prepared by IPA and for the same reason. The elute is collected with the proper absorbance of the particular protein when the peaks is started going up the elution collection become started and it gets end when it comes to its normal stage, after collecting the elute around sample is given to the QC for check the purity and concentration of the sample.

This step is for cleaning the column rather preparing the column so that it can use in next time. This step is called regeneration. A final wash with high ionic strength buffer regenerates the column and removes any molecules still bound. This ensures that the full capacity of the stationary phase is available for the next run, NaCl, NaOH, and sodium acetate is transferred to regenerated the column. And it get stored by the storage buffer.

- 2nd Step: This step is denoted as unblocking step. During protein expression in the upstream citraconic anhydride is used to masking the amino acid by forming a reversible bond in alkaline condition, so that when the trypsine digestion will happen after refolding the protein does not get cleaved. SCP at the selective site of the two chain and also the SOD complex. But there are various site of SCP which are prone to trypsine digestion, if this sites will cleave it will cause the inactivation of insulin. To prevent the unwanted cleavage of lysine which is also a site for trypsine digestion it get blocked by citraconic anhydride, in the purification step it is needed to remove the citraconic anhydride and remove the masking to increase the purity. Citraconic anhydride works at high pH such as grater than 10 so to release this in unblocking step the pH of the elute get down into less than 3 by HCL and leave it for incubation. After the sample is sent to QC for Check the purity and concentration.
- 3th step: In this stage filtration of the sample is done by filter for increasing the purity level of the sample, and the filtered sample is send to QC to Check the purity and concentration.
- 4th step: After incubation of protein, zinc chloride is added into the elute and bring up of the pH grater than 5 by NaOH. Basically metal ions are used for precipitation of protein molecule and further developed other step in the process such as in formulation of protein and may have the utility in

purification of protein. In such processes, the main thing that should take care is the main structure of the sample get maintained and the metal ion complexation is reversible. The precipitates were dissolved, yielding complete recovery of native protein. Proteins has a specific metal binding sites in their structure and require specific molar ratios of zinc to protein to initiate precipitation. Beside this Zinc complexes and zinc-induced precipitates, are use measured the solubility of both the protein by infrared and circular dichroism spectroscopies. The soluble zinc complex of had minor tertiary structural alterations, whereas zinc binding did not alter the tertiary structure of protein. These studies indicated that metal-induced precipitation provides a method to maintain proteins in their native state in precipitates, which may be useful for purification, storage, and formulation, the zinc chloride form a hexamar with the protein and its weight get increased, and leave the sample for 3 hour. Afer the sample is sent to QC for Check the purity and Concentration.

- 5th step: Centrifugation is done to make the elute more concentrate and to increase the purity of the sample. Before centrifugation it is important to bring the temperature down, the temperature should be less. Because during centrifugation heat is generated and which can cause the denaturation of the protein so the protein should be in cool temperature, if the supernatant has more than 1.5 OD after centrifugation it should be collect in a container with the zinc pallet, and the sample is sent to QC for Check the purity and Concentration.
- 6th step: After zinc centrifugation Q-load is prepared for Chromatography 2 with the zinc pallet. For Q-load preparation EDTA is used as a buffer which has above 8pH. Here EDTA is act as a chelating agent, it help to chelate the metal ion and by which the metal ion can not bind with the protein. At first the zinc pallet get dissolved in the buffer and after this condition the pH of the load bring down to 2.9 by HCL for help to properly dissolve the pallet, and then again the pH of the load get increased by NaOH and the pH should be in high range, and the conductivity should be grater than 2ms/cm, after the load is filtered store in a vessel and the sample is sent to QC for Check the purity and Concentration.
- 7th step: Here in this step the second chromatography is done. This one is the strong chromatography. Q sepharose fast flow resin is used here.

At first the column is neutralized by the PuW until the column et the proper pH of column volume. This one is kind of CIP step, this one is used for cleaning the storage buffer, The PuW run is still continuing until the flow through has the pH of PuW.

The next stage is to equilibrate the column with Equilibration buffer, to make the resin in a proper condition so that protein can bind with the resin during loading. The pH of this buffer is greater than 8, but the conductivity range vary between the two type of equilibration buffer. Equilibration-1 buffer has the conductivity range greater than equilibration-2 and the Equilibration-2

buffer has the conductivity range less than equilibration-1. Basically the salt is needed to remove the impurities if there any impurities are still present and to increase the salt concentration in the column.

