International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 3 May - June 2021, pp: 878-893 www.ijprajournal.com ISSN: 2249-7781

Review on Affinity Chromatography

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Date Of Submission: 01-06-2021 Date Of Acceptance: 14-06-2021

ABSTRACT:-Affinity chromatography is a method of separating biochemical mixture based on a highly specific interaction between antigen and antibody, enzyme and substrate, receptor and ligand, or protein and nucleic acid. In addition to its solute preparation and purification affinity chromatography thus also posses considerable potential for investigating the functional roles of the reactants there by purified. Affinity purification can provide significant time savings and several hundred-fold or higher purification, but success depends on the method used. This review describes about the basic principle, procedure and its applications

KEYWORDS:- Affinity Chromatography, Principles, Types, Purification, Separation, Current Techniques, Application.

I. INTRODUCTION:-

Combination of bio affinity and chromatography gave birth to affinity chromatography [(1)]. Affinity chromatography is a type of liquid chromatography that makes use of biological-like interactions for the separation and specific analysis of sample components. [(2,3)] The technique offers high selectivity, hence high resolution, and usually high capacity for the protein of interest. Purification can be in the order of several thousand-fold and recoveries of active material are generally very high. Affinity chromatography takes advantage of specific binding interactions between the analyt of interest (normally dissolved in the mobile phase), and a binding partner or ligand (immobilized on the stationary phase). In a typical affinity chromatography experiment, the ligand is attached to a solid, insoluble matrix--usually a polymer such as agarose or polyacrylamide-chemically modified to introduce reactive functional groups with which the ligand can react, forming stable covalent bonds [(4)].

This technique is especially known as the most specific and effective technique for protein purification. Separation of the biomolecules is based on highly specific biological interactions between two molecules, such as enzyme and substrate [(5)]

Affinity chromatography (also called affinity purification) makes use of specific binding interactions between molecules. A particular ligand is chemically immobilized or “coupled” to a solid support so that when a complex mixture is passed over the column, those molecules having specific binding affinity to the ligand become bound. After other sample components are washed away, the bound molecule is stripped from the support, resulting in its purification from the original sample the basic idea has been widely exploited as a powerful tool for the separation and purification of a wide variety of biological macro-molecules. Its effectiveness for purification rests on the selectivity of interaction, and thus of adsorption,of a biological macromolecule on an affinity adsorbent which is prepared by the covalent immobilization of a specific ligand on solid polymeric matrix [(6)]

Affinity chromatography does not require the molecular weight, charge, hydrophobicity, or other physical properties of the analyte of interest to be known, although knowledge of its binding properties is useful in the design of a separation protocol. [(7)]

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Table No. 1: Typical Biological Interactions Used in Affinity Chromatography [8]

Sr. No	Types of Ligand	Target Molecule
1	Substrate analogue	Enzymes
2	Antibody	Antigen
3	Lectin	Polysaccharide
4	Nucleic acid	Complementary base sequence
5	Hormone	Receptor
6	Avidin	Biotin/Biotin-conjugated molecule
7	Calmodulin	Calmodulin binding partner
8	Glutathione	GST fusion protein
9	Proteins A and G	Immunoglobulins
10	Metal ions	Poly-histidine fusion protein

II. HISTORY:-

In 1910, the German scientist, Emil Starkenstein published an article which described the concept of resolving macromolecule complexes via their interactions with an immobilized substrate. This manuscript discussed the influence of chloride on the enzymatic activity of liver α-amylase and opened the door for the early beginnings of this approach by several researchers

The term affinity chromatography introduced in 1968 by Pedro Cuatecasas, Chris Anfinsen and Meir Wilchek in an article that briefly described the technique of enzyme purification via immobilized substrates and inhibitors. Other early articles described the activation of a Sepharose matrix using a cyanogen bromide (CNBr) reaction and the use of a spacer arm to alleviate steric hindrance. Since 1968, affinity chromatography has become established as a standard laboratory technique for separation of enzymes.[9]

The use of affinity chromatography for the determination of the inhibition constants of enzymes seems to have good prospects. On the basis of the elution volumes of the enzyme eluted from the column with immobilized inhibitor using various concentrations of soluble inhibitor—the inhibition constants can be determined both with bound inhibitors and with the soluble inhibitors employed. [10]

III. FUNDAMENTAL PRINCIPLES OF AFFINITY CHROMATOGRAPHY:-

Separation of adesiredprotein using affinity chromatography relies on the reversible interactions between the protein to be purified and the affinity ligand coupled to chromatographic matrix.As stated earlier, most of the proteins have an inherent recognition site that can be used to select the appropriate affinity ligand. The binding between the protein of interest and the chosen ligand must be both specific and reversible.

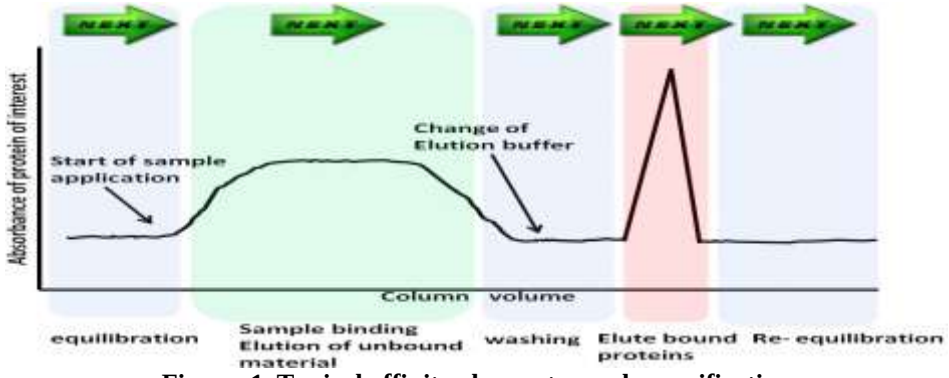

Figure. 1. Typical affinity chromatography purification

Batch Chromatography vs Column Chromatography:-

Binding to the solid phase may be achieved by column chromatography whereby the solid medium is packed onto a column, the initial mixture run through the column to allow settling, a wash buffer run through the column and the elution buffer subsequently applied to the column and

