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An overview of purification methods for proteins

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Abstract
Today proteins are being increasingly used in many industrial processes like food processing, leather, cosmetics and pharmaceutical industries as well as in organic chemistry in the area of enzymatic catalysis.
Proteins are also used for various green synthesis processes. Proteins are required in the purified forms for their application in the Industry as well as in organic chemistry. In fact the purification of proteins is an important step in the protein chemistry. Strategies for protein purification include non chromatographic methods and also some modern chromatographic techniques. This paper describes some of the chromatographic and non chromatographic techniques used for protein purification. Some non chromatographic affinity based purification methods have also been proved worthy for present day industrial needs which are discussed in this paper.

Keywords: Purification, Protein, Industry, Affinity

1. Introduction
Proteins were recognized as a distinct class of biological molecules in the eighteenth century by Antoine Fourcroy and others, distinguished by the molecules' ability to coagulate or flocculate under treatments with heat or acid. Proteins are important compounds as these are associated with several aspects of our life. These are included in our life in the form of detergent, food and fed, cosmetics and other compounds for our daily life requirements. Today proteins are being increasingly used in many industrial processes like food processing, leather, cosmetics and pharmaceutical industries as well as in organic chemistry in the area of enzymatic catalysis.
Proteins are also used for various green synthesis processes. Industry needs proteins for the production of fine chemicals such as pharmaceuticals, agrochemicals, fragrances and flavors, food additives, and consumer care products. Proteins are required in the purified forms for their application in the Industry as well as in organic chemistry. In fact the purification of proteins is an important step in the protein chemistry. Protein purification is carried out in both academic and industrial sectors. Non-chromatographic approaches and chromatographic methods are used for this purpose. [1]
The purification of proteins involves a series of processes for isolation of any specific protein from a complex mixture and removal of unwanted contaminants. Purification of proteins can be preparative and analytical. Preparative purification is applied to produce relatively large quantity of purified protein for industrial usage and for preparation of commercial products like food supplements, and for biopharmaceuticals like Insulin. Analytical purification produces a small fraction of protein for research and analytical purposes like identification, characterization of the function, structure determination and interaction of the specific protein. Separation of one protein from others is the most laborious aspect of the process. Processes and conditions used for purification of one protein may result in the inactivation of another. It is challenging area of protein science to develop simple, low cost, and scalable methods for large scale protein purification with a reasonable degree of separation. Low cost protein purification methods are in high demand for mass production of low selling price enzymes that are important in the upcoming bio-economy [2]. Downstream processing involves various purification methods, bioseparation strategies and practices which are employed to separate a desired or target biomolecule. It is now fairly well known that downstream processing costs constitute a very large percentage of overall production costs [3].

Protein purification is an important research frontier and today a Google search for words 'protein purification' provides about 5,21,00,000 results in 0.30 seconds [4], and a Google search for words 'protein purification industry' gives about 51,40,000 results in 0.53 seconds [5] showing the importance of the topic.

The world market of proteins and their production is growing continuously every day. As per predictions the world market of Protein Crystallization & Crystallography Market is assumed to rise up to worth $1,253 Million by 2018 [6].

Major developments in protein purification have taken place in the area of therapeutic proteins and enzymes production. Proteins are used in pharmaceutical and health sectors as therapeutic drugs, vaccines, hormones as well as clinical research & development. This has shifted the focus towards development of more efficient separation techniques which can yield high purification. Also enzymes are preferred tools in green chemistry to replace hazardous processes and toxic chemicals. So, the trend is towards developing less costly, more efficient and easily scalable separation protocols. Today Industries require fast, cost-effective, ecofriendly and simple separation techniques for partitioning and purification of biomolecules [7].

Strategies for protein purification include non chromatographic methods and also some modern chromatographic techniques. Several pharmaceutically important proteins and enzymes are being produced by conventional as well as recombinant methods. The novel techniques like recombinant DNA technology and tissue culture techniques have made it possible to create variety of proteins in large amounts. Many chromatographic techniques have been proved very effective in protein purifications. They offer very high resolution and give a highly purified product, but there are some drawbacks also associated with them. A brief comparison between chromatographic and non chromatographic separation techniques is given below.

Table 1: Chromatographic vs. non chromatographic separation methods

S. No.	Non chromatographic separation methods	Chromatographic separation methods
1-	Economical	Expensive
2-	Used for large samples	For small samples
3-	Time saving	Time consuming
4-	Considerable degree of purity	High purity
5-	Scalable	Often difficult to scale up
6-	Some of these can deal with crude suspensions	Except expanded bed chromatography, these require clean solutions feed

2. Brief History of protein purification

Protein purification has been performed for more than 200 years. In the early days of protein chemistry, the only practical way to separate different types of proteins was precipitation techniques. Initially in the development phase, the protein separation strategies involved the principles which were used for isolation and purification of organic compounds, like crystallization, extraction and precipitation. Fractional precipitations with salt and organic solvents were also used [8].

