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Purification Techniques For Biological Proteins

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Abstract:

The isolation of biological proteins with highest purity is essential for different *in vitro* bioassays for screening of novel small molecules for their activities. Purification biological proteins are required specific and selective techniques to maintain their purity. There are several techniques have been developed with improved features for selective separation, purification and identification of protein. Here, we described different separation, purification and identification techniques for protein along with their working principles.

1. Introduction

In the 18th century, Antoine Fourcory was identified the proteins as a biological molecule. Proteins are important compounds as they are linked to many aspects of our lives[1].Proteins are necessary in the purified forms for their applicationas an important category of products in foods, biotechnology, pharmaceuticals and cosmetic industriesalong with organic chemistry in the field of enzymatic catalysis[1, 2]. Basically, the proteins may obtain in solid as well as liquid form. Moreover, these are originated from microorganisms, vertebrate animals, and plants[3].

The purification method varies depending on the type of sample, physical and chemical properties of the protein [2]. The mechanical homogeneity is a method for tissue sample preparation, in such method the samples are treated with suitable detergent. With the aid of suitable detergent proteins which are difficult to isolate can be easily get in desired quantity. Moreover, urea and guanidine hydrochloride also known as chaotropic reagents can be used because of their high efficiency of extraction. These reagents cause breakdown of the structural proteinstomakethem watersoluble [3].

There is another method known as centrifugation can be applied to isolate protein from cells. The centrifugation method uses media having different densities is helpful to extract proteins expressed in specific cell. For example, to obtain only immune cells from the body fluid or to

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remove lipid cells or keratinocytes from skin tissue, the removal of liquid-containing cells in the medium of high density can intensify the desired cells. Density was created. High density gradient filtration is also applied to remove unwanted cellular impurities or to receive specific cell organelles[3].

As a result of the several years of scientific researches on protein purification, different techniques have been discovered to achieve the high efficiency and good results. Based on the size, molecular weight, density and solubility, various chromatographic and non-chromatographic techniques has been described. The techniques which are used in large scale industries for protein purification are: western blotting technique, magnetic bed separation, size exclusion chromatography, ion exchange chromatography and so on [1, 4].

The latest advancement in the field of protein purification techniques has been reported in 2017 which is important for high precision structural analysis and industrial production of proteins to meet the requirements of high productivity, high purity and high activity (HHH)[5]. Even, the purification of most proteins or their complexes with required standardsof HHHis a tedious, multistep and continuous process. The colicin E7 DNase (CE7) and immunity protein 7 (Im7) has been described as ultra-high affinity purification system that enables one-phase HHH purification of diverse range of classical biological components, such asDNA/RNA, cellular proteins, protein toxins etcof eukaryotic and prokaryotic origin. These techniques appeared as universal and competent tools for high throughput separation of several biological proteinaceous components which are important for the creation of therapeutic proteins and for modern biological studies [5].

2. Classification of different protein purification techniques

The protein purification techniques, frequently used, are classified on the basis of their working principles into following categories-

(a) Western blotting or immunoblotting
(b) Magnetic beds separation technique
(c) Affinity ultrafiltration technique
(d) Two dimensional Sds-polyacrilamide gel electrophoresis

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(e) Gel filtration chromatography
(f) Ion exchange chromatography
(g) Affinity chromatography
(h) Expended bed absorption
(i) Hydrophobic interaction chromatography

(a) Western blotting or immunoblotting

This approach is primarily based on constructing an antibody-protein complex by binding antibodies to membrane bound protein and distinguishing the required antibody by using certain detection method called gel electrophoresis [6, 7].

The membrane produces a certain band for every protein. By labelling membrane with antibodies which are unique to the required protein, they are incubated and then the unwanted or unbound antibodies are washed off giving the required protein and safe antibody [7].

The green fluorescent protein (GFP) recombinant with histidine is produced in *E. Coli*is an unique protein of interest. The protein output is determined by western blotting (WB). The western blotting technique has been employed in the purification of plasma derived IgG,transferring, fibrinogen and immunoglobulin pool [6].

This technique has been used for detection of multiple proteins having same molecular weight. PCU3 cells having low quantity of total Akt protein and phosphorylated protein are detected followed by stimulation of TGF-β. Even due to the slight change in molecular weight because of phosphorylation, the duplex ability clearly distinguishes the two proteins in forms [6].

The separation of insulin and proinsulinusing western blotting method has two quantification issues: distortion of signal bands and lower sensitivity for insulin. To resolve these problems WB is performed by aid of anti-insulin clone c27c9 monoclonal antibody, which recognizes both insulin and proinsulin.Subsequent staining of PVDF membrane with CBB R-250 immediately after electro-blotting for WB resolves later issue [8].