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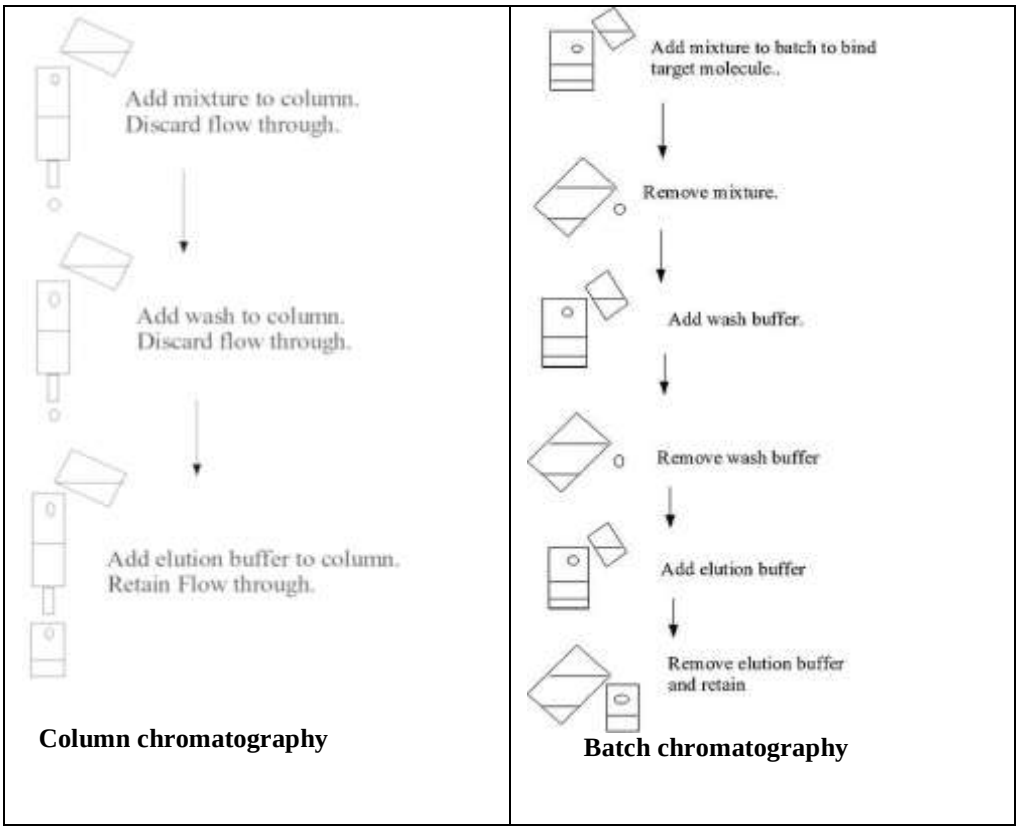
collected. These steps are usually done at ambient pressure. Alternatively, binding may be achieved using a batch treatment, for example, by adding the initial mixture to the solid phase in a vessel, mixing, separating the solid phase, removing the liquid phase, washing, re-centrifuging, adding the elution buffer, re-centrifuging and removing the elute.

Sometimes a hybrid method is employed such that the binding is done by the batch method, but the solid phase with the target molecule bound is packed onto a column and washing and elution are done on the column.

The ligands used in affinity chromatography are obtained from both organic and inorganic sources. Examples of biological sources are serum proteins, lectins and antibodies. Inorganic sources as moronic acts, metal chelates and triazine dyes.

A third method, expanded bed absorption, which combines the advantages of the two methods mentioned above, has also been developed. The solid phase particles are placed in a column where liquid phase is pumped in from the bottom and exits at the top. The gravity of the particles ensure that the solid phase does not exit the column with the liquid phase.[11]



IV. TYPES OF AFFINITY CHROMATOGRAPHY:-

1- Boronate affinity chromatography

Affinity methods that use boronic acid or boronates as ligands are one group of chromatographic techniques that have been used successfully with clinical samples. This group of methods, known collectively as "boronate affinity chromatography", includes one of the earliest reported quantitative applications of affinity chromatography in the clinical laboratory namely, the determination of glycohemoglobin for the assessment of long-term diabetes management Figure (2) [14] [15] [16]. At a pH above 8, most boronate derivatives form covalent bonds with compounds that contain cis-diol groups in their structure. Because sugars such as glucose possess cis-diol groups, boronates are valuable for resolving glycoproteins from non-glycoproteins

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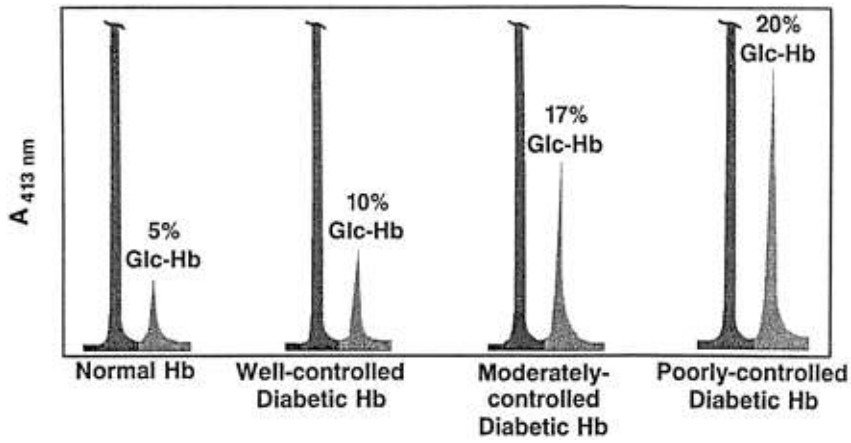

Figure 2- Determination of glycohemoglobin (Glc-Hb) by HPAC for 10-µL samples of diluted whole blood. [17]

2-Lectin affinity chromatography

Lectins are another class of ligands that have been used for the direct detection of clinical analytes by affinity chromatography. The lectins are non-immune system proteins that have the ability to recognize and bind certain types of carbohydrate residues [18]

One clinical application of lectin affinity chromatography has been in the separation and analysis of isoenzymes. This is illustrated in Figure. 3, where an HPLC column containing immobilized wheat germ agglutinin was used to distinguish between the liver- and bone-derived isoenzymes of alkaline phosphatase in human serum [19]

