Chapter

Purification of Proteins: Between Meaning and Different Methods

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Abstract

Purification of protein attracts the scientists' attention toward science in 1926, as Somnar started purification and crystallization of urease from yeast. As years go forward, protein purification strategies updated from using dextrose passing through DEAE-cellulose and ended by affinity chromatography. In this chapter we will describe some of the concepts and the differences between traditional and novel purification methods, in order to point out how a researcher can start the protein purification techniques. Different fundamental purification steps from plant sample will be discussed in this chapter including isolation, concentration, ion exchanger, and affinity chromatography, as well as the important additives that a researcher should add in order to gain a high purification fold.

Keywords: protein, purification, ion exchanger, gel filtration, affinity chromatography

1. Introduction

Purification is an intrinsic objective for profound and exact portrayal for an objective protein, a target protein. As scientists assume that proteins are responsible for bad and good roles throughout everyday life, in this manner we ought to apply more endeavors to definitely comprehend proteins' structure and characters. This objective cannot be achieved else we acquired a highly purified protein which is unfortunately usually present in crowed with different protein communities.

Distinctive fundamental purification steps from plant sample will be discussed in this chapter including isolation of chloroplasts and mitochondria from leaves as well as from plant tissues rich in phenolic compounds. Meanwhile, we cannot ignore purification of protein from microorganisms. Diverse types of affinity chromatography will be also talked about in this chapter. Perusing can discover informative topics, which will urge them to experience protein purification methodology.

This chapter portrays contingent techniques which are previously or now utilized in the purification of proteins from planta. In particular biochemists have devoted effort to finding convenient methods for protein purification in case they are aiming to obtain a precise characterization and dive inside the target protein. Much advancement has been made, utilizing a lot of partiality systems. Especially, proteins as biomicromolecules differ in their chemical and physical properties. The physical properties demand special techniques for studying, for example, mass spectroscopy, gel filtration, ion exchanger chromatography, electrophoresis, and chromatofocusing, while the chemical properties include the composition, covalent and non-covalent bonds, and solubility.

2. Extraction of protein from plant and yeast

It is impossible to obtain a pure plant protein by using a single-step protocol of purification, as plant tissues contain a wide range of proteins and have rigid cellulosic cell wall and phenolic compound which may cause protein degradation.

The extraction process starts with utilizing a known and appropriate weight of fresh or frozen plant sample under liquid N_2. Homogenize this lot in a prechilled mortar with suitable volume of extraction buffer (3 x of the sample). The extraction buffer type and its pH are distinctive to the sample nature. Some additives should be added to the extraction buffer to improve the obtained extract quality. These additives include:

a. Phenylmethylsulfonyl fluoride (PMSF, dissolved in very small amount of propanol just before extraction) to inhibit protease

b. Flavin compounds (FAD)

c. Dithiothreitol (DTT), to preserve the sulfhydryl group

d. EDTA, as chemical chelator especially with phosphate buffer

e. Sodium fluoride to inhibit phosphatase

f. Polyphenylpyrovate (PVP) 25 gm/k gm fresh weight of sample, which is an insoluble compound and binds to phenolic compound present in the sample, the obtained compound discarded by centrifugation

g. 30% glycerol, which may help to stabilize some highly labile proteins

h. Antibiotic such as Hibitane, usually used in protein extracted from rhizomes or underground plant parts in general

Filter the homogenate with nylon mush, then centrifuge at 10,000 g for 10 min using cooling centrifuge. Repeat the process of extraction and centrifugation three times to ensure complete extraction of all proteins in the tissue. Nearly a similar technique was followed in extraction of Rubisco from wheat leaves [1], protein from *Peganum harmala* seeds [2], and chickpea seeds [3]. Total protein content ought to be determined in the crude extracts [4], as well as the activity if the target protein is an enzyme.

In the case of a microorganism, e.g., yeast, a Yeast Protein Extraction Buffer Kit may be used [5]. This buffer is based on organic buffering agents, which utilize mild nonionic detergents, and a proprietary combination of various salts and agents to enhance extraction and stability of proteins. A ready-to-use Zymolyase preparation is also provided.