Now when the column become fully equilibrated it is the right time to loading the protein into the column, the anion exchange chromatography is used here so the resin is positively charged and the protein is negatively charged, so with the bellow conductivity than elution buffer the protein get bind with the resin.

After loading washing is a important stage it is done to clean the loosely bound protein which is present in the column and also to break the hydrophobic bond between impurities and the resin. This buffer also have the same pH range and the conductivity is less than 2 ms/cm. In this step the salt which is present in the buffer get dissociate and help to remove the impurities. This stage is continued for 4 CV of the column volume,

At this stage the protein of interest comes out from the column. Most frequently, proteins are eluted by increasing salt concentration of the buffer. As the ionic get strength increases the salt ions (the Na+ or Cl- ions) start to compete with the bound proteins for charges on the surface of the resin and one or more of the bound protein begin to elute and move down through the column. The proteins which has the lowest net charge at a selected pH will become the first ones eluted sample from the column because ionic strength increases. At the same time, the proteins which has the highest charge at a certain pH will strongly retained with the resin and will be eluted last. For elution the protein the higher the net charge of the protein and the higher the ionic strength that is needed. Using different forms of gradient by controlling changes in ionic strength, proteins are eluted differently in a purified, concentrated form. During elution of the sample, the flow rate of the column should be less cause the Q load is also prepared by IPA and for the same reason. The elute is collected in a suitable range of OD when the peaks is started going up the elution collection become start and it gets end when it comes down the same range from where it start, after collecting the elute around sample is given to the QC for check the purity and concentration of the sample.

When the elute collection is over the column is again regenerated by the NaOH, NaCl, and also sodium acetate. This is important to make the column perfect ready for the second time run, and after regeneration the column is stored in storage buffer.

- 8th step: This one is a step of conversion between pro-insulin to insulin. Pro-insulin is has larger molecular size than the insulin and it is very much similar to insulin in many properties such as solubility, iso-electric point, self associate properties and reactivity with insulin antisera. By this information and other evidence it strongly prove that the conversion of insulin into pro-insulin is very much identical to that of the insulin itself. It is of interest that the connecting peptide much longer than would seem to be required to bride the short 8Å gap between the two A and B chain of insulin.

Although over a portion of the surface insulin monomer the connecting peptide may be folded, which does not completely mask the “active site”, since intact pro-insulin still shows 3-5% biological activity in several system in-vitro. The pro-insulin is consists of a single poly-peptide chain and the range of the length is 78-86 amino acid residue. The variation in the length of mammalian protein occur only in connecting peptide portion which links the carboxyl terminal end of B chain of insulin and the amino terminal of the insulin A chain. All the pro-insulin has the pairs of basic residues of either end of connecting peptide which link the connecting poly-peptide to the insulin chain. This residues are excised during the conversion of pro-insulin to insulin, and the resulting product is the native insulin and reminder of the connecting polypeptide lacking amino or carboxyl terminal basic residues. This peptide is designated as C-peptide. For doing this step a protein is used here which is CPB (carboxy peptidase B).

- 9th Step: This step is denoted as crystallization. Basically in this step the salting in and salting out of the occur with the protein. Protein crystallization has immense potential to be used for separation and formulation applications on an industrial scale. Crystallization under static condition is often limited by the diffusion of molecules from the bulk solution to the growing crystal surface. This results in a lower overall yield of the product and longer crystallization periods. Salt precipitation can be a very powerful technique to purify proteins by precipitation. Here to do the crystallization zinc acetate sodium citrate and citric acid are use. Zinc acetate is preferred salt because it is very cheap, highly soluble in water molecule, and is able to become much more hydrated (interacts with more water molecules) than almost any other ionic solvent. In this step, the salt is either added directly into the sample as a solid condition or else it get added as a powered form into the solution for precipitate the desired protein of interest. At low salt concentrations the addition of more amount of salt in general it tends to increase the solubility of proteins to give the protein an ions shield from the charges of other molecules and this process is termed ‘salting-in’. But at specific condition the ionic strength become too high for the protein and at that condition protein become unable to solubilize into the solution and it get precipitated this is known as salting out. This is happens due to the dissolved salt competes with the proteins for scarce water molecules, and by which the surface tension of water get increased and therefore causing the protein to fold tighter. In this step to form crystal at a specific time there are less protein-water interactions occur which allows for more hydrophobic interactions between protein molecules, causing aggregation and subsequently precipitation, in a crystal form this is known as reduction of surface area. Proteins in the solution can become precipitate out as a function of salt concentration. This is an another way to the purify the protein, to purify specific proteins a specific amount of salt is add into the solution to precipitate out unwanted proteins, then the supernatant get recovered, and by adding a bit more salt to precipitate the desired protein get precipitate and then