In 1840 Felix Hoppe-Seyler prepared the first crystals of hemoglobin. Repeated crystallization was used by Hofmeister in 1889 to purify ovalbumin. During World War II there was an urgent need for blood proteins. Cohn fractionation of plasma was developed for the purification of albumin and other plasma proteins. The method was based on multiple precipitation steps with varying pH, ethanol concentration, temperature, and protein concentration at each step. These precipitation methods are still in use today for example, ammonium sulfate precipitation.

Crystallization was the first technique applied for protein separation in 1926 and Sumner obtained the enzyme urease in crystalline form [9]. This progress was followed up by Northrop and Kuintz [10] and they crystallize several proteins and enzymes.

In 1903 the botanist Mikhail Tswett described his work on separation of plant pigments on a column of calcium carbonate. Later, in 1906, he introduced the term chromatography. In 1924 Theodor Svedberg showed that proteins can be separated by centrifugation. During the next several decades, other important protein separation methods were developed: electrophoresis and affinity chromatography (AC) in the 1930s and ion exchange chromatography (IEX) in the 1940s. Hydroxyapatite, is a mixed-mode ion exchange, was developed in 1956 by Arne Tiselius and co-workers. Selective precipitations methods were also developed during this period. The affinity chromatographic methods were next to them and applied extensively.

Table 2 enlists some of the physical properties which form the basis of common purification methods depending on certain characteristics of the proteins.

Table 2: Separation techniques for proteins and their basis- [11, 16]

Separation Process	Basis of Separation
1. Precipitation Ammonium sulphate Acetone Isoelectric	Solubility Solubility Solubility, pH
2. Phase partitioning (e.g. With polyethylene glycol) Chromatography Ion exchange Hydrophobic interaction Affinity Gel filtration/size exclusion Reverse-phase HPLC Immobilized metal affinity (IMAC)	Charge, charge distribution Hydrophobicity Ligand-binding Size, shape Hydrophobicity, size Metal binding
3. Electrophoresis Gel electrophoresis (PAGE)	Charge, size, shape
4. Isoelectric focussing	Size, shape, density
5. Centrifugation	Size, shape
6. Ultrafiltration	Size, shape
7. Differential Denaturation	Thermal Stability
8. Affinity chromatography	Biological recognition

In the large-scale manufacture of recombinant proteins for industrial and therapeutic use, downstream purification is very costly and can account for up to 80% of the total production cost [12]. This cost can be cut down by using appropriate purification method. The optimal purification scheme is dependent upon the properties of the particular protein which is being purified [14].

3. Techniques used for protein purification- Generally for academic needs the purification of proteins is carried out for the structure establishment and structure-function relationship and here the amount purified for these purposes are in small quantities. Tools like capillary electrophoresis, mass spectroscopy, HPLC and micro sequencing methods serve this purpose pretty well. While For Industrial usage,

proteins are required in larger amounts with purity required at somewhat lower levels, like in food and detergents industries.

The degree of protein purity required depends on the probable use of the protein. For some applications, a crude extract is sufficient. However, for other uses, such as in foods and pharmaceuticals, a high level of purity is required.

For these purifications a series of purification steps is typically used. Each protein purification step usually results in some degree of product loss. Therefore, an ideal protein purification strategy is one in which the highest level of purification is achieved in the fewest steps. The selection of best step to use is dependent on the size, charge, solubility and other properties of the target protein. [15]

In most of the purification schemes described in literature, the protocol is generally a mix of non chromatographic and chromatographic steps. The early steps in purification aim at concentration of the protein sample (both in terms of processing volume as well as increasing the percentage of the target protein in total protein) is non chromatographic steps like precipitation with salt, polymer or an organic solvent. The latter polishing steps are generally chromatographic. [16]

A Typical Protein Purification Scheme used today is shown in the figure below.

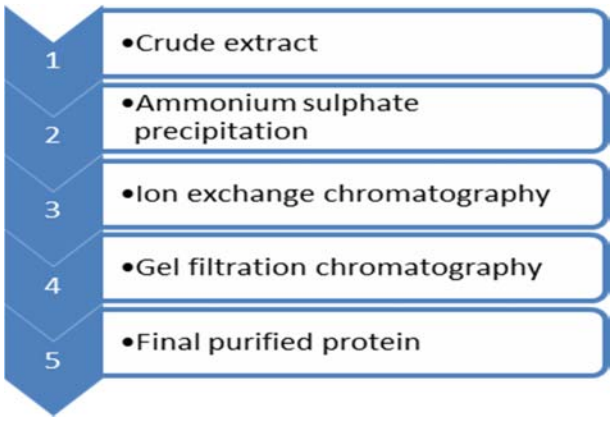

Fig 1: A Typical Protein Purification Scheme

Purification methods for protein are broadly classified into chromatographic and non chromatographic methods.