3. Concentration of crude extract

3.1 Ammonium sulfate precipitation

To concentrate or reduce the total volume of crude extract, solid ammonium sulfate was added to the crude extracts to bring the final concentration to 70% (w/v) or follow up **Table 1** to choose alternative concentration. After complete

	Final percent saturation to be obtained																
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
Starting percent saturation	Amount of ammonium sulfate to add (gram) per liter of solution at 20°C																
0	113	144	176	208	242	277	314	351	390	430	472	516	561	608	657	708	761
5	85	115	146	179	212	246	282	319	358	397	439	481	526	572	621	671	723
10	57	86	117	149	182	216	251	287	325	364	405	447	491	537	584	634	685
15	28	58	88	119	151	185	219	255	293	331	371	413	456	501	548	596	647
20	0	29	59	89	121	154	188	223	260	298	337	378	421	465	511	559	609
25		0	29	60	91	123	157	191	228	265	304	344	386	429	475	522	571
30			0	30	61	92	125	160	195	232	270	309	351	393	438	485	533
35				0	30	62	94	128	163	199	236	275	316	358	402	447	495
40					0	31	63	96	130	166	202	241	281	322	365	410	457
45						0	31	64	98	132	169	206	245	286	329	373	419
50							0	32	65	99	135	172	210	250	292	335	381
55								0	33	66	101	138	175	215	256	298	343
60									0	33	67	103	140	179	219	261	305
65										0	34	69	105	143	183	224	267
70											0	34	70	107	146	186	228
75												0	35	72	110	149	190
80													0	36	73	112	152
85														0	37	75	114
90															0	37	76
95																0	38

Table 1.
Quantities of ammonium sulfate required to reach given degrees of saturation at 20°C [1].

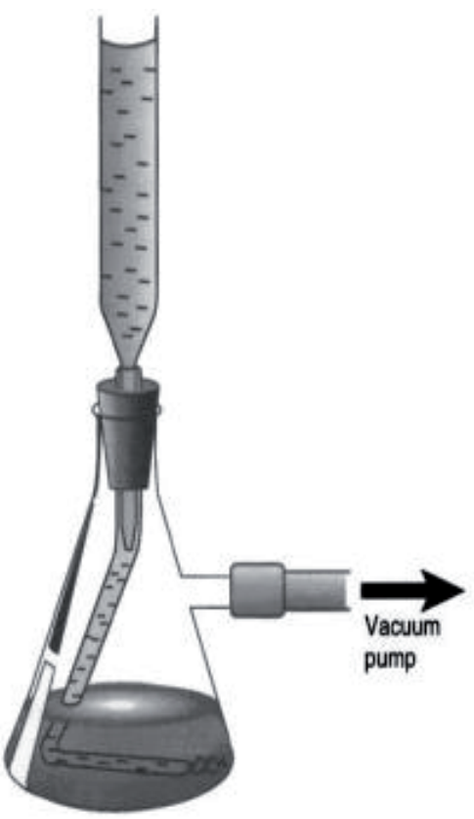

Figure 1.
Experimental arrangement for concentration by forced dialysis [1].

dissolving the added solid ammonium sulfate, the mixture was allowed to stand at 4°C for 24 h, and the precipitate was collected by centrifugation at 10,000 g for 10 min using cooling centrifuge. The precipitated proteins were dissolved in the least amount of extraction buffer. To get rid of excess ammonium ions, dialysis was carried out against the same buffer (see **Figure 1**).

3.2 Fractional precipitation with acetone

The optimum precipitation range for the particular protein fraction is between 37.5 and 50% (v/v). Appropriate and known volume of cruse protein extract is chilled in an ice-salt bath. For each 1 ml of protein solution, add 0.60 ml of acetone (dropwise with constant stirring). After addition of acetone is completed, continue stirring for 10 min with constant control of temperature. The developed precipitate is collected by centrifuging the acetone-protein mixture at 3000 *x*g for 10 min. The precipitated protein is removed and recovered in least volume of extraction buffer. The supernatant volume is measured and 0.25 ml of acetone/ml of protein solution is added further. As in the previous, the precipitated protein by centrifugation is dissolved. To drain off the remaining acetone, the centrifuge tubes over filter paper are inverted. The pellet is suspended in small volume of buffer, and it is kept for the next step of purification [6].