pellet is stored as a form of precipitated protein. The salt precipitation can only affects the solubility of proteins and does not denature them, the recovered sample can then be stored in the salt solution for prolonged periods of time without having any tension about the bacterial contamination because the high salt content inhibits any microbial contamination or protease activity. The salt precipitation only reduces the solubility of proteins and does not denature them, then after that te proteins can be concentrated by removing the remaining salt from the solution to re-solubilize the pallet in standard buffers or a low concentrate salt solution. Hydrophobic interaction chromatography can then be used to further purify the protein solution.

- 10th Step: After creating the protein crystal the next step is to run the one more column but here the column is not the ion exchange column this one RPHPLC. The reason behind the use of one more column is to increase the purity of the protein, because the resin particle are not same in case of first and second chromatography so when the bed form the particle are not present uniformly in the bed so there will be a problem for moving and binding of the protein with the resin. This one is totally depends on the hydrophobicity.

Before transferring the protein of interest rather insulin two different buffer is transfer in the column one of them increase the salt concentration and the other one create a hydrophobic environment in the resin so that when the protein will go it can bind with resin via hydrophobic bond.

So then after all these equilibration of the column has done the next step is to transfer the load. But as we can see that our protein is in crystal form so it can not run in the column in this form so it need come in a suitable form for loading. Actually there are one more time centrifugation is done and by which we get the pallet and the pallet get dissolved in bowl slurry or supernatant, cooled WFI, and IPA, and the pH of the load is adjust by the HCL. Now it become in that form that it can e run in the column.

During the equilibration the concentration of the IPA is less but after loading the concentration of the IPA or else Buffer B gradually increase and the concentration of buffer A is gradually decreasing. This step is also known as washing of the column or wash of the impurities. All this also done in a suitable flow rate because the density of IPA is greater than the water so it create more pressure and for this the back pressure will also become higher and if the back pressure will cross the ultimate level it can disturb the bed and the effects will come onto the protein.

After washing the impurities the next step to elute out the protein of interest. In this case the concentration of the buffer in high level and the buffer B in very low from the first situation or than the equilibration, and for this at a specific time the IPA of the buffer B create the hydrophobic environment than the resin as create and for this protein get repulsed from the resin and

bind with the IPA of the buffer by hydrophobic bond, and the elute comes out the collection of elute fraction is done from a specific ascending absorbance to descending absorbance, and if the collected fractions of before and after the specific absorbance has a good absorbance in 280 nm then it will also consider also the main fraction and it get conformed by QC department by checking the purity and the concentration of the fractions. At the same time the concentration and purity of the final fraction is also checked by QC. All these is done in the down flow position of the column.

The next step is to regenerate the column this is also done via IPA and ammonium sulphate rather the buffer B. Here no need of buffer A to regenerate the column because the RPPLC works on hydrophobicity. And the column is then stored in 50% IPA in CWFI for the next run.

- 11th Step: The next step is TFF to concentrate the elute. For doing TFF first it need to adjust the pH of the elute and dilute the elute in a dilution buffer, and the pH is adjust by NaOH the pH range is grater than 7.5, after that it get stored in 4°C to bring its temperature bellow 15°C. During this period the condition of the TFF is adjusted to concentrate the protein the main reason to concentrate the protein is to remove the salt, impurities, and other factors which should not present and by this the volume become less of the protein and the protein reach its deserving purity level. The process still continued until the permeate get the conductivity of the dilution buffer. It should be observed during the TFF that temperature should not cross 7°C. Because in high temperature the protein get denaturated and also in high temperature different kind impurities can form i.e, A21des-amido.
- 12th Step: Here once again the crystallization is done. Mix the buffer with the filtrate sample and maintain the temperature less than 8°C. The crystal will start forming after some time and then it keep at least 7 hour incubation in cool temperature.
- 13th Step: This one is the last process of purification and here finally the collection of the crystal is done. In this an another filtration is done which call Nutsche filter. After collecting the purified and activated protein two time wash is done with CWFI this is done to remove any particle, salt, and other impurities if it is present. Then to remove the water residue air is blown upon the protein and finally keep it in a LAFU, to make it in powdered form which is the ultimate product the powdered form of the protein is also called API which is ready to be marketed.