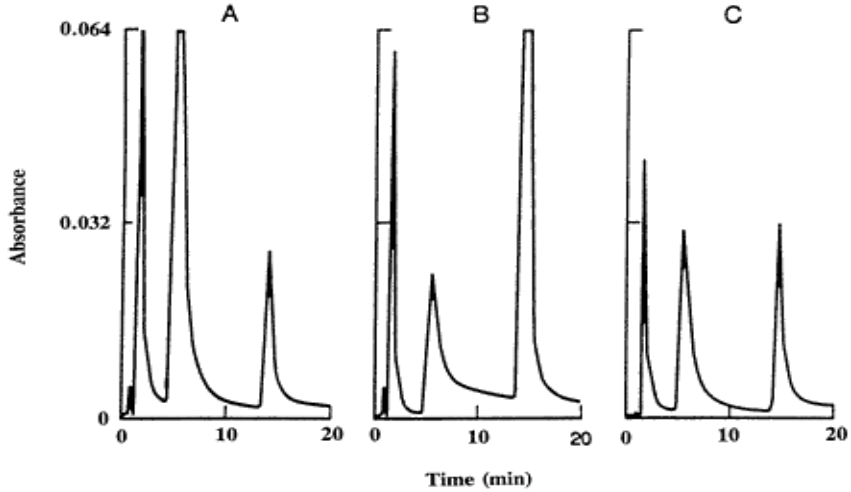

Figure- 3 Determination of liver and bone-derived isoenzymes of alkaline phosphatase by HPAC, using an immobilized wheat germ agglutinin column for 50-µL injections of serum from patients with liver (A) or bone (B) disease, and healthy individuals (C).

3- Protein A or Protein G Affinity Chromatography

A third class of ligands that have been used in direct analyte detection by affinity chromatography are antibody-binding proteins such as protein A and protein G. These are bacterial cell wall proteins produced by Staphylococcus aureus and group G streptococci, respectively. [20] [21]

These ligands have the ability to bind to the constant region of many types of immunoglobulins. Protein A and protein G bind most strongly to immunoglobulins at or near neutral pH, but readily dissociate from these solutes when placed in a buffer with a lower pH. [21] [22]

The ability of protein A and protein G to bind to antibodies make these good ligands for the

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analysis of immunoglobulins, especially IgG-class antibodies, in humans. The first clinical uses of these ligands in an HPLC system were methods based on immobilized protein A for the analysis of IgG in serum samples [23]

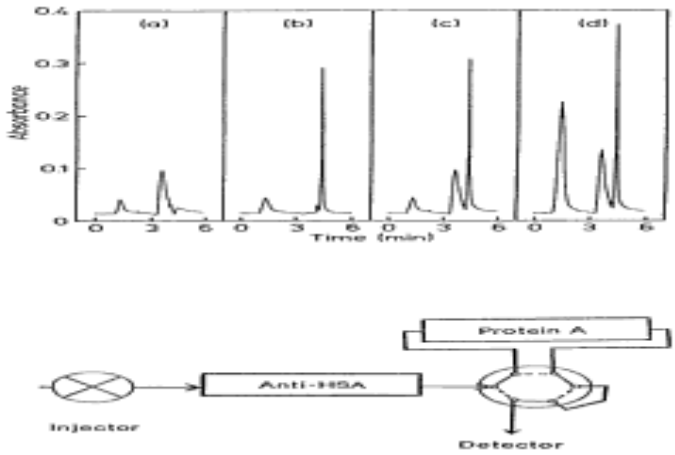

Figure- 4 Chromatograms (top) and valve switching system (bottom) for 10-µL injections of an HSA calibrator (a), an IgG calibrator (b), a mixture of HSA and IgG (c), and a 1:5 dilution of serum (d) on a dual column HPAC system containing anti-HSA antibodies and immobilized protein A.

4- Immuno-affinity chromatography

All the types of affinity chromatography, those that use antibodies or antibody fragments as ligands make up the largest and most diverse group of affinity methods in clinical testing. This is a combined result of the specificity of antibodies and the relative ease with which they can be obtained to a wide variety of analytes.

The term "immunoaffinity chromatography" (IAC) is used for an affinity chromatographic method in which the stationary phase consists of an antibody or antibody-related reagent. When such a technique is performed as part of an HPLC system, the resulting method can be referred to as "high-performance immuno-affinity chromatography" [13] [24]

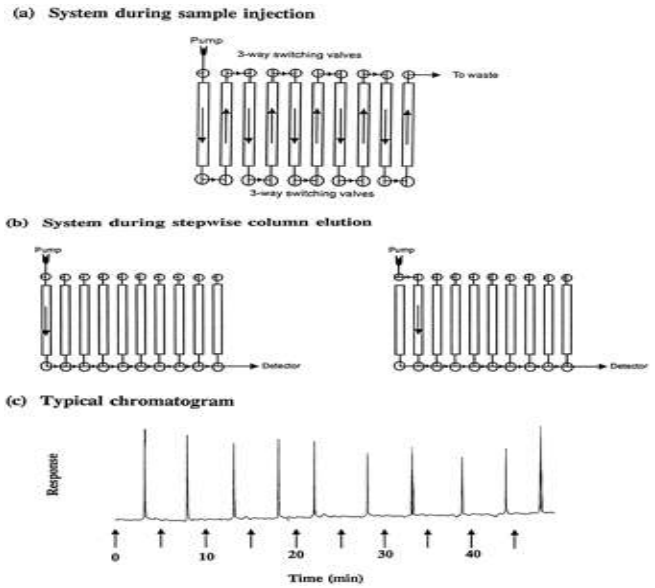

Figure- 5 Diagram of a recycling immunoaffinity system for the determination of multiple analytes during sample application (a) and stepwise analyte elution (b) from each column in the system; and chromatogram (c) showing results obtained for the analysis of a calibration mixture.

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V. AFFINITY PURIFICATION:-

Affinity chromatography is a method for selective purification of a molecule or group of molecules from complex mixtures based on highly specific biological interaction between the two molecules. The interaction is typically reversible and purification is achieved through a biphasic interaction with one of the molecules (the ligand) immobilized to a surface while its partner (the target) is in a mobile phase as part of a complex mixture. The capture step is generally followed by washing and elution, resulting in recovery of highly purified protein. Highly selective interactions allow for a fast, often single step, process, with potential for purification in the order of several hundred to thousand-fold.