(b).Magnetic separation technique

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The fundamental principle of magnetic separation is that when the sample encompassing the target compounds are mixed with magnetic carriers that possesses either aaffinity immobilized or a lipophilic molecules (ligand) or separating/ion exchanging groups with magnetic biopolymer possess good affinity for separating components[9]. The target compound sample can be as milk, wastes derived from cultivation media, wastes derived from fermentation industry, cultivation media for microbes, crude cell lysates etc.When the incubation period is surpassed and the magnetic particles bounded to the target component the entire magnetic complex can be eliminated efficiently from the mixture sample *via* asuitable magnetic separator [9].

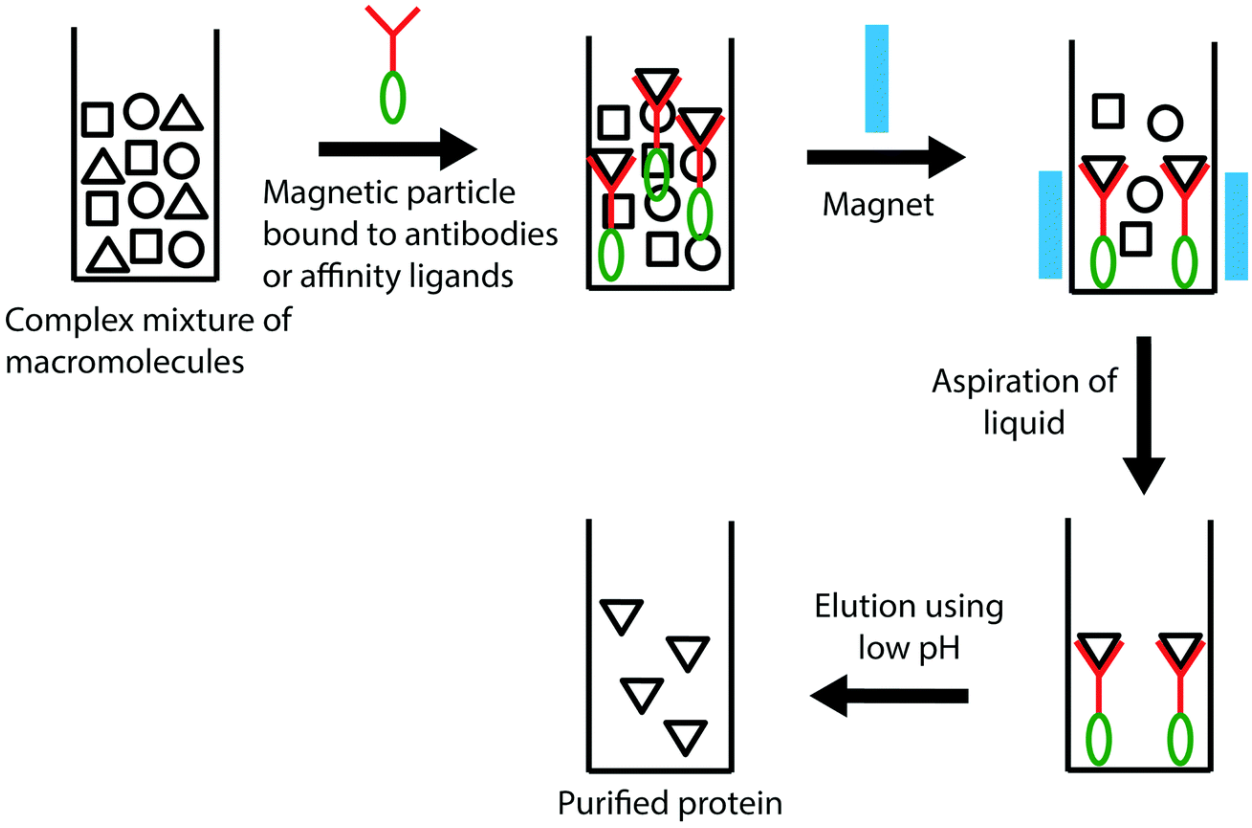

Figure-1: Working principle of magnetic separation technique [10].

In many incidences magnetic beads show low and nonspecific binding to non-target components present in sample mixture. In such case preclearing is done to eliminate molecules having high nonspecific binding activity. If preclearing is desired, then molecule will not be coated with the affinity ligand but coated with magnetic beads[9, 11].

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This technique has been used for the purification of histidine-tagged cell-wall-associated protein from *Staphylococcus aureus* that is found in *Lactococcuslactis*. By using this method 90% of target proteincan be isolated[8].Some beads have been used in a special design for removal of endotoxin, which are contaminants and produced by Gram- negative bacteria and can cause inflammation when injected into mammals and humans. Polymyxin B immobilized on beads permits to dispose of endotoxin without difficulty and effectively from samples of interest[12].