2.**Background and Rationale**:

So in this industry the main material of production is insulin protein, which is help to decrease the glucose level in blood. Insulin is hormone which acts on diabetes mellitus type-1, diabetes mellitus type-2, gestational diabetes. Normally in human body the secretory gland of this hormone is the beta cells of the pancreatic islets. It is a peptide hormone, but when the secretion of this hormone become less in human being then the glucose level get increased and and to bring it in it's natural amount the

doctor advice us to take insulin. There are lots of way to produce insulin in-vitro but the most acceptable method is the recombinant DNA technology but in this when the protein get over expressed the next step is to purify it because there are lots of other protein which may be harmful for human being, and the product is not in the proper insulin form so it need to some modification or else purification by which it can convert the pro-insulin into a active insulin.

The main agenda behind the purification of protein is that there are lots other protein which is consider as the impurities and if it get directly inject into human body firstly it does not work properly because it is still not in that form to decrease the glucose level and second one is all these will consider as an antigen in human body and for these there will be the activation of immunogenicity. Which is fetal for human being, as well as the importance protein purification is the Purified proteins are required for many experimental applications, including structural studies and in vitro biochemical assays. Accept this the purification of protein is very important because,

- Detailed studies on the function of the protein
- Determination of structure of the protein
- Generate antibodies
- Amino acid sequence determination of this protein

Proteins can be obtained from tissue or, more often, by their over expression in a model organism, such as bacteria, yeast, or mammalian cells in culture. The process protein purification involves isolating proteins from the source, based on differences in their physical properties. The purification scheme of a protein can be done by a variety number of steps. Proteins are purified according to their,

- Charge,
- Size,
- Hydrophobicity,
- Molecular recognition (affinity).

Here the purification is done based on the charged of the protein. Ion exchange chromatography separates proteins based on differences in net surface charge, which is highly pH dependent as well as concentration of salt .The iso electric point (pI) of a protein is the pH at which it carries no net charge, if the protein get higher pH than it's pI that means the protein is negatively charged and if the pH of the protein is less than it's pI that means the protein is positively charged. Same goes for conductivity when the salt get dissociate in the column it form a positive ion and a negative ion when and according to the charge of protein the ion help to remove the protein from resin which results the elution of the purified protein of interest. Ion exchange chromatography is two type anion and cation exchangers. Anion exchangers employ a positively charged resin interacting with an exchangeable anion, while in cation exchangers the resin is negatively charged. A "strong" anion exchanger, such as

Q-Sepharose is positively charged across a broad pH range, whereas a “weak” anion-exchanger, such as chromatography-1 cellulose will lose its charge above pH 9. Therefore, column equilibration pH is an important parameter in ion exchange chromatography as it can affect protein charge and, depending on the resin, the charge on the column. Both of these affect protein adsorption and separation.

The logic behind the process is to remove the impurities. The formation of impurities is totally depends on the process conditions. Protein cannot become stable in room temperature or in high temperature it acts rather behave good in cold atmosphere. During the process time there are lots of another protein get generated due to improper handling or else the absence of favorable condition, which is structurally very much similar to insulin but their working property is not same as insulin i.e, A21Desamido, A21 Threonine, HMWP, Related proteins etc.

- A21 Des is produced due to high temperature, in high temperature the amino group change into hydroxyl group and it form the A21 desamido, and if it get entire into the human being immunogenicity will occur which may be fatal for human. But still due to some reason or improper handling there some impurities generate but there is range up-to this the impurity is acceptable the range is less than 2%.

- HMWP (High Molecular Weight Protein) is also one kind of impurities. It is produced due to high temperature. When the temperature get increased hydrogen-bond formed between the C-termini of B chains and the dimer is an inactive form of insulin, insulin protein can acts properly when it is in monomeric form. But due to handling error there are some impurities can form which is analysed by size exclusion chromatography. The appropriate range of the HMWP in a sample is 0.8-1.8%.

- Related Protein: Some proteins are having few properties like desired protein structure, conformation, S-S formation, amino acid sequence, functional group change, aggregation. It get separated by RPHPLC. Due to handling error this proteins are form. Unwanted chemical reaction in functional group and reactive group in peptides and buffer components, i.e, Improper folding (folding intermediates formed due to improper S-S bond), functional group change in amino acid in peptides. The proper bonding in formed between the 6th position of A chain and 7th position of B chain and 20th position of A chain and 19th position of B chain. But the improper handling is formed in the 6th and 11th position of A chain, 7th and 20th position in A chain and 7th and 19th position of B chain, and other improper bond also formed by which related proteins are formed.