3.1. *Chromatographic methods*

The term "chromatography" was coined by M. Tswett. Liquid chromatography techniques in which substances in a moving liquid or mobile phase are separated or "partitioned" between the mobile phase and a stationary phase were developed in the early 1940s and following years. The early liquid chromatographic techniques were performed in large columns with "soft" supports (silica gels or cellulose) under atmospheric conditions and resulting separations were achieved slow yielding lower resolutions.

Currently, commercially available media for chromatographic applications offer faster, high resolution separations for a wide range of applications, including several biological molecules like proteins, amino acids, and DNA. One or more chromatographic steps are often needed to yield a high level of purity in protein purification. However, these processes are more expensive and have certain limitations [17].

The different chromatographic purification techniques are briefly described below-

3.1.1. Affinity chromatography (AC)

It separates proteins on the basis of a reversible interaction between protein and a specific ligand attached to a chromatographic matrix. Affinity chromatography offers high selectivity, high resolution, and usually high capacity for the target protein. It is frequently applied as the initial step of a two-step purification protocol, followed by a chromatographic or polishing step to remove remaining impurities. The target protein is specifically and reversibly bound by a ligand. Unbound material is washed away, and the bound target protein is recovered by changing conditions to those favoring desorption. Desorption is performed by using a competitive ligand, or by changing the pH, ionic strength, or polarity. Samples are concentrated during binding, and protein is obtained in purified and concentrated form.

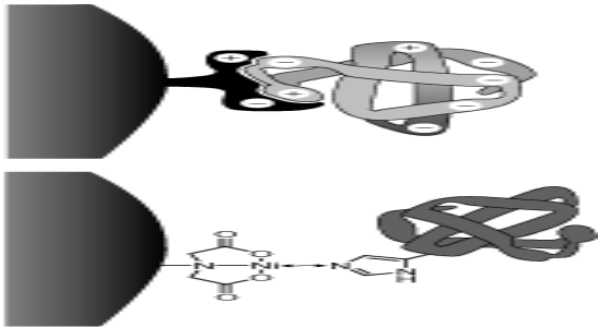

Fig 2: Affinity Chromatography Surface bound with Epoxy, aldehyde or aryl ester groups.

Affinity chromatography is useful for "polishing" or completing the protein purification process. Since proteins have high affinity towards the specific chemical groups / ligands, these attach covalently and bind to the column matrix while non bound proteins pass through the column. During Affinity chromatography, biological interactions like electrostatic or hydrophobic interactions, Vander Waals' forces and hydrogen bonding take place between ligands and the target proteins [18].

3.1.2. Ion exchange chromatography (IEX)

Ion-exchange chromatography separates proteins with differences in their surface charge to result a very high resolution separation with high sample loading capacity. The separation is based on the reversible interaction between a charged protein and an oppositely charged chromatographic medium. Proteins bind while they are loaded into column. Conditions are then modified so that bound substances are eluted differentially. Elution is performed by increasing salt concentration or changing pH in a stepwise manner. Generally samples are eluted with common salt (NaCl), using a gradient elution. Target proteins are concentrated during binding and collected in a purified, concentrated form.

In Ion-exchange chromatography, columns are of anion exchange or cation exchange type. Anion exchange columns contain a stationary phase with a positive charge which attracts negatively charged proteins. Cation exchange columns have negatively charged stationary phase attracting positively charged proteins. Most of the proteins have an overall negative or positive charge depending on their isoelectric point (pI) at a given pH; it makes them possible to

interact with an opposite charged chromatographic matrix. If the net charge of the protein is positive at a pH below pI value, the protein binds to a cation exchanger. At a pH above the pI value the net charge of the protein is negative and it binds to an anion exchanger.

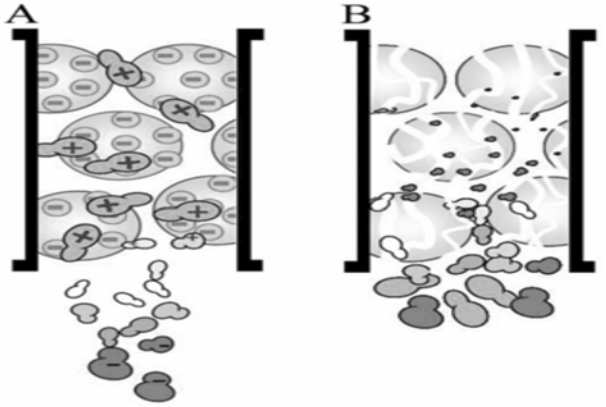

Fig 3: Illustrations of two classical chromatographic methods
A. Ion Exchange Chromatography. The charges of a protein are used for purification.
B. Size Exclusion Chromatography. Protein size is used for fractionation. [19]