Recent advances in magnetic bead separation are based on use of aptamer modified magnetic beads.Aptamersas a binding ligands part of magnetic beads are mostly used to separateanalytefrom complex matrices. Magnetic beads can also be decorated with antibodies and peptides for separation with specificity.However,aptamerdecoratedmagnetic beads offer significant advantages over antibody or peptide decorated magnetic beads such as prolonged shelf life, wide dynamic range, high stability, and low cross reactivity[13, 14].

(c) Affinity ultrafiltration technique (AUT)

The AUT combines processing of high volume and high selectivity of membrane filtration, which can be appliedfor purification of proteins. In AUT the protein to be isolated is converted into a complex with a soluble polymeric macroligand or an insoluble microparticlewith protein-specific affinity ligands bounded covalently. It is based on the principle that, when solutionof protein mixture is passed through the ultrafiltration membrane the complex is trapped and free protein is passed through the membrane. The complexed protein may be either desired or undesired depending on complexing reagent. If complex protein is desired one than it can be further treated with suitable reagent to obtained free protein. If complex protein is undesired one then desired protein can be obtained by removal of solvent from the filtrate containing pure protein in free form(**Fig. 2**) [13, 15].

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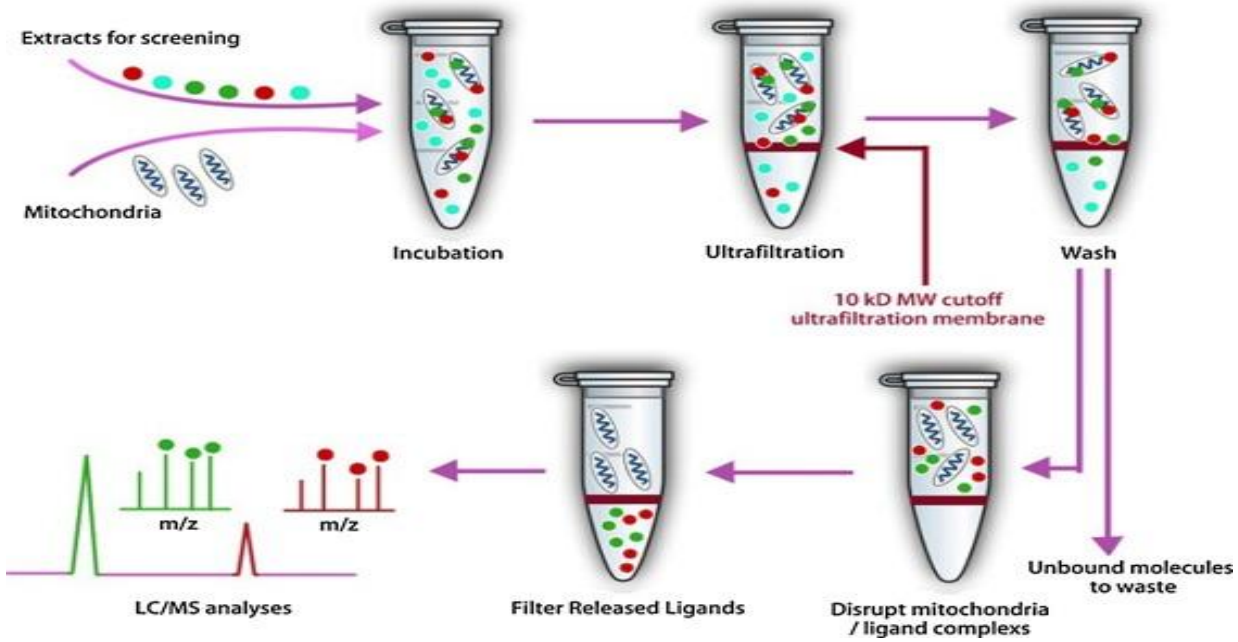

Figure-2: Operating process of affinity ultrafiltration technique [15].

In continuous ultrafiltration processing, the tangential flow filtration (TFF)is used for purification ofdesired protein. The bound-protein is trapped by a filtration membrane under the influence of pressure and the passing unwanted proteins through the membrane [16].The system is then supplemented with agents that dissociate the target protein to separate it from macroligand. It is used for purification of large volume solutions [17]. Recently, Chen *et al.*have been used affinity ultrafiltration in combination of (LC/MS) liquid chromatography mass spectroscopy for rapid separation and identification of small active molecules that bind to desired biological targets at the same time [18].

Phospholipase A2 (PLA2) is known for itslipolyticactivityand hydrolysis ofphosphoglycerates into the corresponding lysophospholipid and fatty acid at the sn-2 position of the acyl ester bond. The macro ligand is used to purify PLA2 from the pancreas of bovine by using ultrafiltration technique. The PLA2 is isolated with 79-fold purified and high activity yield of 76.3% [19].Similarly, Male *et al.*havealready been developed a methodology using affinity ultrafiltration to isolate urokinase from a mixtureor raw urine containing peroxidase and urokinase[20].