3. Achievement:

Since from last six month I got an opportunity to know how an industry produce run. During this period I got opportunity to learn,

- <u>How to pack the column</u>: The art of packing a column is to distribute all the resin particles in the column in such a way that to obtain an homogeneous packed bed in a reproducible manner.

To pack the column the following things are needed:

- A column
- A fixed or adjustable bottom frit
- A top frit which can move with the column by the adapter
- Resin which is normally present in a semi liquid form and dissolve in 20% ethanol.

For packing the column first it need to bring the resin in a slurry form, the slurry is prepared by re-suspending the resin in the packing buffer. By dividing te volume of the settled gel from the total volume of the slurry we can get the concentration of slurry and the slurry concentration is adjusted as follows:

- Re-suspend the resin slurry into a vessel and after that transfer the homogeneous slurry to a cylinder.
- Leave the slurry for overnight incubation to settle (>12 hours) for best results.
- Determine the volume of the settled resin, and adjust the concentration of slurry to 30–50 % by adding or removing packing buffer.

Now before starting the column packing it is very important to make sure that total volume of column is sufficient to contain the whole slurry volume and if not then it need to add a reservoir on top of the column. Ensure that the column is leveled prior to packing and the bottom frit is wet in the column with buffer. To remove any air bubbles allow the buffer to drain for a few seconds. Then the column outlet is plugged by leaving around 1-2 cm of buffer in the bottom of the column. The resin slurry is then homogenized by re-suspend it. After that carefully pour the resin slurry down along the inside wall of the column, to avoid the air which may be being trapped if the slurry pour down directly into the column. Then the wall of the column is cleaned by the packing buffer. Then immediately place the flow adapter of the column onto the resin slurry. But it should be noted that there should be no trapped air between the flow adapter and the buffer. Then Open the column outlet, and start the pump slowly to flow packing buffer through the column. To form a perfect bed and prevents the uneven packing of the column bed the flow rate has to increase slowly by this the hydraulic shock of the column can also be prevented. The flow rate can be decreased in several incremental changes. These increments will be determined by the size of

the column and target flow rate. Then shut off the pump, and close the column outlet, when the bed has become fully formed, the entire bed should reside in the lower column section if using a packing reservoir. Using a pump and siphon the supernatant from the upper section of the column. The upper reservoir and the coupling ring is then removed. Carefully place the flow adapter into the column, approximately 2-3 cm away from the fully formed bed. Avoid introduction of air into the column. Secure the flow adapter in place, slowly increasing the pump, and open the column outlet. The bed will compress further and. The pump should not get stopped until the compression is completed and then close the column outlet. Then slowly and carefully loose the flow adapter seal and lower the adapter near to the resin bed. But it should be noted that the resin bed should not disturbed when moving the flow adapter. Then again transfer the buffer by gradually increasing flow rate so that if there some compression need to occur it should be done.

In this way the column get packed but it is still not be in a condition to run actually after packing it is important to check that the packing is proper or not and to check it we have to do HETP (Height Equivalent Theoretical Plates).The number of theoretical plates are also known as column efficiency. It is basically a mathematical concept which can be calculated by,

$$\mathbf{N= 5.545(t_R/W_h)^2}$$

Where, N= Number of theoretical plates, t_R= Retention Time, W_h= Peak width at half height (in units of time). Column is become more efficient by the higher number of plate and the column which has lower number of plates are considered to be a less efficient. A column with a high N number will have a narrower peak at a given retention time than a column with a lower N number. This Theoretical plate are important for some specific set of conditions. Specifically, isothermal temperature conditions are required because temperature programs result in highly inflated, inaccurate plate numbers. The plate numbers are also used to calculate the retention factor (k) of the performed test solute and it should be greater than 5. Less retained peaks result in inflated plate numbers. The same temperature conditions and peak retention (k) are required for compare the theoretical plate numbers between columns.

The height of theoretical plates is also an another name of column efficiency, which is denote as H. It is calculated by:

$$H= L/N$$

Where, H= Height of theoretical plates, L= Length of the column, N= Number of theoretical plates. The shorter each theoretical plate, the more plates are "contained" in any length of column. If the column has higher plates per meter that means the column has higher column efficiency. If the HETP result comes between 0.8-1.8% that means the packing of the column is accurate so then the column is ready to run.