4. Ion-exchange chromatography

4.1 Principles of chromatography

When a sample is applied to any two-phase system (e.g., liquid-liquid, liquid-solid), a molecule may partition between the two phases. With partition coefficient [7],

$$K = C_s/Cm$$

where C is sample concentration in separation phase (s) and in mobile phase (m). When a mixture of several components is applied to such two-phase system, each component of the mixture will have its own individual partition coefficient.

4.2 Components of liquid chromatography

The chromatography system has two components: one is buffer (represents the mobile phase), and the other is hydrated polymers. Buffers should be degassed and filtered before using, especially in the case of high-resolution chromatography. It pumped from a reservoir onto the column. In FPLC and HPLC, high-precision pumps drive mobile phase through the system. The solid phase should be mechanically stable, chemically inert, and widely ranged in their type, efficiency, and

Packing	Composition	Application
DEAE/CM-cellulose	Polysaccharide (cellulose)	Ion exchange chromatography, especially early in purification scheme
Glutathione-agarose	Polysaccharide (agarose)	Affinity chromatography and purification of GST fusion proteins
IDA-agarose	Polysaccharide (agarose)	IMAC (see Section 2.4.6)
Sephacryl S-300	Polysaccharide (dextran/ bis acrylamide)	Gel filtration of proteins in Mass range 10–1500 kDa
Sephadex G-25	Polysaccharide (dextran)	Desalting of protein extracts by gel filtration
Superose 12	Polysaccharide (agarose)	Gel filtration FPLC in the Mass range 1–300 kDa
C-18 Silica	Silica	Reversed-phase HPLC of tryptic peptides
POROS	Poly(styrene-divinylbenzene)	Perfusion chromatography
DEAE/CM-MemSep	Polysaccharide (cellulose)	Membrane-based ion exchange chromatography of proteins

Table 2.
Some stationary phases used in chromatography [7].

application. **Table 2** shows some of the widely used stationary phases; all of them are commercially attainable packed columns.

By the beginning of the chromatographic separation method, the loaded sample is eluted from the column using the mobile phase. This may be achieved in three ways:

a. Continuous flow elution (mobile phase composition and flow rate remain constant).

b. Batch flow elution (the adsorbed sample is selectively eluted by using a range of mobile phase) and stepwise introduction of different mobile phases varied in pH, polarity, and salt concentration.

c. Gradient elution (the adsorbed material separated by two or more mobile phase compositions) is continuously varied.

The chosen way for elution is depending on the behavior of sample components on the stationary phase (**Figure 2**).

4.3 Types of ion chromatography and elution ways

Amino acid sequence determines the net charge of protein. Ion-exchange chromatography separates proteins either on the basis of their charge type (cationic or anionic) or charge strength (e.g., strongly anionic from weakly anionic). Like most column chromatography techniques, ion-exchange chromatography requires a stationary phase which is usually composed of insoluble, hydrated polymers, such as cellulose, dextran, and Sephadex. **Table 2** indicates some of the commonly used ion exchangers [1]. The main concept is that the anion like a negatively charged protein exchanges with chloride ions, while the cation like a positively charged protein exchanges with potassium or sodium ions. This process can later be reversed by washing with chloride ions in the form of NaCl or KCl solution. After loading the crude solution that contained the target protein, some of the proteins are bounded; however, others are not. Elution is carried out by washing with buffer which contained an