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(d) Two dimensional SDS-polyacrylamide gel electrophoresis technique

This technique is based on the separation of protein molecule by two different method followed by 1st dimension and 2nd dimension. The purification of protein molecules in 1st dimension is isoelectric point dependence. There are different techniques that are helpful for the isolation of protein under the specific pH gradient which allows the intense band recovery such as immobilized gradient electrophoresis (IPEG),non-equilibrium pH gradient electrophoresis (NEPHGE), and isoelectric focusing (IEF) etc. The separation of protein in 2nd dimension is determined by the molecular weight through SDA laemmli as well as Tristricine buffers [21-23].

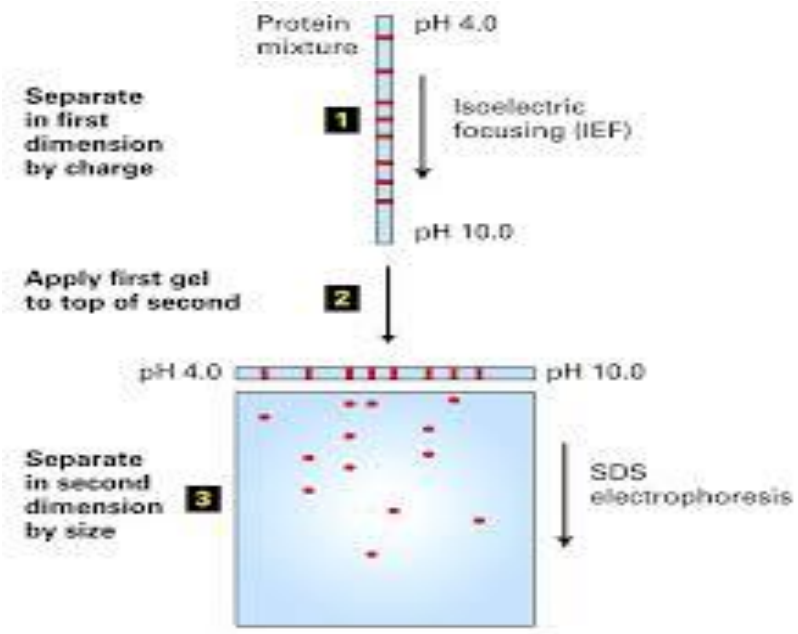

Figure-3: Working of 2D-SDS polyacrylamide gel electrophoresis[24].

The recent advancement in 2D-SDS polyacrylamide gel electrophoresis has been introduced as differential imaging gel electrophoresis (DIGE) method by Flavo(**Fig. 3**) [24]. It is technique designed to increase the sensitivity and reproducibility with multiplexed fluorescent dyes (cyanine dye) labelled with protein sample. Two dimension-DIGE is mainly relies on running of less than three samples. Two dimensional-DIGE has important applications in the field of biomarker discovery for the diagnosis of cancer related disease, and also employed for clinical laboratories in determination of level of protein expression [24].

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Schagger and von Jagow have been initially introduce a convenient technique known as blue native polyacrylamide gel electrophoresis that can easily separate the enzymatic complexes present in active membrane of protein under the moderate conditions[25]. In this technique, before loading of protein sample,it should be ensured that the anionic dye Coomassie brilliant blueG-250(5% w/v) has been mixed properly with the protein sample. These dyes have capability to impose negative charge around the surface of protein. These activate the charge shift which enhances the solubility process of proteins, particularly the primary proteins of the proton membrane of the mitochondrial membrane, providing an overall analysis of the proteomics of the membrane. The dye is bound to the complex mixture of protein and migrate both of them during the process of electrophoresis. Finally, Coomassie dye used to stain the gel before completeanalysis of mass. This technique is useful to determine individual compounds with inflammation protein, integrin and histone protein complexes in the placenta [25].

(e) Gel filtration chromatography technique (size exclusion chromatography)

The basic principle of gel filtration chromatography or size exclusion chromatography is to separate the protein molecules (macromolecule) through dextran material on the basis of their different molecular sizes. This method is suitable for removal of salts from protein solutions and determination of molecular weights of proteins. The stationary phase of gel-permeation column consists of an inert material and traps the molecules within small pores. The molecules having variable size and shape are continuously allowedto pass from the column at constant flow rate. The molecules with large size can not permeate and diffuse into gel particles and thus remain in the space or pores between gel particles. While molecules smaller in size rapidly diffused into gel particles to retain there for longer period of time than lager molecules. Thus, large molecules move quickly to pass through the column as compared to molecule smaller in size. The smaller molecules spread on the pore and move from column with proportional to the longer retention time [26]. Different nonionic species can also be isolated by size-based mechanism over ion exchangers [27].

