Small Molecules in Affinity Chromatography of Proteins

History

In 1910, α-amylase was the first protein purified via reversible binding to a column of its natural substrate, insoluble starch (Starkenstein, 1910). The column-bound α-amylase was then eluted with a solution containing chloride salts; today, we know this procedure as affinity chromatography.

Affinity chromatography is based on the specific interaction of binding pairs (affinity pairs), which often, but not always, includes a target protein and its specific ligand. The first generation of binding pairs were mainly enzyme/substrate pairs, such as porcine elastase/elastin or pepsin/edestin (Matos, 2020). The term 'affinity chromatography' itself, was coined in a report detailing the purification of a staphylococcal nuclease, α-chymotrypsin and carboxypeptidase A on matrices, containing their respective, covalently-linked, competitive inhibitors (Cuatrecasas, 1968).

Affinity chromatography options for target proteins other than enzymes followed shortly thereafter. An early example of lectin-carbohydrate-based affinity chromatography was the use of a carbohydrate dextran as a ligand in the isolation of the lectin concanavalin A (ConA) (Aggarwal, 1965). More recently, lectin-carbohydrate-based affinity chromatography has also been used in the isolation of a coronavirus spike glycoprotein (McPherson, 2016; see figure 4).

Boronic acids are another early example of affinity purification that does not involve the binding of the target protein to another protein (part) itself. Boronic acids are reactive towards cis-diols, and thus vicinal cis-diols in carbohydrates. This is another classic discovery (Böeseken, 1949) that is exploited today, particularly in the purification of glycoproteins (Liu, 2006). Another milestone was reached when Protein A, coupled to Sepharose-4B for the purification of immunoglobulin G (IgG) antibodies, became commercially available (Hjelm, 1975). The underlying principle - the high reactivity of *Staphylococcus aureus* Protein A (SpA) towards antibodies in rabbit serum which causes precipitation, was already discovered 3 decades earlier (Verwey, 1940). Today, antibody purification has developed into a billion-dollar industry.

The focus of this review is on the role of small molecules in the affinity purification of proteins, in particular in industrial applications.

The Basic Principle in the Affinity Chromatography of Proteins

Definition

Affinity chromatography is a liquid chromatography method in which a biological agent or biomimetic ligand is used for the selective retention of complimentary compounds (Hage, 2005).

Affinity chromatography takes advantage of the selective interaction between a target protein and its ligand, and typically consists of three individual steps (figure 1, below):

- ### Loading

The crude protein extract containing the target protein is applied to a column containing the respective ligand immobilised on a matrix of choice.

- ### Washing

A wash buffer is applied to the loaded column to flush out any proteins that bind non-specifically to the matrix. The target protein remains bound to the matrix-immobilised ligand.

- ### Elution

The reversible binding between the target protein and the ligand is interrupted by application of an elution buffer, which contains an element that disrupts the interaction of the affinity pair. Consequently, the target protein is released from its resin-immobilised ligand. Different types of competing compounds can be used, depending on the type of affinity chromatography (see table 1), here are some common approaches:

    - Applying a high concentration of a small molecule in an elution buffer that mimics or equals the ligand. The small molecule competes with the ligand for binding sites in the target protein and subsequently, the target protein is released from the column.

    - For some affinity chromatography types, simple changes in physical conditions that separate the affinity pair are also used, such as, addition of high salt concentration or changes in pH of the elution buffer.