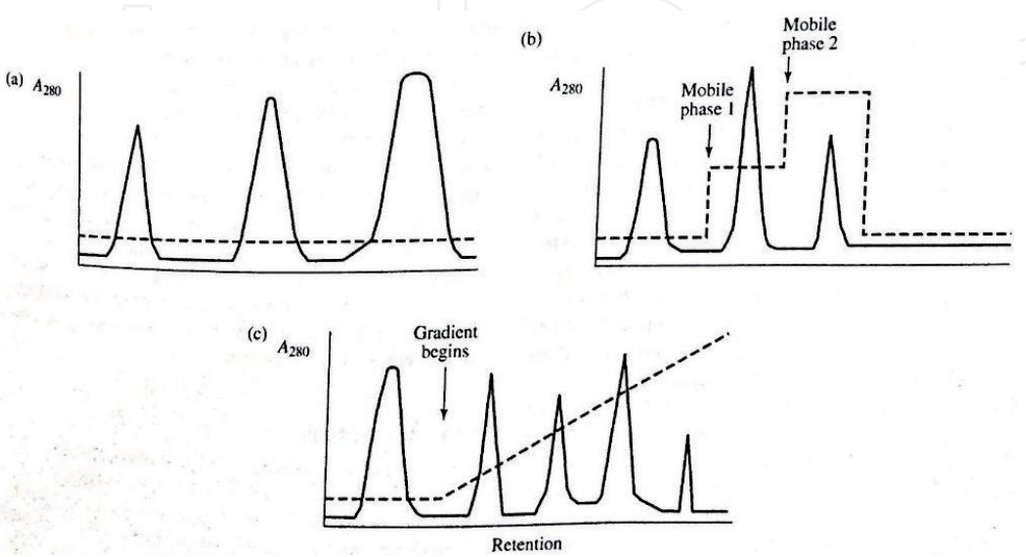

Figure 2.
Elution from stationary phase. Mobile phase is shown as dashed lines. (a) Continuous flow elution. (b) Batch flow elution. (c) Gradient elution, where the adsorbed material is separated by the application of two or more buffers in which mobile phase composition is continuously varied [7].

electrolyte. During elution step, weakly bound proteins are firstly removed, followed by more strongly bound proteins. The opposite is correct for the cation, which like a positive charged protein exchanges with sodium or potassium ion. As an example, in the purification of uricase from soybean, DEAE-cellulose which is an anion exchanger is used. DEAE-cellulose column was prepared and pretreated as recommended by Peterson and Sober [8]. Column was equilibrated with 50 mM phosphate buffer pH 7.5, 1.0 mM EDTA, and 10.0 M mercaptoethanol. The protein was eluted with a stepwise gradient from 0.0 to 0.5 M KCL solutions prepared in the same buffer used in column equilibration. This column was successfully used for purification of uricase extracted from leaves and root nodules of soybean [9]. It is worth to measure uricase activity and protein content in each eluted fraction. Soluble protein was routinely determined spectrophotometrically at 280 nm. Separated peaks may be collected by fraction collector. For more perfection, some researcher may also use 205–220 nm to ensure not missing proteins or peptides which have poor aromatic residues. Elution profile is represented by constructing a plot between detector signal and the retention of sample contents (eluted fractions vs. OD_{280}).

5. Gel filtration chromatography

5.1 For protein purification

Shape and mass of proteins are unique. Gel filtration is also known as size exclusion in which the stationary phase is made up of a gel consisting of beads, which have narrow and fixed size pores (**Figure 3**). So, the proteins below the size pore will diffuse out from the column. Each type of gel filtration has certain cutoff value. By loading the sample, the smaller components pass through the bed and retain faster than the larger ones. The greatest component in the size is eluted freely from the column just after the void volume. Hence, the fraction range will depend uniformly on the population of beds in the column.

Sephacryl S-100 and Sephadex G-100 columns were used extensively for this step. These columns were equilibrated with extraction buffer such as 50 mM phosphate buffer pH 7.5, 1.0 mM EDTA, and 10.0 mM mercaptoethanol. The protein was eluted with the same buffer. Enzyme activity and/or protein content should be assayed for each of the eluted fractions.