Affinity chromatography can be used in a number of applications, including nucleic acid purification, protein purification from cell free extracts, and purification from blood as well as can provide significant time savings and several hundred-fold or higher purification, but success depends on the method used. Thus, it is important to optimize the purification protocol to achieve efficient capture and maximum recovery of the target. By using affinity chromatography, one can separate proteins that bind to a certain fragment from proteins that do not bind that specific fragment.[10] Because this technique of purification relies on the biological properties of the protein needed, it is a useful technique and proteins can be purified many folds in one step. [31]

Steps Involed In Purification:-

Step 1: An immuno-adsorbent is prepared. This consists of a solid matrix to which the antigen (shown in blue) has been coupled (usually covalently). Agarose, sephadex, derivatives of cellulose, or other polymers can be used as the matrix.

Step 2: The serum is passed over the immunoadsorbent. As long as the capacity of the column is not exceeded, those antibodies in the mixture specific for the antigen (shown in red) will bind (noncovalently) and be retained. Antibodies of other specificities (green) and other serum proteins (yellow) will pass through unimpeded

Step3:A reagent ispassed into the column to release the antibodiesfromthe immunoadsorbent. Buffers containing a high concentration of salts and/or low pH are often used to disrupt the noncovalent interactions between antibodies and antigen. A denaturing agent, such as 8M urea, will also break the interaction by altering the configuration of the antigen-binding site of the antibody molecule. [12]

Step 4: Affinity medium is re-equilibrated with binding buffer. The eluate is then dialyzed against, for example, buffered saline in order to remove the reagent used for elution.

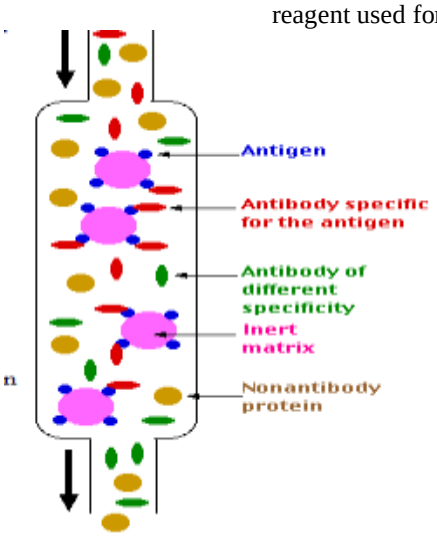

Figure 6: Steps for Purification in Affinity Chromatography

VI. SEPARATION PROCEDURE IN AFFINITY CHROMATOGRAPHY:-

A sample containing the compound of interest is applied to the affinity column in the presence of mobile phase which was prepared in suitable pH, ionic strength and solvent composition for solute-ligand binding. This solvent which is referred as the application buffer presents the weak

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mobile phase of an affinity chromatography. While the sample is passing through the column compounds which are complementary to the affinity ligand will bind. However other solutes in the sample will tend to be washed off or eluted from the column as non-retained compounds.

After all, non-retained components are washed off the column, binding solute or together with ligand as solute-ligand complex are eluted by applying a solvent. This solvent which is referred as elution buffer represents the strong mobile phase for the column. Later all the interested solutes are eluted from the column, then application buffer is applied and the column is allowed to regenerate prior to the next sample application.[8]

Separation procedure in affinity chromatography can be simply illustrated as shown in Fig 7

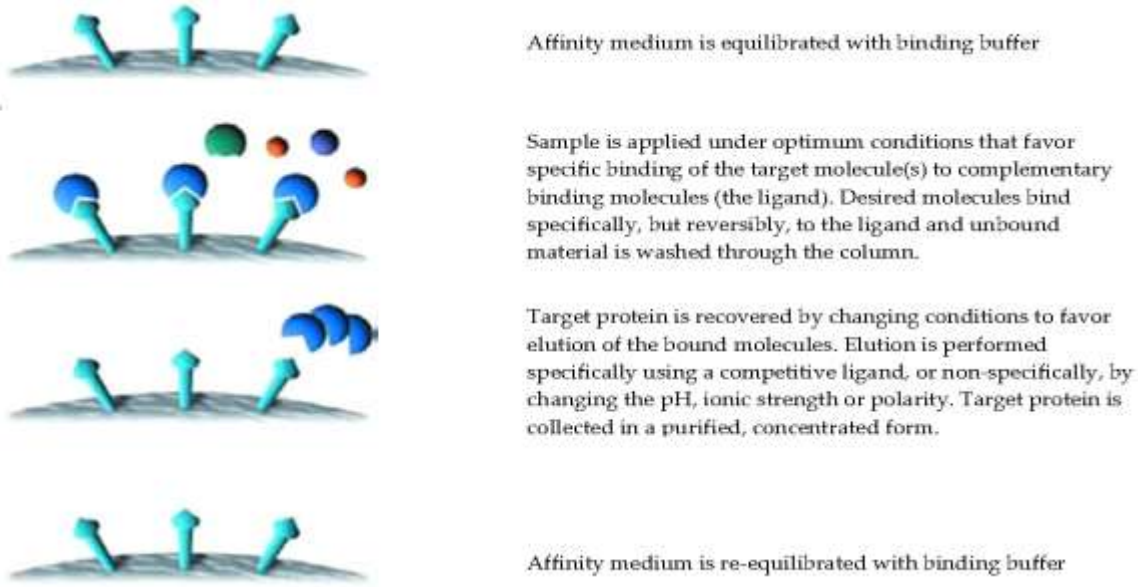

Figure 7 Separation procedures in affinity chromatography

The content apart from the analyte passes through the column without or with weak binding to the ligand while the analyte is retarded. After the analyte is obtained generally by using an elution buffer, the column is regenerated by washing with the application buffer in order to prepare the column for the next injection.[13]

In the Figure 8, a typical scheme of an affinity chromatography application is shown.