During the handling of column I did not need to face any big trouble but during the running of the column it should be take care that the air can not pass through the column if it happen then the air create a pocket or the blank region inside the column by this the loading of the protein can not be done properly, for this proper elution will not come and the elute may e diluted, So to run chromatography it is very important to take care. Beside this during running the column back pressure of the column can not cross its limit because by this there will be a disturbance occur in the column bed which should not happen, and to control the back pressure the flow rate should be maintain, flow rate and back pressure is very much dependent on each other.

- How to prepare buffer: Buffer preparation is not a big deal but during preparation it should be observed that the pH and Conductivity of that particular buffer is maintain properly if it is not done then the buffer will not give accurate result, and it also should observed that the component of the buffer is calculated and added accurately. In this case temperature is also a main things because in high temperature the pH will become low and in low temperature the pH will be high and if the buffer is not prepare in proper temperature or else room temperature it may cause the problem in the buffer and there are lots of buffer where endothermic and exothermic reaction is happen. So during exothermic reaction we have to wait until it comes to the room temperature by providing a chilling water, same goes for endothermic reaction in that case we do not need to provide any heat it will come to the room temperature automatically. In case of that buffer where IPA is used there also care should be take during adjusting the conductivity. In case of any buffer preparation the main thing is the volume make up if the volume is not adjust accurately then the concentration of that buffer will change and this will become a big problem.

- CIP: CIP (Cleaning In Place) is done after each and every steps of a batch. Because if cleaning is not done properly then the composition will change between two solution or sample. CIP of vessel and tank

is normally done by PuW, but in case of that vessel or tank where protein is present the CIP s not done by only with PuW to clean tat vessel and tank at first we have to transfer process for some time then alkali because alkali helps to denaturate the protein and then it is wash with Puw until its pH come 7 and conductivity bellow 0.5. During CIP it should be noted that not only the vessel are getting clean also the pipe line get cleaned, there should not present any hold-up.

- GDP: GDP(Good Documentation Practice) is a major part of any kind of industry, because it is the only written prove for our product. It carries every single details of our product, this is needed when the product is export then for help to believe the buyer that our product is perfect for sale, and also important during audit because without any written prove auditors will not believe any thing, beside this documentation is also important because if there is any abnormalities occur with any batch then to analysis the problem the previous batch record is needed to compare the new batch with the old one. There are lots of rules to maintain the documentation:

- All documentation should be done via blue ball pen.
- If there is any correction require in any line then the incorrect ward should be cut by one single line.
- In one page there maximum 3 cut will be acceptable.
- After cut the incorrect ward there must be one signature is need by whom the problem is happen.
- Each and every document should be done by QA controlled paper.
- In any page there can not be present any empty space in this space a horizontal line should be draw by writing NA between the line and at the end there must be signature is need by whom the line is drawn.

These are some basic and very important rules people have to follow during work in any industry. There are some other rules are also present.

- cGMP: The full form of cGMP is current Good Manufacturing Practice. This one is another important set of rules which each and every one has to follow in the industry. The rules are describe in the bellow:

For API manufacturing industry it should be observed then the RM should be weight under appropriate conditions then do not affect their suitability for use. Weighing and measuring devices should be suitable accuracy for the intended use. If a material is subdivided for later use in production operations, then in the container the information should present like the name of the material, name of ongoing batch, date, time e.t.c.

If there any deviation happen the it should be clearly documented and explain. In case of any critical deviation investigation should done as soon as possible.

Either on the individual units of equipments or by appropriate documentation, computer control system the processing status of major units of equipments indicated.

All the sample should be clearly labeled so that any one can distinguish the material.

Cleanliness is the major things because to avoid any kind of contamination the floor, vessel, tank, equipments, should clean and there are different kind of solution which is use to clean the floor.

Personal cleaning is also a main things as the people are working in a bio-pharma industry, personal cleaning is main things. Besides that before get entry into the production department at first the person has to wash his hand then open off the shoes and wash the hand with hand sanitizer and then wear a clean apron and shoe and head cap after that before get entry into the final purification area the person has to wear a proper gown, gloves, and head cap, and nose mask and then again change the shoe and again wash the hand with hand sanitizer and the person is ready to take entry into the production area. The personal hygiene is to avoid any kind of contamination.

If air is re-circulated to production areas, appropriate measure should taken off to control risks of contamination and cross contamination.

The environment should be exact in the production area means the pressure, temperature, humidity, all these things should be proper in condition.