3.1.3. Size-exclusion or Gel filtration (GF) chromatography

It separates proteins on the basis of differences in their molecular size. This is suitable technique for final polishing steps in purification while sample volumes have been reduced. Sample volume significantly affects resolution in gel filtration chromatography. Samples are eluted isocratically, i.e. using single buffer without gradient. Buffer conditions are changed to suit the sample protein or the requirements for further purification, analysis, or storage. Purified proteins are collected in the selected buffer. Size-exclusion chromatography separates larger proteins from smaller ones, as larger molecules travel faster through the cross-linked polymer of the chromatographic column and do not fit into the pores of the polymer while smaller proteins do, and take longer time to travel through the chromatographic column. Eluate is then collected in a series of tubes separating proteins based on elution time. Gel filtration chromatography is useful tool for concentration of protein samples; since the target protein is collected in smaller elution volumes than initially added amount to the column. An advantage of gel filtration chromatography is its suitability for biomolecules that are sensitive to pH changes, concentration of metal ions, and harsh conditions.

3.1.4. Hydrophobic interaction chromatography (HIC)

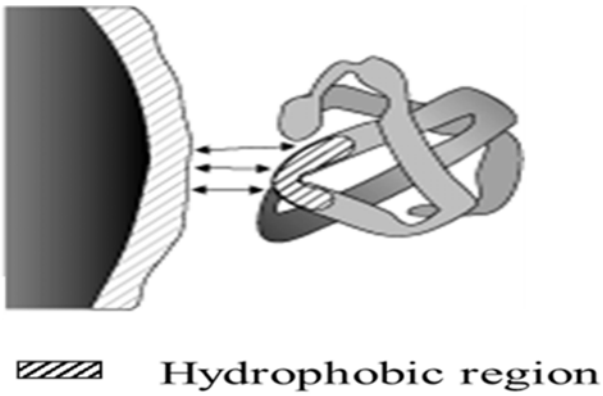

Fig 4: separation of proteins by Hydrophobic Interaction Chromatography [20]

Hydrophobic Interaction Chromatography separates proteins with differences in their hydrophobicity. This technique is suitable for the intermediate or capture step in a purification protocol. Separation is based on the reversible interaction between a protein and the hydrophobic surface of chromatographic medium. This interaction is enhanced by high ionic strength buffer, making HIC an excellent following step after ammonium sulfate precipitation or elution in high salt during ion-exchange chromatography. Samples in high ionic strength solution bind while they are loaded onto a column. Conditions are then altered so that the bound substances are eluted differentially.

Elution is usually performed by decreasing salt concentration. Changes are made stepwise or with a continuous decreasing salt gradient. Generally samples are eluted with a decreasing ammonium sulfate gradient. Target proteins are concentrated during binding and collected in a purified and concentrated form.

3.1.5. Reversed phase chromatography (RPC)

RPC separates proteins and peptides on the basis of difference of their hydrophobicity i.e. reversible interaction with the hydrophobic surface of a chromatographic medium. This is a highly selective technique but requires the use of organic solvents. The figure below shows the binding of protein to the ligand.

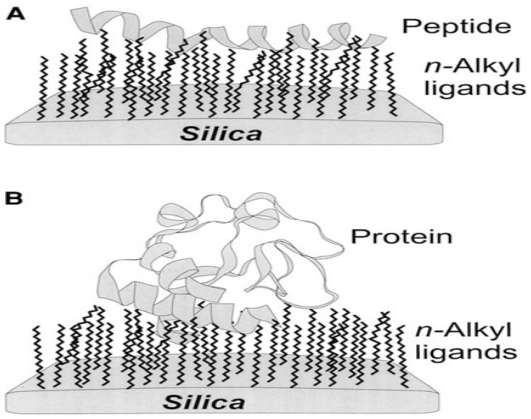

Fig 5: binding of protein in RPC Reference [21]

As some proteins are permanently denatured by solvents and lose their functionality during RPC, this method is not recommended for all applications, especially where retaining the activity of target protein is necessary. This method is frequently used for purity check analyses when activity and tertiary structure are not a focus. Using RPC, the recovery of activity and native tertiary structure of protein is often compromised. Proteins tend to denature and bind strongly to the RPC medium, and it becomes very difficult to elute. RPC is excellent in the polishing phase, when the majority of protein impurities have been removed, particularly for small target proteins that are less commonly denatured by organic solvents.