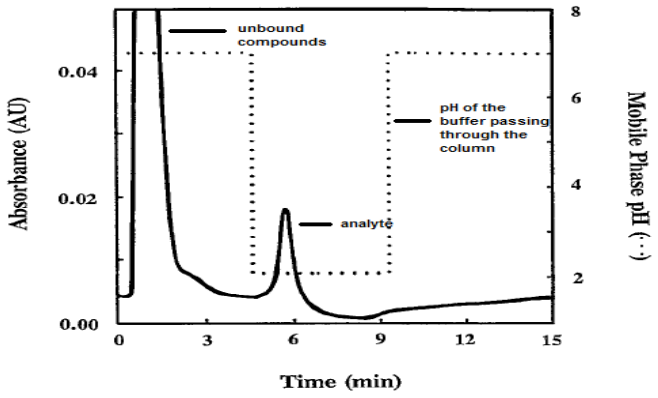

Figure 8: Scheme of an affinity chromatography application

VII. CURRENT TECHNIQUES INVOLVING AFFINITY CHROMATOGRAPHY:-

Affinity chromatography is currently being used for a wide variety of applications ranging from the study of drug-protein binding

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interactions to the depletion of high abundance proteins to enhance the detection/quantification of dilute proteins. Affinity chromatography can be used to study drug-protein binding interactions. Frontal analysis, zonal elution, and the Hummel-Dreyer method can be used to measure drug-protein binding constants, to quantify kinetic properties of the various interactions, to quantify allosteric interactions, and to identify drug binding sites. More information about the measurement of drug-protein binding constants can be found in two review articles. Information on quantifying kinetic properties of drug-protein interactions can be found in a review. A discussion on the quantification of allosteric interactions by affinity chromatography can be seen in an article. Additional information on the identification of drug-binding sites can be found in a review article. When trying to analyze low abundance proteins, it is often necessary to remove high abundance proteins prior to analysis. This removal effectively enriches low abundance proteins and allows more of them to be identified and quantified. Removal of the top 7 or top 14 high-abundance proteins has been shown to result in a 25% increase in identified proteins. Moreover, affinity chromatography is widely used in many 'omics' studies (e.g. proteomics, metabolomics and genomics) and is currently used in tandem with other methods to develop high-throughput screening methods for potential drugs.

VIII. APPLICATIONS OF AFFINITY CHROMATOGRAPHY :-

1-Immunoglobulin purification (antibody immobilization):-

Antibodies can be immobilized by both covalent and adsorption methods. Random covalent immobilization methods generally link antibodies to the solid support via their free amine groups using cyanogen bromide, N-hydroxysuccinimide, N,N'-carbonyldiimidazole, tresyl chloride, or tosyl chloride. Alternatively, free amine groups can react with aldehyde or free epoxy groups on an activated support.

As these are random immobilization methods, the antibody binding sites may be blocked due to improper orientation, multi-site attachment or steric hindrance.Site-specific covalent immobilization of antibodies can be achieved by converting the carbohydrate residues located in the Fc region of the antibody to produce aldehyde residues which can react with amine or hydrazide supports.Site-specific covalent immobilization of antibodies can be achieved by converting the carbohydrate residues located in the F_c region of the antibody to produce aldehyde residues which can react with amine or hydrazide supports. Another site-specific immobilization of antibodies can be accomplished by utilizing the free sulfhydryl groups of F_{ab} fragments. These groups can be used to couple the antibody fragments to an affinity support using a variety of established methods including epoxy, divinylsulfone, iodoacetyl, bromoacetyl, thiol, maleimide, TNB-thiol, tresyl chloride, or tosyl chloride methods

Antibodies can also be immobilized by adsorbing them onto secondary ligands. For example, if an antibody is reacted with hydrazide biotin, the hydrazide can react with oxidized carbohydrate residues on the F_c region of the antibody. The resultant biotinylated antibody can then be adsorbed onto an avidin or streptavidin affinity support. This type of biotin immobilization allows for site-specific immobilization of the antibody and can be performed using commercially available biotinylationkits.

Alternatively, antibodies can be directly adsorbed onto a protein A or protein G support due to the specific interaction of antibodies with protein A and G. Immobilized antibodies on the protein A or G support can easily be replaced by using a strong eluent, regenerating the protein A/G, and re-applying fresh antibodies. Generally, this method is used when a high capacity/high activity support is needed. If a more permanent immobilization is desired, the adsorbed antibodies may be cross-linked to the support material using carbodiimide or dimethyl pimelimidate.

2-Recombinant tagged proteins:-

Purification of proteins can be easier and simpler if the protein of interest is tagged with a known sequence commonly referred to as a tag. This tag can range from a short sequence of amino acids to entire domains or even whole proteins. Tags can act both as a marker for protein expression and to helpfacilitate protein purification. In general, the most commonly used tags are glutathione-S-transferase (GST), histidine fusion (His or polyHis tag) and protein A fusion tags other types of fusion tags are also available including maltose-binding protein, thioredoxin, NusA, GB1 domain for protein G, and others. The decision to use any of these tagging methods depends mainly on the needs of of the researcher. Table 2 compares GST and 6 tags and may help when deciding which tag is appropriate for a particular purification.

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GST tag	$(His)_6$ tag
Can be used in any expression system	
Purification procedure gives high yields of pure product	
Selection of purification products available for any scale	
Site-specific proteases enable cleavage of tag if required	Site-specific proteases enable cleavage of tag if required. N.B. enterokinase sites that enable tag cleavage without leaving behind extra amino acids are preferable
GST tag easily detected using an enzyme assay or an immunoassay	$(His)_6$ tag easily detected using an immunoassay
Simple purification. Very mild elution conditions minimize risk of damage to functionality and antigenicity of target proteins	Simple purification, but elution conditions are not as mild as for GST fusion proteins. Purification can be performed under denaturing conditions if required.Neutral pH but imidazole may cause precipitation. Desalting to remove imidazole may be necessary
GST tag can help stabilize folding of recombinant proteins	$(His)_6$ -dihydrofolate reductase tag stabilizes small peptides during expression
Fusion proteins form dimmers	Small tag is less likely to interfere with structure and function of fusion partner
Mass determination by mass spectrometry not always accurate for some $(His)_6$ fusion proteins	

Table 2. Comparison of GST and His tags for protein purification. The information from this table is summarized from the Amersham recombinant protein handbook

3-GST Tagged Purification:-

Separation and purification of GST-tagged proteins is possible since the GST tag is capable of binding its substrate, glutathione (tripeptide, Glu-Cys-Gly).When glutathione is reduced (GSH), it can be immobilized onto a solid support through its sulfhydryl group. This property can be used to crosslink glutathione with agarose beads and, thus, can be used to capture pure GST or GST-tagged proteins via the enzyme-substrate binding reaction. Binding is most efficient near neutral physiological conditions (pH 7.5) using Tris saline buffer and mild conditions to preserve the structure and enzymatic function of GST. As a result of the potential for permanent denaturation, denaturing elution conditions are not compatible with GST purification. In addition, upon denaturation or reduction, the structure of the GST fusion tag often degrades. Following a washing step to remove unbound samples, the bound GST-fusion protein can be recovered by the addition of excess reduced glutathione since the affinity of GST for free glutathione is higher than the affinity for immobilized glutathione. The free glutathione replaces the immobilized glutathione and releases the GST-tagged protein from the matrix allowing its elution from the column.