Before start of any kind of solution preparation the pH probe, conductivity probe should be calibrated first by the engineering person.

During handling of protein extra care should be taken to avoid any kind of contamination.

- SIP: The full form the SIP is Sterilization In Process. During final purification cleaning by PuW is not enough so to avoid any kind of contamination the cleaning is done by steam of water which is called SIP. Sterilization is a critical process in the bio-pharma industry for the control of microbial populations. While most prevalent in the manufacture of sterile products it can be used in a variety of settings where microbes have potential impact on product.

- TFF: The full form TFF is Transient Flow filtration. It is a kind of ultra filtration method. It is a rapid and efficient method for separation and purification of proteins. To concentrate and desalt the sample solutions TFF is used, and the range of the volume is from 10 mL to thousands of liters. It can be used to fractionate large from small proteins. The importance of using of TFF is, the set up of the TFF is very easy and also the handling. The TFF device has to connect with a pump and pressure gauge with tubing and a few fittings, then add the sample to the reservoir, and the begin any filtration process by using TFF. It is a very fast and efficient process and much faster than dialysis. Filtration of higher concentrations of

sample can be achieved in less time than when using centrifugal devices or stirred cells. Two steps of purification can done in one system these are Concentrate and diafilter, and also with in a very less time as well as not to loss any product. It can be use for scale up as well as for scale down. By using this the sample can concentrate 10 ml and at the same time it can increase the volume as large as thousands of liters. It is economically very chef because after one time use the cleaning process can do with it and can reuse the filter but before reusing a simple integrity test it has to conform that the membrane and seals are intact. Total sample volume, the required process time, and the desired final sample volume upon this parameters the cassette has choosed, and the channel is depends on the Sample concentration and viscosity to run the process, and it works best with the high viscosity sample.

- Concentration: Concentration is a simple process in which the removing fluid from a solution is done while retaining the solute molecules. The concentration of the solute and the volume of the solution are directly proportional in nature (i.e., halving the volume effectively doubles the concentration). To concentrate a protein sample, choose an ultrafiltration (UF) membrane with an MWCO (molecular weight cut-off) that is substantially lower than the molecular weight of the molecules to be retained. This technique is important to assure complete retention and to higher recovery of the target molecule.

- Diafiltration: For washing smaller molecules through a membrane and leaves larger molecules in the retentate without changing the ultimate concentration this process is used as the purification process. It can be used to remove salts or exchange buffers as well as to remove ethanol or other small solvents or additives. It is two type first one is continuous diafiltration and the second one is dis-continuous diafiltration. In case of continuous diafiltration process the diafiltration solution which may be water or buffer is added to the sample feed reservoir at the same rate as filtrate is generated. By this method reservoir sample volume is usually stay constant, however the small molecules (e.g., salts) will freely permeate through the membrane are and washed away. As compare to the Discontinuous diafiltration the continuous diafiltration requires less filtrate volume to achieve the same degree of salt reduction, but in case of second one which is discontinuous diafiltration, before concentrate the solution to the starting volume the sample first get diluted.

- Nutsche Filter: The Nutsche filter is work either on vacuum or pressure. This is use for filtration purpose in industry it is a very sophisticated machine with tight process control on parameters such as pressure, temperature and pH. For handling the flammable material, toxic, corrosive materials since these are autoclaved and designed for use in hazards in ex-proof environments when extremely safe operation are

required this kind of filters are generally used. It is available in all size and suitable for fast filtering the sample, and produce wet cake. The components of this filter are,

- Vessel
- The filter floor and cloth, woven mesh screen
- The discharge mechanism

The filter has some advantage, this filter only occupy a minimum space, it can be use for variety of case, the downstream as well as upstream, the slurry washing process is more efficient than insitu displacing washing, when the cake tends to crack smoothing avoids the wash liquid, air or as purge from by-passing due to all this advantages there are some disadvantages like the system takes a long time to form the cake, some time the sticky cake does not part readily from the filter medium. Though it has a lot of advantages it require maintenance like,

- The upper vent of this filter should be open for free evacuation of air
- The condition of the filter medium, cloth, must be done periodically to ensure that they are not damaged
- The cleanliness of the filtrate sight lass that enable the inspection of filtrate clarity
- The pressure relief valve which is present on the top must be checked for emergency functioning
- The swing type locking bottle that attach the head and cylinder on the smaller machines to the filter flow
- The interlock that disable opening the cake discharge valve or opening when the vessel is still under pressure.