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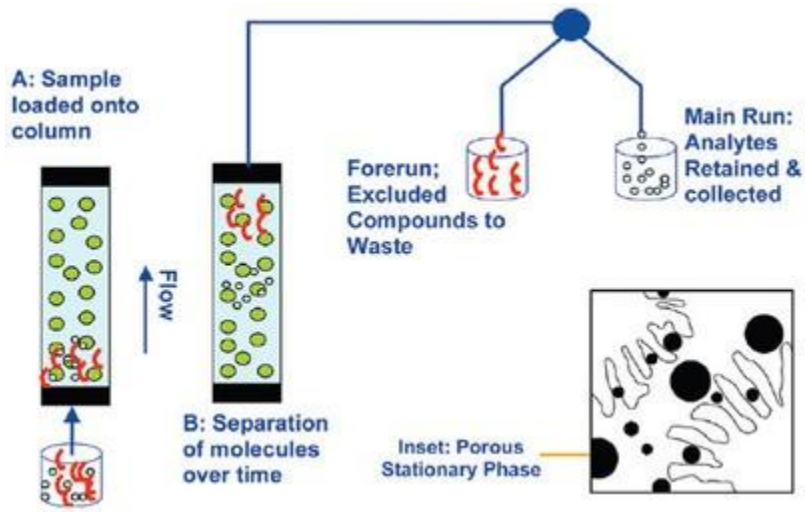

Figure-4: Working of gel filtration chromatography[28].

Gel permeation chromatography (GPC) is also a size exclusion chromatography (SEC) technique.In this technique molecular separation takes place by hydrodynamic volume in solution (molecular size) which differs from molecular weight. When sample is passes through a porous stationary material then ability to permeate the porous resin is determined by size of the molecules being solvated. The mobile phase used for purification of molecules is an organic solvent. The solvent is able to solvate and move the molecules on the basis of their molecular size. This method has been employed to determine distribution of different proteins or polymers present in sample. Generally, it is used to clean up proteins(high molecular analytes)fromundesired low molecular weight components such toxic, pesticides, plant and animal residues etc[28].

The quantitation of aggregates in protein-based therapeutics is important for their immunogenicity and efficacy. The connection of SEC and light dispersion detectors enables to obtain information or confirm the molecular weights in a better way.SEC associated with multiangle light scattering (MALS) can provide information about concentration, shape and size of sample. It has been established that SEC-MALS provides information about proteins, protein-dimmers, and high order aggregates of proteinssuch as human IgG aggregates [27].

This technique has been used to measure the concentration of the growth factor of fibroblasts in localized formulations. During the formulation and storage of protein based therapeutics, SEC

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methods are generally used to determine the formation of protein oligomers. It has been reported the formation of aggregates in frozen powder cakes from bovine serum albumin and BGG (BGG) globulins by SEC and NMR in solid state protons[28].

The size exclusion chromatography also developed to investigate the recovery of active interferon-γ andL-asparaginase/superoxide dismutase during encapsulation of protein aspolylactic-coglycolic (PLGA) biodegradable microspheres and as poly (alkyl cyanoacrylate) particles, respectively. Similarly, SEC with off-line ESIMS has been used for direct analysis of the decapeptideLVV-hemorphin-7 peptide contentspresent in samples of cerebrospinal fluid [29]. This approach can be used to purify biological RNA-protein complexes originated from synthetic methodology,*in vitro* RNA transcription and recombinant protein [30].

(f) Ion exchange chromatography

This technique is one of important chromatographic method for protein purification. Charge-charge interaction in between charged protein groups, and the immobilized charges of ion exchange resins or solid support matrix is the basic principle of ion exchange chromatography[31]. The matrix contains opposite ionic chargesfrom protein that is being separated. Separation of protein from column is affected byionic strength of buffer media, concentration of ion salt and changing pH. The matrix has opposite ion charge as that of protein to be separated [26]. There are two types of ion exchangers are available one is the anion exchange contain positive charged stationary phase which have affinity towards negative charged proteins. Moreover, the second is cation exchange exhibit negatively charged stationary phase that attached with positive charged proteins [32].

Whenever the protein is bounded with the column matrix, at this step the pH of buffer will be less than isoelectric point of the protein which is going to purify. The cation-exchange resins are generally containing negatively charged such as sulfate ions (known as S-resins) whereas the anion-exchangers have positively charged quaternaryammonium ions. However, in some of the cases it may also contain either primary or secondary or tertiary amino groups. Due to lack of specificity this technique is not able to give single step purification [31].