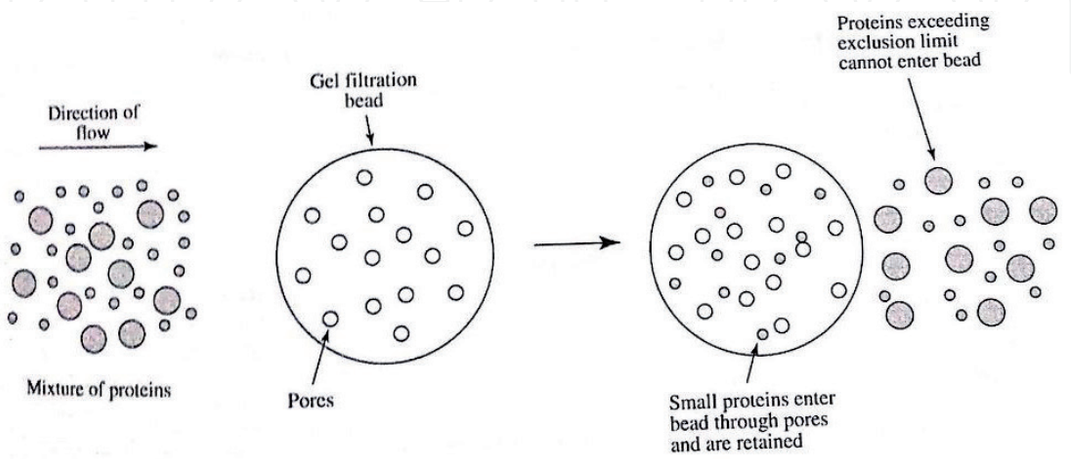

Figure 3.
Gel filtration chromatography of proteins. The proteins smaller than the bead exclusion limit may enter the bead. Larger proteins are excluded. Therefore, proteins elute in inverse order of native mass [7].

5.2 For mass determination

The relative native molecular mass of purified proteins was determined after fractionation on Sephadex G-100 (23 x 1.5 cm) equilibrated with 50 mM phosphate buffer pH 7.5, 1.0 mM EDTA. The marker proteins of known low molecular mass used such as:

aprotinin (6500), cytochrome c (12,400), carbonic anhydrase (30,000), and albumin bovine serum (66,000) Da. The void volume (V_o) was determined with dextran blue (2,000,000) Da. Purified enzyme samples of 2.0 ml volume contained about 2 mg protein/ml was applied to the same column. The protein was eluted from the column with the same buffer used for equilibration. At a flow rate of 1 ml/2.5 min, 2.5 ml fractions were collected. A calibration curve was constructed by plotting log molecular masses versus V/V_o, where (V) was the elution volume and (V_o) was the void volume.

5.3 For removal of low molecular components

Gel filtration may be used in routine way to remove salts. Sephadex G-10 and Sephadex G-25 are commonly used to desalt samples, and hence, it makes the exchange process more rapid and minimizes the dilution of protein sample.

6. Affinity chromatography

The principle of this technique is to fix a small molecule which is called "ligand" on the column stationary phase just before sample loading (**Figure 4**). Affinity is bispecific recognition and distinguishing of biomolecules. **Figure 5** shows a schematic diagram of the steps involved in an affinity-based separation [10].

There are many types of this technique, the most used of them are:

6.1 Substrate or inhibitor affinity

In this technique an enzyme will be recognized by its substrate or inhibitors or cofactors. As a result of the highly specific nature of this ligand to the target protein, only this protein will be bound to the stationary phase and hence purify with high quality. On the other hand, the other protein molecules will go through the chromatography system. At the end, the target protein will be eluted from the ligand. In the case of closely related proteins or isozymes, a variation of this approach should be applied to create an affinity gradient, where the concentration of ligand is gradually increasing in the mobile phase. Hence, each isozyme is unique in its K_m's for the substrates or K_i's for the inhibitors.

6.2 Metal affinity

In this technique, metals are binding to a stationary phase, and metal-binding regions of proteins bind to this ligand. The most common metal used in this technique is Cu, Zn, Ca, Ni, and Fe which are covalently bound to iminodiacetic acid (IDA) and then to the stationary phase, i.e., agarose or silica. A critical problem faced by the researchers during using this technique is that some amino acids, i.e., histidine, cysteine, and tryptophan, may be gradually leached from the stationary phase due to the weakness of interaction with IDA. Elution of the target protein is usually displayed by decreasing pH gradient.