➢ **Evaluation:**

- After loading the sample in chromatography-1 how much purity increase is describe in the bellow:

Sample	Volume(ml)	OD	TA	Purity(%)	Concentration (mg/ml)	Specific Protein (gm)
Load	42000	2.20	92400	35	0.16	7
LFT	42000	0.50	21000	NA	NA	NA
WFT-1	15000	0.67	10050	NA	NA	NA
WFT-2	15000	0.45	6750	NA	NA	NA
Elution	8000	5.20	41600	55	0.83	6.7
EFT	4000	0.30	1200	NA	NA	NA
Reg-1	10000	0.20	2000	NA	NA	NA
Reg-2	10000	0.13	1300	NA	NA	NA
Reg-3	10000	0.07	700	NA	NA	NA

The main purpose of the chromatography-1 stage is capture and concentrate the protein. According to the table after binding 7 gm of specific protein through 42000 ml load sample on chromatography-1 resin, the elute gives 6.7 gm of specific protein in 8 L volume. Purity of the load was 35% which has increased up to 55% after elution purity and specific protein was analyzed using analytical HPLC system.

- After loading the sample in Chromatography-2 how much purity increase is describe in the bellow:

Sample	Volume(ml)	OD	TA	Purity(%)	Concentration (mg/ml)	Specific Protein (gm)
Load	10000	4.20	42000	55	0.65	6.5
LFT	10000	0.20	2000	NA	NA	NA
WFT	15000	0.53	7950	NA	NA	NA
Elution	3800	7.50	28500	85	1.57	6.0
EFT	2500	0.42	1050	NA	NA	NA
Reg1	10000	0.23	2300	NA	NA	NA
Reg-2	10000	0.16	1600	NA	NA	NA
Reg-3	10000	0.08	800	NA	NA	Na

The main purpose of the Chromatography-2 stage is to remove the intermediate of the protein. According to the table after binding 6.5 gm of specific protein through 10000 ml protein sample on Q sepharose resin, the elute gives 6.0 gm of specific protein in 3800 ml volume. Purity of the sample was 55% which has increased up to 85% after elution and purity and specific protein was analyzed using analytical HPLC system.

- After completing the chromatography-2 and CPB digestion the next step is RPHPLC, and in this stage the detail of the protein is given bellow:

Sample	**Volume**	**OD**	**TA**	**Purity (%)**	**Concentration (mg/ml)**	**Specific protein (gm)**
Load	7000	7.00	49000	85	0.84	5.9
LFT	7000	0.67	4690	NA	NA	NA
WFT	3000	0.41	1230	NA	NA	NA
Elution	1500	8.45	8450	99.95	3.6	5.4
EFT	495	0.10	49.5	NA	NA	NA
Reg-1	800	0.04	32	NA	NA	NA

The main purpose of the RPHPLC stage is to polishing the protein. According to the table after binding 5.9 gm of specific protein through 7000 ml protein sample on chromasil resin, the elute gives 5.4 gm of specific protein in 1500 ml volume. Purity of the sample was 85% which has increased upto 99.95% after elution and purity and specific protein was analyzed using analytical HPLC system.

- In this following sections the table has shown the purity of the final API:

Sample	Volume	OD (/ml)	TA	Purity (%)	Concentration (mg/ml)	Specific Protein (gm)
Crystallized Product	500	8.65	4325	99.97	10.6	5.3
Wet cake	10.6gm	NA	NA	99.97	NA	5.3
Final API	5.36gm	NA	NA	99.97	NA	5.36

This one is the last step where the purified and concentrated products come out. According to this table after doing the diafiltration the final product is 500 ml volume the OD of the final product is 8.65 and after doing lyophillization the powered product has come out which is 99.97% pure and the volume is 5.36 gm which is finally ready to marketize.

➢ 4. Complement in corporate:

The main agenda is to get acquainted with industrial environment and to get exposure to the purification technique applied in industrial biotechnology industry.

So I got exposure to various chromatography technique and other purification steps which are important for enhancement of purification so that the final molecule in the form of API meets regulatory requirements as well as is suitable for human use.

Since purification involves lots of commercial input in terms of money and machine, therefore, other new techniques such as continuous chromatography techniques can be checked for an alternate wherein we can maximizes the purity level and can substantially reduce the operating cost.

Further to improvise on the purity level, process steps need to be handle with utmost care this can also save the extra effort required to decrease the impurity level which can substantially reduce the cost.

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