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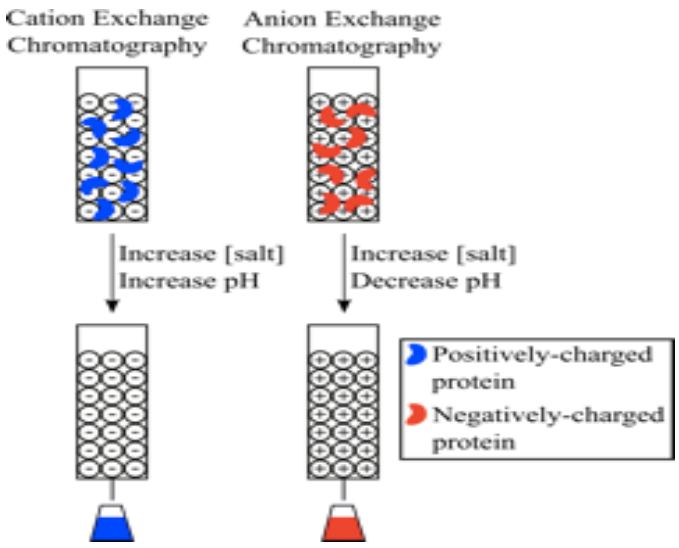

Figure-5: Proteins bound to a charged stationary phase[32].

In the above fig. 5, at low ionic strength the proteins become combined to stationary phase which contains some charge. The protein can be successfully captured by adjusting the ionic strength of buffer media and pH. Depending on the difference in charge property, it allows the separation of ionizable molecules [32]. This is technique has been used for the purification of different proteins and enzymes such asbase excision repair (BER) enzyme (human *N*-methylpurine DNA-glycosylase), GST-BRCA1, mAPE1, hOGG1 and hOGG1 by adjusting the pH with respect to isoelectric points of proteins [31].

There are various parameterswhich must be optimized in ion exchange chromatography in order to improve separation such as composition and concentration of salt, type of ligand, buffer pH, , gradient flow rate etc. If IEX with MALS (multi-angle light scattering) is combined then it providedfine separation capabilities with high resolution of IEX chromatography.Thus, it allows direct molar mass measurement for every individual peak from elution profile [33].

(g) Affinity chromatography technique

Affinity chromatography is based on recognition of a target molecule by ligand bound to column matrix. Thus, separation relies on reversible interaction between target protein molecule and affinity ligand of matrix. Separation depends on specific biomolecularinteractions between two

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molecules such as enzyme-substrate interactions, receptors-antigen interactions orantigen-antibody interactions etc[34].

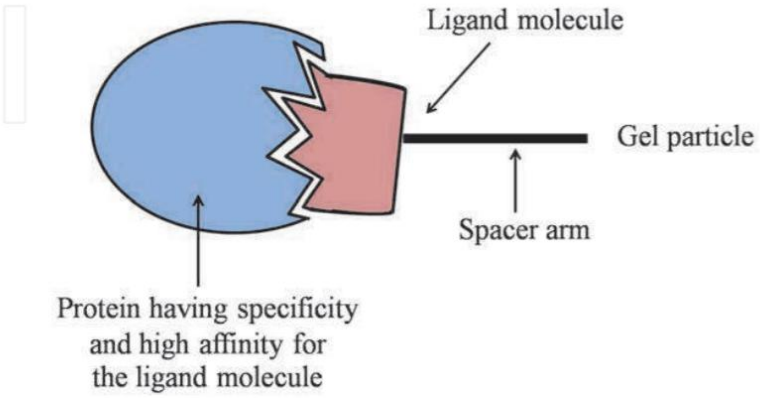

Figure-6: Separation of protein by affinity chromatography[4].

The reversibility of interactions is essential to purify interacting molecules so that target molecules can be dissociated from ligand after washing out of unbound components of sample. The ligand in a solid matrix functions as a stationary phase while the target molecule is a part of mobile phase. Thus,in affinity chromatography the ligand molecule is immobilized on a matrix for separation of its partner molecules (fig. 6)[4].

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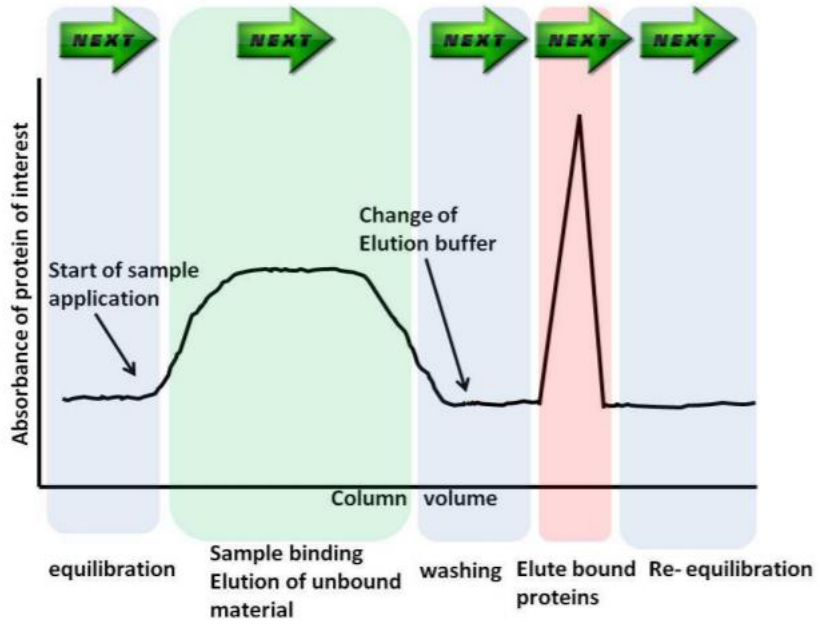

Figure-7: Operating procedure of affinity chromatography[34].