3.1.6. High performance liquid Chromatography (HPLC)

High performance liquid chromatography is advanced form of column chromatography. In HPLC, solvent is forced to drip under high pressure near 400 atmospheres making it to flow faster. It can purify very much smaller particles giving larger surface area for interaction between stationary phase and the molecule. This offers much efficient separation of the component of the mixture [22]

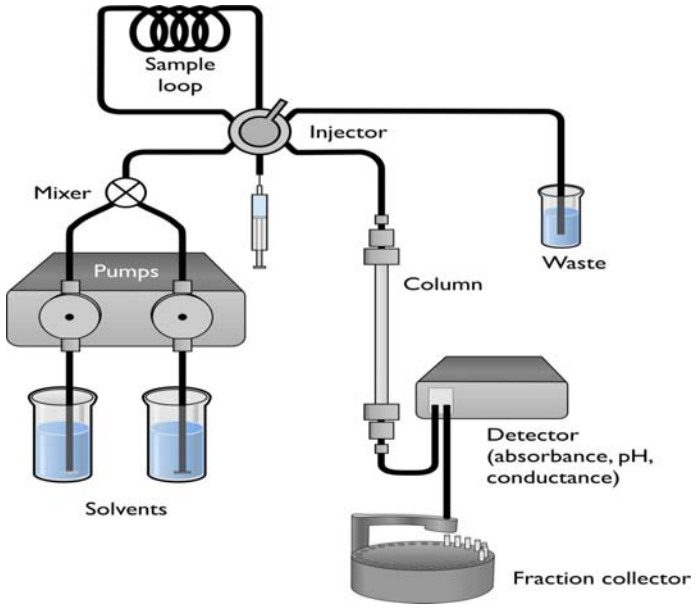

Fig 6: Set up for High performance liquid Chromatography [23]

Chromatographic methods are considered trusted method for purifying both native and recombinant proteins that is why chromatography has been called the workhorse of protein purification. [24]

Some modern chromatographic techniques are being used in combination of other methods for purification of proteins/ enzymes. The following table shows some earlier chromatographic methods used for protein purification. [25]

Table 3: Chromatographic Purification Techniques for proteins

S No.	Technique	Protein purified
1	Radial flow chromatography	Endonuclease VIII
2	Ion exchange chromatography	a. Vibronectin-binding surface protein b. Tubulin
3	Hydrophobic interaction chromatography	α -N-acetyl-galactosamidase
4	Reversed phase liquid chromatography	Sanguinarine
5	Perfusion chromatography	a. Early light-inducible proteins b. BRCA 1 protein
6	Membrane affinity chromatography	a. Polyclonal antibodies b. Human chorionic gonadotrophin
7	Electrokinetic chromatography	Tylosin
8	Capillary electro-Chromatography	Basic proteins
9	Displacement chromatography	Dairy whey protein

Chromatographic methods have some limitations like their inability to handle crude or viscous extracts which contain impurities that can cause column plugging, column fouling, or lowered flow rates. Other drawback of chromatographic methods is uncertainty of scaling-up at higher levels. Chromatographic methods are time consuming as well as costly because columns are expensive. In recent times there is a trend to apply one or more Chromatographic methods or a combination of purification techniques for protein purifications. Some of the early steps are purifying steps while the next other are polishing steps.

3.2. Non Chromatographic methods

Non chromatographic separation methods offer a considerable amount of purified target molecule at large scale. Selective precipitations and extraction are some commonly used operations as early steps in protein purification. Precipitations using reversible soluble –

insoluble polymers and Selective heat denaturation are effective methods. Membrane based separations are other viable options. Two phase extraction and extraction into reverse micelles have also been proved worthy. These non chromatographic methods are now being exploited with full capacity.

If the objective is to obtain purified protein in reasonable amount, some non chromatographic affinity-based processes are worth considering as a practical option. In the area of affinity-based separations, the new strategies have evolved by combination of existing methods. Table 4 enlists some non chromatographic affinity-based separation methods.

Table 4: Non Chromatographic affinity-based Purification protocols for proteins [26]

S no.	Technique	Protein/ enzyme purified
1	Affinity Precipitation	a. Wheat germ lectin b. Peanut phospholipase D
2	Affinity cross flow filtration	a. Concanavalin A b. β-galactosidase
3	Aqueous two phase extraction	Calf intestinal alkaline phosphatase
4	One line dialysis	Alkaline phosphatase
5	Capillary electrophoresis	α-lactalbumin
6	Three phase partitioning	a. Cellulase b. Horse radish peroxidase c. Protease d. α-amylase e. Invertase

3.2.1. Crystallization

Crystallization is an efficient protein purification method. However, purification by crystallization is relatively rare. Protein purification by crystallization has many advantages like high yield, high purity in single step, greater scaling up possibilities and the product is highly concentrated protein crystal slurry ready for further formulation. Some industrially important proteins, like xylose, isomerase, cellulase, and protease, have been purified by crystallization in large-scale.