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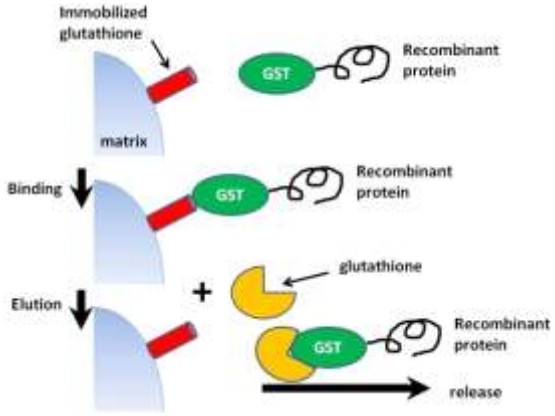

Figure 9 GST- tagged protein immobilization.

4- His-tagged protein purification:-

Recombinant proteins which have histidine tags can be purified using immobilized metal ion chromatography (IMAC). TheHis-tag can be placed on either the N-or C-terminus. Optimal binding and, therefore, purification efficiency is achieved when the His-tag is freely accessible to metal ion suppor.

Histidine tags have strong affinity for metal ions (e.g. Co^{2+}, Ni2+, Cu2+, and Zn2+). One of the first support materials used immobilized iminodiacetic acid which can bind metal ions and allow for the coordination complex with the His-tagged protein. One difficulty with iminodiacetic acid supports is the potential for metal ion leaching leading to a decreased protein yield. Modern support materials, including nickel-nitrilotriacetic acid (Ni-NTA) and cobalt-carboxymethylasparate (Co-CMA), show limited leaching and, therefore, result in more efficient protein purifications. The coordination of a His-tag with a Ni-NTA support can be seen in Figure 11. Once the tagged protein is bound by the immobilized chelating agent, it can be eluted by introducing a competing agent for the chelating group (imidazole) or an additional metal chelating agent (EDTA).

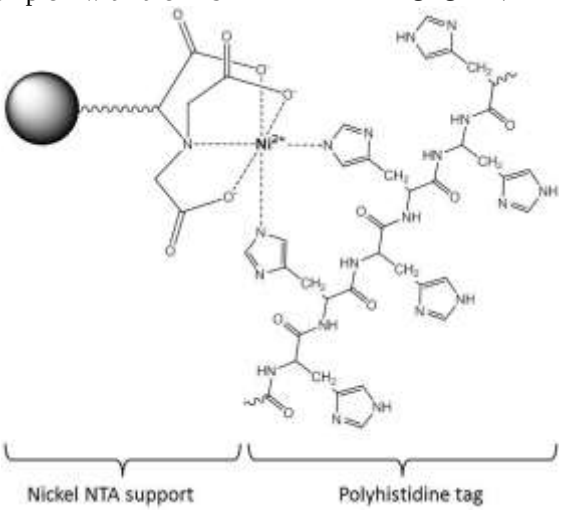

Figure 10. Showing the complex formed between the poly-histidine tag and a nickel NTA support.

One advantage of using His-tags for protein purification includes the small size of the affinity ligand. Due to the small size, it has minimal effects on the folding of the protein. In addition, if the His-tag is placed on the N-terminal end of the protein, it can easily be removed using an endoprotease. Another advantage of using His-tag purification methods is that polyhistidine tags can bind proteins under both native and denaturing conditions. The use of denaturing conditions

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becomes important when proteins are found in inclusion bodies and must be denatured so they can be solubilized.

Disadvantages of using His-tag protein purification include potential degradation of the His-tag, dimerand tetramer formation, and coelution of other histidine-containing proteins. First, when a few histidine residues are proteolytically degraded, the affinity of the tagged protein is greatly reduced leading to a decrease in the protein yield. Second, once a protein has a His-tag added to its structure, it has the potential to form dimers and tetramers in the presence of metal ions. While this is often not a large problem, it can lead to inaccurate molecular mass estimates of the tagged protein. A third disadvantage of protein purification using His-tags is coelution of proteins that naturally have two or more adjacent histidine residues.

5-Protein A, G, and L purification:-

Proteins A, G, and L are native or recombinant proteins of microbial origin which bind specifically to immunoglobulin's including immunoglobulin G (IgG). IgG represents 80% of serum immunoglobulins. The most popular matrixes or supports for affinity applications which utilize protein A, G,or L is beaded agarose. Native and recombinant protein A can be cloned in Staphylococcus aureus. Recombinant protein G (cell surface protein) is cloned in Streptococcus while recombinant protein L is cloned from Peptostreptococcus magnus. Both protein A and G specifically bind the F_c region of IgG while protein L binds to the kappa light chains of IgG.

The most popular matrixes or supports for affinity applications which utilize protein A, G, or L is beaded agarose (e.g. Sepharose CL-4B; agarose crosslinked with 2,3- dibromopropanol and desulphated by alkaline hydrolysis under reductive conditions), polyacrylamide, and magnetic beads.

All three proteins bind extensively with the IgG subclass. In general, protein A is more suitable for cat, dog, rabbit and pig IgG whereas protein G is generally more preferable when purifying mouse or human IgG. A combination of protein A and G is also applicable for purifying a wide range of mammalian IgG samples. Since protein L binds to the kappa light chain of immunoglobulins and these light chains exist in other immunoglobulins (i.e IgG, IgM, IgA, and IgE), protein L is suitable for the purification of different classes of antibodies. The binding characteristics of antibody binding proteins (A, G and L) to a variety immunoglobulin species is summarized in Table 2. IgGs from most species bind to protein A and G near physiological pH and ionic strength. To elute purified immunoglobulins from protein G sepharose, the pH should be less than2.7.