As shown in fig. 7, the affinity purification is a stepwise process. Initially, samples in binding buffer are appliedby maintaining the conditions for maximum binding of desired molecules (proteins) with affinity ligand. Then,unbound substances are washed out by suitable buffer solution without dissociationof the bounded (desired) molecule from affinity support. Whenever required, the detergent level or salt concentration may be adjusted for minimization of non-specific binding interactions. Finally, bound (desired) molecules are elutedbydissociation with an elution buffer in a desorption step through breaking the binding interactions. It can be done by using a competitive ligand in a specific manner or by changing elution conditions (such as ionic strength polarity and pH) in a non-specific manner. At the end of elution process the desired molecules are collected as concentrated andpurified forms [34].

The aim of technique is to describe the interaction between biological molecules either enzymes or proteins. In affinity chromatography,there are distinctive kinds of affinity ligands, for example, chemical substrate or inhibitor, biomimetic dye, protein, immunizer, antigen, hormones etc. The dyes (biomimetic monocholoro and dichlorotriazinyl) are used for the protein as well as enzyme purification because theycan easily capture most of the proteins and thusthey are mostly

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employed in dye-ligand affinity chromatography[35].Bead agarose (Sepharose) is a functional insoluble carrier that has been used successfully for selective refining of antibodies and enzyme antigens [36]. Earlier, Fernandez *et al.* has been used protein G-HPLC affinity chromatography for quantitative separation of nimotuzumab from cell culture supernatants due its high specificity and affinity for human IgG antibodies[37]. Similarly, protein A affinity chromatography, containing staphylococcal protein A with hexameric ligand, for determination of IgG2 antibody from culture supernatant and process samples due to its very high binding affinity and equilibrium capacity [38]. However, hexa-histidine tags(His-tags) may create problem in the purification process by catalyzing hydrolysis of ester functionalities in the desired molecules[39]. Recently, Reiter *et al.* have been reported separation of HIV-1 gag virus-like particles using flow-through heparin affinity chromatography from HEK 293 cell culture supernatant with yield of 54% in the flow-through and 15% in elution[40].Liangsupree*et al.* have been developed a new affinity chromatographic methodology for a rapid isolation of low-density lipoprotein particles from plasma using chondroitin-6-sulfate and anti-apoB-100 antibody[41].

(h) Expanded bed absorption

Expended bed adsorption (EBA) is a simple and rate limiting step technique for the purifications of biological proteins obtained directly by raw sample. The settled bed in expanded bed adsorption is expanded through equilibration buffer. The crude mixture of soluble proteins cells, cells debris and contaminants are move via expended bed. The adsorbents are only can give the target proteins. While changing the elution buffer, maintaining the upward flow will absorb the target protein into the extended bed mode. Instead, the adsorbed particles settle rapidly if the flow is reversed and the proteins can be absorbed by an isolation barrier. After filtration, the cleaning in place(CIP) (e.g. phosphate-free alkaline detergent) solution is used for the pre-cleaning of the absorbent material [36, 42, 43].

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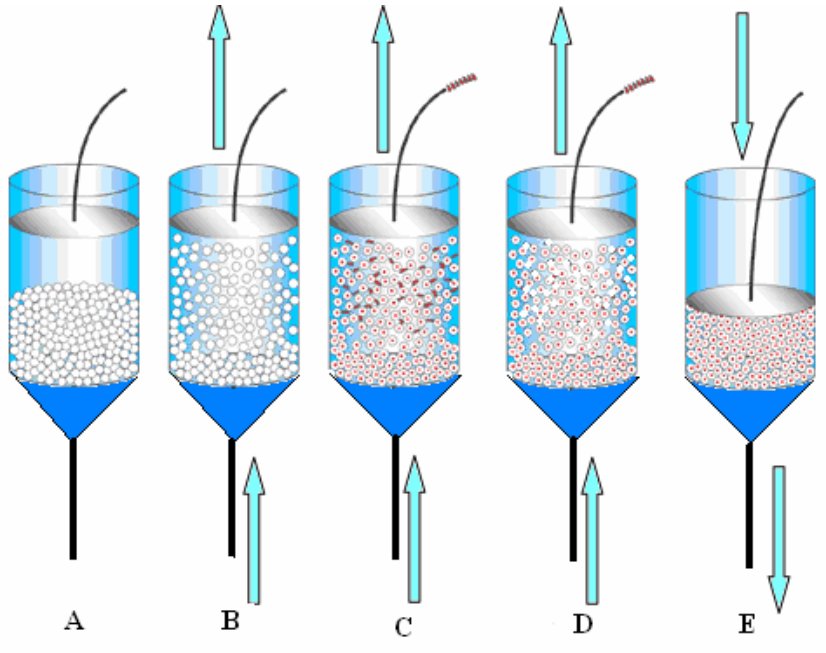

Figure-8: Working procedure of expended bed adsorption[44].