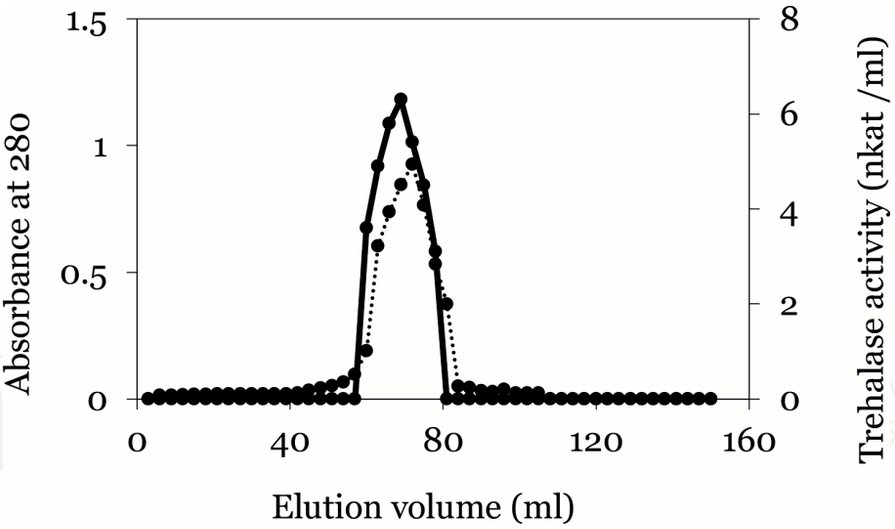

Figure 4.
A typical elution profile for the behavior of trehalase of seeds of T. aestivum *on Sephadex G-200 column (30 × 1.5 cm) equilibrated with 100 mM sodium citrate buffer (pH 5.5). The flow rate was 1.5 ml min^{-1} and 3 ml fractions were collected. Solid line indicates the absorbance; the dotted indicated the enzyme activity [9].*