Species	Protein A	Protein G	Protein L*
Human	Strong	Strong	Strong
Mouse	Strong	Strong	Strong
Rat	Weak	Medium	Strong
Cow	Weak	Medium	Strong
Goat	Weak	Strong	No binding
Sheep	Weak	Strong	No binding
Horse	Weak	Strong	Unknown
Rabbit	Strong	Strong	Weak
Guinea pig	Strong	Weak	Unknown
Pig	Strong	Weak	Strong
Dog	Strong	Weak	Unknown
Cat	Strong	Weak	Unknown
Chicken	Unknown	Unknown	Unknown

Table 2. Binding affinity for proteins A, G, and L with a variety of immunoglobulin species.

*Binding affinity based on total IgG binding, L proteins binds to Kappa light chains while Proteins A and G bind to FC region.

6-Biotin and biotinylated molecules purification:-

If a biotin tag can be incorporated into a biomolecule, it can be used to purify the bio-molecule using a streptavidin or avidin affinity support. One way is to insert a biotinylation sequence into a recombinant protein. Biotin protein ligase can then be used to add biotin in a post-

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Volume 6, Issue 3 May - June 2021, pp: 878-893 www.ijprajournal.com ISSN: 2249-7781

translational modification step. Biotin, also known as vitamin H or vitamin B_7, is a relatively small cofactor present in cells. In affinity chromatography it is often used an affinity tag due to its very strong interactions with avidin and streptavidin. One advantage of using biotin as an affinity tag is that it has a minimal effect on the activity of a large biomolecule due to its small size (244Da).

Streptavidin is a large protein (60 kDa) that can be obtained from Streptomyces avidiniiand bind biotin with an affinity constant of 10^{13} M–1. Avidin is a slightly larger glycoprotein (66 kDa) with slightly stronger binding to biotin (1015 M–1). Both avidin and streptavidin have four subunits that can each bind one biotin molecule. To purify biotinylated biomolecules, streptavidin is immobilized onto a support material and used to extract the biotinylated molecules out of solution. Both avidin and streptavidin may be immobilized using amine- reactive coupling chemistries. In addition, avidin can also be immobilized via its carbohydrate residues.

Due to the strong interaction between biotin and (strept)avidin, harsh elution conditions are required to disrupt the binding. For example, 6 M guanidine hydrochloride at pH 1.5 is commonly used to elute the bound biotinylated biomolecule. This prevents the recovery of most proteins in their active form. To overcome this difficulty, modified (strept)avidin or modified biotin may be used to create a lower affinity interaction. In one study chemically modified avidin had relatively strong binding ($>10^9$ M–1), but was also able to completely release biotinylated molecules at pH 10. In addition, at any pH between 4 and 10, a 0.6 mM biotin solution could be used to displace and elute the biotinylated molecules.

Biotin is also used in isotopically coded affinity tags (ICATs) which can be used to compare the protein content in two different sample. The ICAT consists of two labels, one which contains deuterium (heavy) and one which contains only hydrogen (light). The two labels (light and heavy) are added separately to the cell lysates being compared. Since the reagent contains a thiol-specific reactive group, it will covalently bind free cysteines on proteins. The labelled lysates are combined, digested with trypsin, and then isolated on a streptavidin column. After a second separation step, the labelled proteins are analyzed using mass spectrometry. The change in protein expression between the two cell lysates can then be quantified and related to the different conditions applied to the two sets of cell lysates.

7-Affinity purification of albumin and macroglobulin contamination:-

Affinity purification is a helpful tool for cleaning up and removing excess albumin and α2-macroglobulin contamination from samples since these components can mask or interfere with subsequent steps of analysis(e.g. mass spectrometry and immunoprecipitation). One purification method which can be used to remove these contaminants either before or after other purification steps is Blue sepharose affinity chromatography. In this method, the dye ligand is covalently coupled to sepharose via a chlorotriazine ring. Albumin binds in a non- specific manner by electrostatic and/or hydrophobic interactions with the aromatic anionic ligand. The most commonly used dye is Cibacron blue F-3-GA which can be immobilized onto sepharose to create an affinity column. This dye is capable of removing over 90% of albumin in the sample . [25,26]

8-Enzyme isolation:-

There are a number of areas related to affinity chromatography that have also been of great interest in pharmaceutical and biomedical analysis. One such area is in the use of affinitycolumns for enzyme isolation. This approach may use affinity ligands that are substrates, inhibitors, cofactors, or proteins that are associated with the biochemical pathways of the target enzyme.[27,28].

9-Lectin affinity chromatography:-

Lectin affinity chromatography is one of the most powerful techniques for studying glycosylation as a protein post translational modification. Lectins are carbohydrate binding proteins that contain two or more carbohydrate binding sites and can be classified into five groups according to their specificity to the monosaccharide. They exhibit the highest affinity for: mannose, galactose/N- acetylgalactosamine, N-acetylglucosamine, fucose, and N-acetylneuraminic acid. In this affinity technique, protein is bound to an immobilized lectin through its sugar moeities (N-linked or O-linked). Once the glycosylated protein is bound to the affinity support, the unbound contaminants are washed away, and the purifed protein eluted.

Currently, many lectins are commercially available in an immobilized form. Among them,

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 3 May - June 2021, pp: 878-893 www.ijprajournal.com ISSN: 2249-7781

Concanavaline A (Con A) Sepharose and wheat germ agglutinin (WGA) are the most popular for glycoprotein purification. As shown in Table 3, several different types of lectin may be used in affinity chromatography.