3.2.2. Gel Electrophoresis

Gel electrophoresis is a method to separate protein according to their size and charge properties. The partially purified proteins from the chromatographic separations are further purified with non denaturing polyacrylamide gel electrophoresis (PAGE), or native gel electrophoresis. In PAGE, the proteins are driven by an applied current through a gelated matrix. The protein's movement through the gel depends on the charge density (charge per unit of mass) of the molecules. The molecules with high charge density migrate rapidly through the gel. The size and shape of protein are two important factors that influence PAGE fractionation. The acrylamide pore size acts as a molecular sieve to separate different sizes of proteins. The larger the protein, the slower it migrates as it becomes more entangled in the gel. Shape is also a factor because compact globular proteins move faster than elongated fibrous proteins of comparable molecular mass.

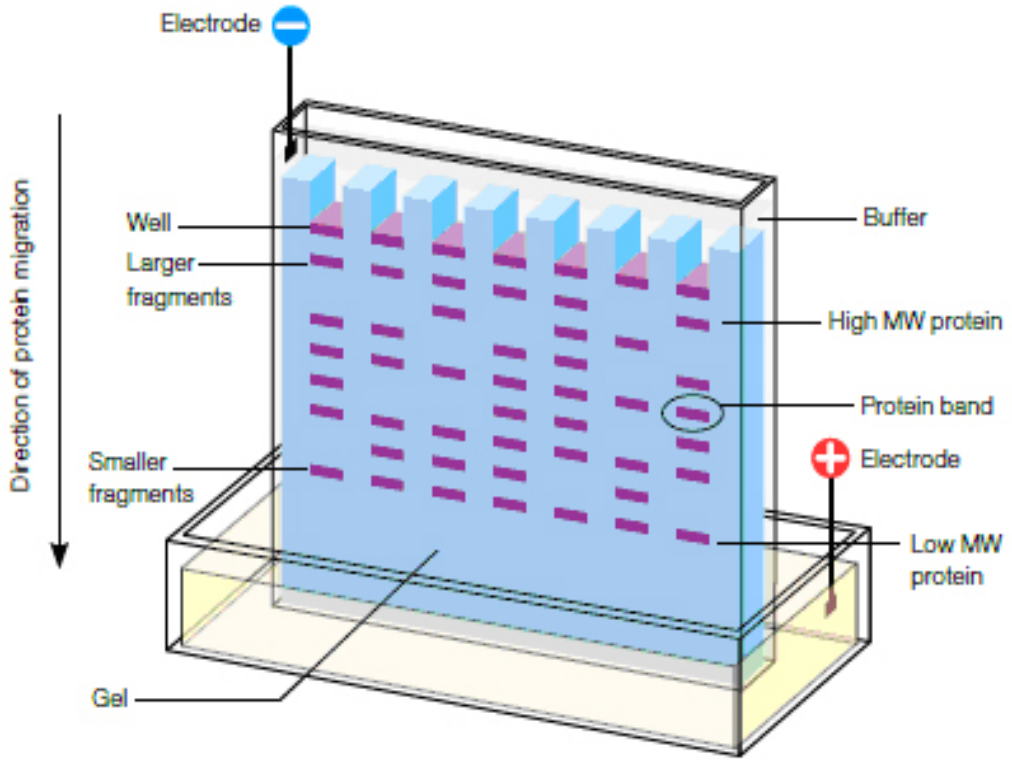

Fig 7: Instrument for SDS PAGE [27]

PAGE is usually carried out in the presence of the sodium dodecyl sulfate (SDS). A protein treated with SDS will usually eliminate the secondary, tertiary and quarternary structure of protein. Most of the proteins retain their biological activities after running PAGE. SDS-PAGE can be used to determine the molecular mass of the mixture of proteins by comparing the positions of the bands with proteins of known size.

3.2.3. Affinity precipitations

This purification approach combines the convenience of precipitation with selectivity property of bio affinity. Addition of a suitable smart polymer-affinity ligand conjugate to a crude broth captures the target protein. This complex can be precipitated by a suitable stimulus. This leaves all other contaminating proteins and other materials in solution. The precipitate can be separated and the target protein dissociated and recovered. This process does not require any costly equipment as in affinity chromatography. It is easy to scale up. Some smart polymers have shown inherent affinity for variety of useful proteins/ enzymes. Eudragit™ S-100 for xylanase alginate for amylases, pectinase and lipases has been successfully exploited for affinity precipitation of the enzymes [28].

3.2.4. Two-phase affinity extractions

In Two-phase affinity extractions process, a water soluble polymer like poly ethylene glycol (PEG) is either mixed with another water soluble polymer like dextran or with a salt solution. Two water rich phases, PEG-dextran or PEG-salt are formed. Any protein would partition in both phases to a varying degree depending upon a complex set of its properties. Thus, a crude mixture of proteins in aqueous solution along with suspended impurities tends to separate into insoluble impurities at the bottom and different proteins partitioned into two phases. The method is easy and can be scaled up. However, its applications at industrial level are limited for different reasons like low selectivity, and limited fold purification. The polymers are also costly sometimes. In recent years, the modified version of two-phase affinity extraction has been successfully applied for purification of proteins.