In the working of EBA,initially the column in which the adsorbent is poured will be remains close together and that leaves minimal room for large clumps and aggregates (fig. A).After the insertion of equilibration buffer from below, the adsorbent is coverts in the fluid and when its sedimentation velocity will be equal to the velocity of the liquid moving on upward direction the adsorbent makes an stable concentration gradient (fig. B).After injecting the sample, all the particles and cell debris are able to move easily throughout the bed adsorbent and exit from the uppermost tip of the column. Like any chromatographic step, the adsorbent is heavily washed to restrict the unspecific interactions into the particles and adsorbents. Now, the compounds were retained in the column and interact with the beads(fig. C). Then compound eluted into the adsorbent, the flow brings in opposite direction and column is packed (fig. E).The final part of the procedure will be done in packed as well as fluidized bed mode (fig.D) [44].

Earlier studies have been reported that EBA can be utilizes in isolation of rHSA (recombinant human serum albumin) from yeast fermentation media. Monoclonal antibody(MAB) product can also be successfully purified which is obtained from animal cell culture. Transgenic protein is produced from cow milk, which may be needed to purify because the cow milk contains other

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proteins and lipids. As a result, EBA technique is an ideal choice for processing such feedstock and simplifies the whole process to reduce the cost[45-48]. Recently, de Sa Leitao*et al.* have been reported purification of 503 antigen *Leishmania i. chagasi* using EBA technique [49].

2.9.Hydrophobic interaction chromatography

Another chromatographic technique for protein isolation is hydrophobic interaction chromatography (HIC) [44]. This method is relying on the hydrophobic interaction between a micro molecule (such as biological molecule, and the stationary phase) the same as reversed-phase liquid chromatography (RPLC)[44]. The hydrophobic ligand which is a stationary phases in this method are fixed with a matrix [4]. The non-polar groups of the proteins are generally made up of amino acids like valine isoleucine and phenylalanine and these are exhibit the hydrophobic nature. Moreover, the non-polar parts of protein surface are shows the hydrophobic interaction with hydrophobic groups on the matrix of column, the matrices are crossed linked agarose or silica [4, 44]. However, the relative affinity through the stationary phase is determined by the non-polar patches of the protein surface [44].

The recent applications of His have been reported for purification of enzyme monoterpenecyclase which is obtained from rauwolfia serpentine. It can also purify the enzymes like L-tyrosine decarboxylase (from *Eschscholtzia California*), 6a-hydroxymaackiain 3-O-methyltranferase (from *Mlycine Max*) and 17-O-deacetylvindiline 17-O-acetyl transferase (from *Catharanthusroseus*) [50].

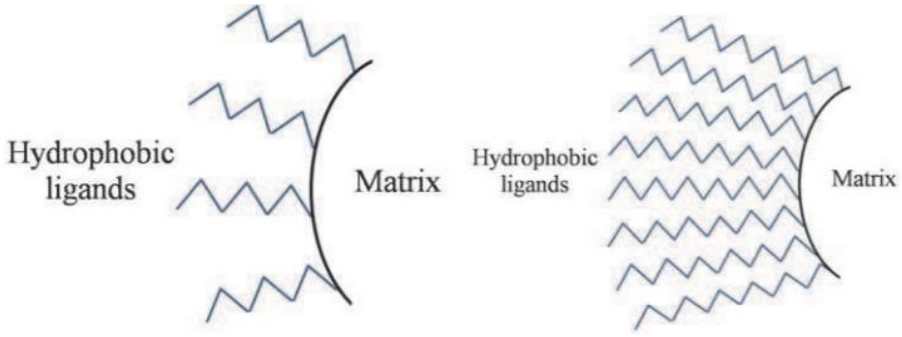

Figure-9: Compare the structure of stationary phase using in HIS and RPLC [4].

Conclusion

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The study of various protein purification techniques concluded that these techniques give better yield of proteins as well as enzymes from a complex mixture, usually cells, tissues or whole organism. They play significant role in development and progression of commercial products such as nutritional proteins, biopharmaceuticals etc. separation steps usually exploit differences in protein size, physico-chemical properties, binding affinity and biological activity. Analytical purification produces a relatively small amount of protein for a variety of research including proteins structure, post translation modifications and function. Finally, this report provides a cumulative and updated description of various protein purification methods.

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