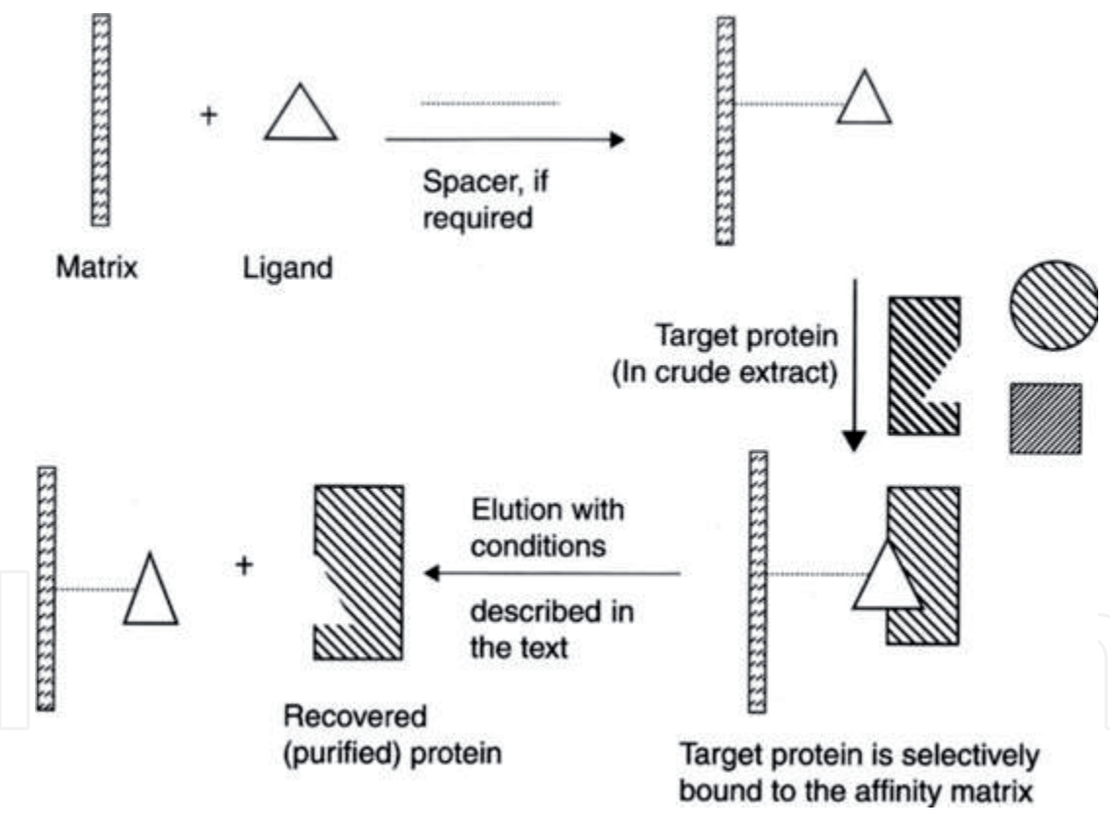

Figure 5.
Schematic diagram of the steps involved in an affinity-based separation [1].

6.3 Immunoaffinity

Immunoaffinity chromatography (IAC) is a type of liquid chromatography that uses a highly specific binding of antibodies for an antigen which is representing the target protein. The application of IAC includes immune-extraction, immune-depletion, chromatographic immunoassays, and post-column immune-detection [11]. These different applications are coming from the tight and selective interactions between antigens and antibodies. Versatile applications of IAC by using either immobilized antigens or antibodies have been used on purification of wild-type

and recombinant mutant amidases from *Pseudomonas aeruginosa* [12]. Antibodies are isolated as an antibody class for immunodiagnostics and biopharmaceutics, and the higher levels of purification are beneficial for the diagnostic methods and for the therapeutic applications. With respect to the importance of purification of antibodies, more and more attention has been paid to IAC, which is an efficient protein separation method based on the interaction between target proteins and specific immobilized antibodies [13, 14]. Monoclonal antibodies (MAbs) are useful for the treatment of a wide array of indications including autoimmune diseases, infectious diseases, cardiovascular diseases, transplant rejection, and cancer. In this respect, the IgG class which plays a most pronounced role in the clinical application was extremely purified using specific antibodies (hyperimmune IgG) [15, 16]. With respect to the importance of purification of antibodies, more and more attention has been paid to IAC, which is an efficient protein separation method based on the interaction between target proteins and specific immobilized antibodies. The less cost and low risk as well as the more safety prompted research activities to focus on novel synthetic ligands (synthetic mimic ligands of proteins A and L). Immunodiagnostically useful *Mycobacterium tuberculosis* H37Ra protein antigens ES-31, ES-43, and EST-6 were isolated from detergent soluble sonicate (DSS) antigen using monospecific antibodies by affinity chromatography. ES-31, ES-43, and EST-6 antigens purified from both culture filtrate and DSS antigen showed similar seroreactivity with overall sensitivity 85, 80, and 75%, respectively [17]. Shuai Sheng and Fansheng Kong reviewed the advantages, applications, as well as drawbacks of IAC in the separation and purification of antibodies and antigens [18].

6.4 Fusion tag protein purification

When proteins are expressed recombinantly, additional amino acids, a functional domain, or a whole protein is often appended to aid in the purification. A standout among the most widely recognized combination labels is His, which is a short chain of six to nine histidine buildups (known as the 6xHis or polyHis tag). Application of 6xHis in purification gives 1000-fold of binding specificity than that of IDA-agarose [7]. The later will bind to metal ions such as nickel or cobalt. Another fusion tag, glutathione S-transferase (GST), which binds tightly to reduced glutathione (GSH), is mostly used. Unlike His and GST tags, most of these other tags are called epitope tags because they require specific antibodies (e.g., immobilized anti-HA antibody) for purification. Epitope labels are irregularly used as it is generally far reaching contrasted with straightforward ligand media, for example, nickel or glutathione agarose. In this way, antibody combination labels are broadly accessible for utilization of single-step liking cleaning.