3.2.5. Aqueous two phase systems (ATPS)

Aqueous two phase systems (ATPS) offer an attractive alternative meeting present day requirements as well as the criteria for industrially compatible procedures. This method is increasingly receiving importance in protein purification industry. The advantage of using ATPS technique is that it reduces the number of initial downstream steps and clarification, concentration, and partial purification can be integrated in one unit [29]. Scaling-up processes based on aqueous two phase systems are simple. An aqueous two-phase system is an aqueous, liquid–liquid, biphasic system which is obtained either by mixing of aqueous solution of two polymers, or a polymer and a salt. Generally, the former is comprised of PEG and polymers like dextran, starch, polyvinyl alcohol, etc. This polymer-salt system results in higher selectivity in protein partitioning, resulting in an enriched product with high yields in the first extraction step. Apart from the large-scale purification of extracellular proteins, the ATPS can be applied for concentration of proteins, separation of membrane proteins, and as a substitute for microfiltration, ultrafiltration and chromatography protocols.

3.2.6. Polyelectrolyte precipitation

Precipitation methods are usually employed for processing large quantities of feed material proteins, offering modest enrichment and high specificity at a low cost early step in the purification process. Including a precipitation step early in the purification process can significantly decrease the number of steps needed in purification process by removing large amounts of impurities and concentrating the product feed. Precipitation processes can be scaled-up generally for commercial and industrial operations. Some common precipitants are acids or bases (isoelectric precipitation), salts ('salting out' with ammonium sulfate), organic solvents, polyelectrolytes, protein-binding dyes, and macroligands. "Selective" precipitation methods such as polyelectrolyte precipitation, protein-binding dyes, and macroligands offer better purification than "non-selective" methods like isoelectric precipitation, ammonium sulfate precipitation, or organic solvents precipitations. Polyelectrolyte precipitation has been identified as a feasible method for an initial, non chromatographic purification step.

3.2.7. Chromatofocusing

It separates proteins according to differences in their isoelectric point (pI). Chromatofocusing is a powerful method and can resolve very small differences in pI (down to 0.02 pH units) and thus separate very similar proteins. However its capacity is lower and it is ideal be used for use with partially pure samples. A pH gradient is generated on the column with interaction of buffer and chromatography medium. Proteins with different pI values migrate at different rates within the column as the pH gradient develops, continually binding and dissociating while being focused into narrow bands and finally eluted. The protein with the highest pI elutes first, and the protein with the lowest pI value elutes last. The upper limit of the gradient is defined by the pH of the start buffer, and the lower limit of the gradient is defined by the pH of the elution buffer.

3.2.8. Three Phase partitioning

Three phase partitioning (TPP) is one of the recent methods, which has been developed for separating proteins into a midlayer and has been successfully employed for purification of a number of proteins and enzymes. Three phase partitioning is an emerging purification method, which is a simple but elegant non chromatographic process for purification and concentration of proteins. It is easily scalable and can be used directly with crude suspensions. It can handle gram quantities of crude inputs in relatively small volumes. It was initially used as a downstream method for isolating the enzymes cellulases [27]. The schematic representation of TPP is shown in Figure 8.