Acronym, Organism and source	Metal ions required	Sugar specificity	Elution conditions	Useful for binding
Con A (Canavalia ensiformis; jack bean seeds)	Ca^{2+}, Mn^{2+}	α-Man > α-Glc	0.1–0.5 M α-MeMan	High-Man, hybrid, and biantennary N-linked chains
LCA or LCH (Lens culinarus; lentil seeds)	Ca^{2+}, Mn^{2+}	α-Man > α-Glc	0.1–0.5 M α-MeMan	Bi- and triantennary N-linked chains with Fuc α1-6 in core region
PSA (Pisum sativum;peas)	Ca^{2+}, Mn^{2+}	α-Man	0.1–0.5 M α-MeMan	Similar to LCA/LCH
WGA (Triticum				GlcNAc- and Sia-terminated
vulgaris; wheat	Ca^{2+}, Mn^{2+}	ß-GlcNAc	0.1–0.5 M	chains, or clusters of O-GlcNAc;
germ)			GlcNAc	succinylated form selectively
				binds GlcNAc>Sia
HPA (Helix				Proteins with terminal α-
promatia; albumin	-	α-GalNAc	0.1–0.5 M	GalNAc or GalNAcα-O-Ser/Thr
gland of edible			GalNAc	(Tn antigen)
snail)				
UEA-I (Ulex			0.1–0.5 M L-	Sugar chains with terminal α-
europaeus; furze	-	α-L-Fuc	Fuc or	Fuc, especially in α1-2 linkage,
gorse seeds)			methyl-α-L-	but much less with α1-3 or α1-6
			Fuc	linkages
LBA (Phaseolus lunatus; lima bean)	Mn^{2+}, Ca^{2+}	Terminal α-GalNAc	0.1–0.5 M GalNAc	Proteins with blood group A structure GalNAcα1-3(Fucα1-2)Gal–

Table 3. Some examples of lectins used for glycoprotein purification modified from current protocols in protein science.

Lectin affinity columns can be prepared by immobilizing lectins with different specificities toward oligosaccharides to avariety of matrices, including agarose, silica, monolithic stationary phases and cellulose. These immobilized lectins are invaluable tools for isolating and separating glycoproteins, glycolipids, polysaccharides, subcellular particles and cells. In addition, lectin affinity columns can be used to purify detergent-solubilized cell membrane components. They also are useful for assessing changes in levels or composition of surface glycoproteins during cell development and in malignant or virally transformed variants. In subsequent chapters, more detailed examples of lectin affinity purification can befound.

10-Protein and Peptide Separations:-

Affinity chromatography has a wide range of applications for protein purification, such as immunoaffinity, purification of immunoglobulins, purification of glycoproteins, DNA-binding proteins, receptor proteins, enzymes, cell isolation, and nucleotide isolation.

International Journal of Pharmaceutical Research and Applications
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Affinity chromatographyis a specific purification technology based on biological function or individual chemical structure. The application of this technique is in separation of active biomolecules from denaturated or functionally different forms in the isolation of pure proteins present at low concentration and also for removing specific contaminants.(29).

11-Reversed phasechromatography:-

Reversed phase chromatography is a kind of affinity interaction between a biomolecule dissolved in a solvent (mobile phase) that has some hydrophobicity (e.g. proteins, peptides,and nucleic acids) and an immobilized hydrophobic ligand (stationary phase). Reversed phase chromatography is generally more suitable for separating non-volatile molecules. The term "reversed phase" was adopted because the binding occurs between a hydrophobic ligand (octadecyl; C18) and molecules in a polar aqueous phase which is reversed from normal phase chromatography [where a hydrophilic polar ligand binds to molecules in a hydrophobic nonpolar mobile phase].

In general, the macromolecules (e.g. protein or peptides) are adsorbed onto the hydrophobic surface of the column. Elution is achieved using a mobile phase which is usually a combination of water and organic solvents (such as acetonitrile or methanol) applied to the column as a gradient (e.g. starting with 95:5 aqueous:organic and gradually increasing the organic phase until the elution buffer is 5:95 aqueuos:organic). The macromolecules bind the hydrophobic surface of the column and remain until the concentration of the organic phase is high enough to elute the macromolecules from the hydrophobic surface.

When using reversed phase chromatography, the most polar macromolecules are eluted first and the most nonpolar macromolecules are eluted last: the more polar (hydrophilic) a solute is, the faster the elution and vice versa. In summary, separations in reversed phase chromatography depend on the reversible adsorption/desorption of solute molecules with varying degrees of hydro-phobicity to a hydrophobic stationary phase.

As illustrated in Figure 12, the initial step of reversed phase separation involves equilibration of the column under suitable conditions (pH, ionic strength and polarity). The polarity of the solvent can be modified by adding a solvent such as methanol or acetonitrile and an ion pairing agent such as formic acid or trifluoroacetic acid may be added. Next, sample is applied and bound to the immobilized matrix. Following this step, desorption and elution of the biomolecules is achieved by decreasing the polarity of the mobile phase (by increasing the percentage of organic modifier in the mobile phase). At the end of the separation, the mobile phase should be nearly 100% organic to ensure complete removal of all bound substances. Once everything has eluted from the column, the initial mobile phase is reapplied to the column to re-equilibrate the column for a subsequent sample application.

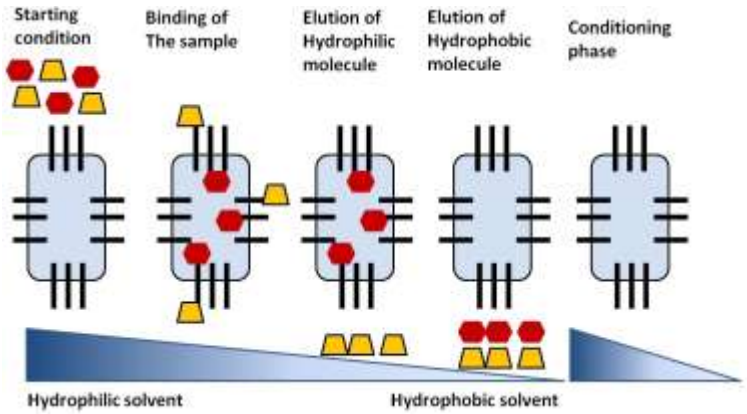

Figure.12. Steps of a of reversed phase chromatography separation.

12-Investigating protein-protein interactions:-

Affinity chromatography provides one important method for identifying and characterizingtheintermolecularinteractions.Wheni nvestigatingprotein-protein interactions, affinity chromatography typically involves linking one protein to an insoluble matrix and then incubating that matrix with a solution containing possible binding partners. This solution could be as simple as homogenous solution containing a single

recombinant protein or complex, After incubating the immobilized substrate protein with its potential binding partner(s) and washing away material associated non specifically the binding partner are the eluted and detected by any variety of methods from chromatographic detection.(29).

13-Funds

This work was supported by JSPS (Japan Society for Promotion of Science) Grant-in-Aid for scientific research (B) to Dr. SamehMagdeldin (23790933) from Ministry of Education, Culture, Sports, Science and Technology of Japan. The funders had no role in the decision to publish or in the preparation of this chapter.(29)

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