6.5 Heme-tagged protein

The addition of affinity tags to the amino- (N) or carboxyl- (C) terminus of a target protein for purification purposes can dramatically reduce the amount of preparation time, reduce the number of purification steps needed, and increase the yield of pure protein [19–22]. Recently, the combination of heme tag-HIS has been found more convenient as the target protein was visible and easy to be detected. For this purpose, as the heme chromophore grants the fusion protein with an intense red or red brown color, visible detection significantly reduces the time and effort associated with protein expression and handling during all purification and chromatographic steps. Heme tags are composed of a heme-binding peptide (HBP) that is linked to the heme chromophore by two stable thioether bonds [23]. HBPs are used for HIS affinity purification. HBPs are typically small, consisting

of 14–20 amino acid residues. The critical element of a HBP is a Cys-X-X-Cys-His (CXXCH) heme-binding motif within the sequence, with X representing any amino acid residue [24]. The basis of the heme-tag-HIS affinity purification method is the reversible coordination between the heme-iron open coordination site and the side chain of histidine immobilized on Sepharose beads. Later on, the target protein can be eluted from the column using an imidazole-containing buffer, a low pH (≤5) buffer, or a high pH (≥8) buffer.

6.6 Avidin-biotin systems

Biotin or vitamin H is a small molecule (MW 244.3) that is present in humble amounts in all living cells. The acidic side of this vitamin can be incorporated with various reactive groups. Once biotin is attached to a molecule, the molecule can be captured for detection, immobilization, or affinity purification using conjugates or supports based on avidin or streptavidin proteins, which bind strongly and explicitly to the biotin group. Native and recombinant derivatives of avidin and streptavidin proteins are promptly accessible in a wide assortment of altered, labeled, and immobilized forms. The "avidin-biotin system" has been adapted for use in many kinds of research applications for detection or purification. As the avidin-biotin affinity interaction is so strong, therefore it is usually impractical to elute biotinylated targets that have been captured to immobilize avidin or streptavidin support. However, modified versions of biotin labeling reagents have been developed, such as cleavable biotin, iminobiotin, and desthiobiotin; these provide readily reversible interactions with streptavidin, making them useful tools for soft-release applications.

6.7 Cellulose binding model

Cellulose binding module (CBM) has been distinguished as a potential tool for the fast and easy assembling of recombinant proteins [21, 25]. CBM is an attractive affinity tag for protein purification because of its high capacity and specific adsorption to cellulose. In addition, it can be efficiently adsorbed to cellulose in most buffers and eluted under non-denaturing conditions. There are many advantages of this technique such as low cost, appropriate physical properties, applicable for most proteins, and high stability in the presence of buffers. All of these make it an ideal matrix for large-scale affinity. Moreover, cellulose is easily procured and safe for many pharmaceutical and human purposes. The method of purification of the recombinant protein with a CBM3 tag was developed in both *Escherichia coli* and yeast and has been proven successful in purifying several proteins [26, 27]. This study introduced the recombinant protein expressed in yeast. EGFP was used to demonstrate the well-ordered purification procedure. A self-cleaving intein-based tag removal method for CBM3 was also introduced [28].

6.8 Affinity precipitation

A novel affinity precipitation developed by Arnold and Chen [6] was very promising. One-step binding and precipitation resulted in >95% recovery yield directly from crude extract and a 22.7-fold purification, giving a specific activity of 420 U/mg. The soybean peroxidase isolated using this affinity precipitation meets or exceeds the quality specifications of reagent grade products. The molar ratio of ligand to target fucosylated protein is key for the recovery yield.

Affinity precipitation is an alternative approach with the potential to overcome the challenges associated with affinity chromatography, as it retains the specific

interaction of affinity ligand with the protein of interest while avoiding column operation [29].

7. Conclusion

As there is no restricted catalog for purification steps of proteins to follow it up, the researcher should design its own protocol according to the aimed target and the final quality that is being looked for. Gel filtration and ion exchangers are suitable for broad mass and low-cost aim. However, affinity chromatography with its versatile types is the only technique for highly purified and selective protein yield. All purification techniques have the same major steps that include equilibration of a column, adsorption of a sample, washing to remove unbound materials, and elution of bound protein target protein and ended with regeneration of the media.

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