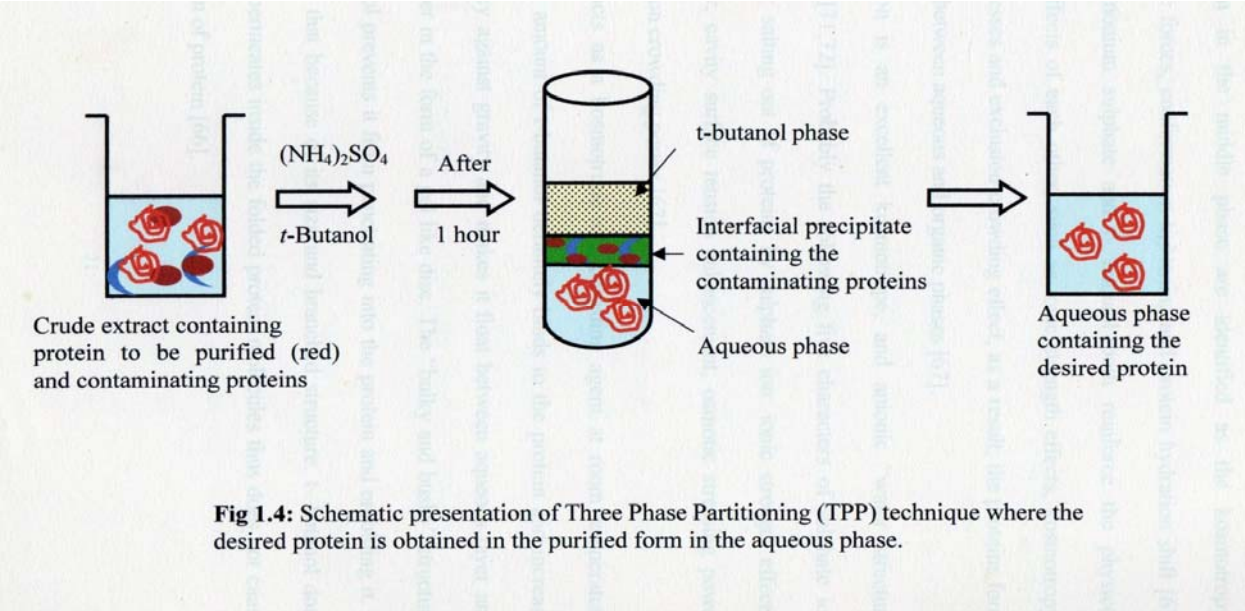

Fig 1.4: Schematic presentation of Three Phase Partitioning (TPP) technique where the desired protein is obtained in the purified form in the aqueous phase.

Fig 8: Schematic representation of TPP System [26]

TPP employs collective operations of principles involved in numerous techniques such as conventional salting out technique, Morton's n- butanol extraction method, isoionic precipitation, cold cosolvent precipitation, osmolytic and cosmotropic precipitation of proteins. TPP technique has following advantages as compared to other separation methods:

1) TPP is a rapid and time saving procedure, containing lesser steps. Milliliters of crude samples can be dealt within 5- 10 minutes.
2) TPP is a concentrative or dewatering step, unlike chromatography, which dilutes the protein; even the concentration factors of 100 folds are also achieved.
3) TPP is a depigmenting process and removes unwanted compounds like lipids, phenolic compounds and some detergents from protein samples.
4) It is flexible in selecting pH, temperature, type of alcohol, and salt.
5) It can be operated at room temperature, as compared to other purification methods, i.e. no need to shifting the temperature.
6) TPP is an economical method, as ammonium sulphate and t-butanol are not expensive chemicals.

A number of proteins and enzymes have been purified from different plant and animal sources using TPP for example- Cellulase, Lipase, Peroxidase, Catalase, Protease, α- Amylase, Invertase, β- Glucosidase, Amyloglucosidase, Protease inhibitor, Mannitol dehydrogenase and alcohol dehydrogenase [27]. In most of the cases good yield and many fold increase in specific activity along with Retrieval of nearly all activity of proteins have been reported. Today modification of TPP like Two Step TPP, Metal – affinity based TPP, Affinity macroligand facilitated three phase portioning (MLFTPP) have also been successfully applied [29].

4. Concluding remarks

In 2004, the journal editorial of "Biotechnology and Bioengineering" carried an article "The importance of Bioseparations: Giving credit where it is due" stating, "And finally the explosion in new high level expression systems for production of recombinant proteins has decreased up-stream processing costs to the point where product concentration and purification steps are now dominating the overall manufacturing cost for many protein therapeutics and most industrial enzymes" [30]. This comment has become more relevant with the passage of time.

Scientists working in the academic sector need purified proteins for understanding their structure, metabolic pathway, mechanism or signal transduction. Here economics of protein production is not a matter of concern. For industrial requirements the cost, time and similar factors are worth considering. For production of pharmaceutical proteins and synthesis of chiral compounds etc. non chromatographic approaches are not adequate and in such cases one or more chromatographic step (generally affinity chromatography) is required [2]. Most of the industrial proteins and enzymes for application in detergents, food processing sector are required in larger amounts with fairly low purity level and here chromatographic methods are rarely used. Non chromatographic separation methods like ATPS, Three phase partitioning, Multi Ligand Facilitated Three Phase Partitioning (MLFTPP), participation with smart polymers like PEG are the modern techniques which can deal the feed of suspended impurities thus eliminating the need of costly chromatographic techniques [31].

Today it is realized that decreasing purification steps is desirable to obtain high yields and cost effectiveness, it resulted in use of affinity based separations in non chromatographic modes and generally very early in the purification protocols. The input feed in affinity chromatography needed as a clear solution to prevent choking the column. In some non chromatographic modes of affinity based separations, this constraint is not there. This eliminates the need for centrifugation which is a necessary and costly step at industrial level. Infact, the availability of affinity based non chromatographic methods is rapidly changing the scenario of protein purification industry. Participations are back in the mainframe as an essential step of purification schemes.

Appropriate usage of non chromatographic with chromatographic approaches is required for purifications depending on need of the purified product. Non chromatographic affinity based separation methods are efficient as well as economic, hence offers a good option for purifications at a large scale. These are capable for present day needs of the industries. The challenges in protein purification that still remain make it worthwhile to gain solid knowledge about protein purification so that the available methods can be selected and applied in an optimal way.

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