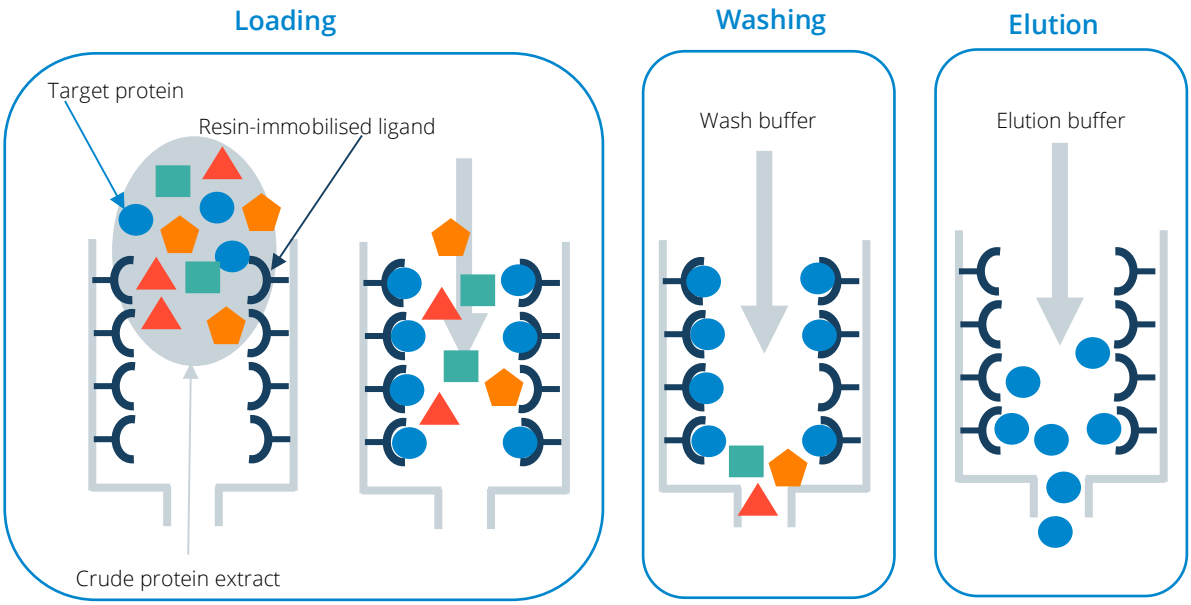

Figure 1. The three steps of protein affinity chromatography.

Depending on the elution method and need for downstream work, a final step is required to separate the target protein from the high concentration of salts or small molecules present in the elution buffer. This can include using desalting columns or dialysis (not shown).

Common Affinity Pairs and Elution Methods in Protein Purification

The various methods of protein affinity purification can be classified into two groups, depending on the type of ligand used:

Group 1:

The immobilised ligand is a protein. For instance, a glycoprotein target purified on a lectin column or via protein A chromatography.

Group 2:

The immobilised ligand comes from a non-protein class of molecules. In this case, the ligand can be of low or high molecular weight. For instance, cellulose for CBD-tagged proteins, immobilised mannose for concanavalin A, or a Ni^{2+}-complex for His-tagged proteins.

Method	Affinity pair	Stationary phase	Elution method	Reference
Proteins as immobilised ligands (Group 1)				
Protein A chromatography	- Protein A - Immunoglobulin G (IgG)	Protein A immobilised on a natural or synthetic support (polysaccharides, synthetic polymers, controlled-pore glass)	Decrease pH from neutral to acidic	Ramos, 2019
Lectin affinity chromatography (LAC)	- Lectin - Glycan (free or as glycoprotein)	Lectin immobilised on a polysaccharide	Solution of lectin-binding sugar	Freeze, 2001
Purification of tag-free protein	- Subtilisin prodomain - Subtilisin protease	Subtilisin protease immobilised on agarose	Fluoride-containing solution	BioRad Profinity eXact™
Streptavidin-biotin chromatography	- Streptavidin/avidin - Biotin-tagged protein	Avidin or streptavidin resin (monomeric with $K_d = 10^{-7}$)	Biotin solution	Promega
Calmodulin-binding peptide (CBP)	- Calmodulin-binding peptide (CBP) - Calmodulin	Calmodulin affinity resin	Removal of Ca^{2+} required for CBP-calmodulin bond	Stratagene Simcox, 1995
Non-protein molecules as immobilised ligands (Group 2)				
Metal affinity chromatography (mainly His-Tag)	- Sequence of 2-10 terminal His residues - Immobilised transition metal	Immobilised transition metal such as Ni^{2+} (most common), Co^{2+}, Cu^{2+}, Zn^{2+}, Ca^{2+}, Fe^{3+}	Imidazole solution	Kimple, 2013 Lilius, 1991
Glutathione S-transferase (GST) based affinity chromatography	- GST - glutathione or reduced glutathione	Glutathione - sepharose	Glutathione solution	Smith, 2000
Maltose-binding protein (MBP) based affinity chromatography	- MBP - Cross-linked amylose resin	Amylose resin	Maltose solution	Maina, 1988 diGuan, 1988
Intein-chitin binding domain (CBD) based affinity chromatography	- Intein/CBD fusion protein - Chitin	Chitin matrix	Dithiothreitol solution (induction of self-cleavage)	Chong, 1997
Boronic acid-based affinity chromatography	- Boronate - Cis-diols in Glycoproteins	Boronate matrix	Acidic buffer or competing cis-diol (mannitol, sorbitol)	Mallia, 1981
Cellulose-binding domain (CBD)-based affinity chromatography	- Cellulose-binding domain - Cellulose	Cellulose	Cellobiose solution	Levy, 2002

Table 1. Affinity purification methods involving proteins as immobilised ligands. *For the sake of brevity, the references chosen are limited to one.

A number of tags that are otherwise used as reporter groups for protein expression and protein-protein binding have also been used for affinity purification. For instance, chloramphenicol acetyl transferase-tagged fusion proteins (CAT-tagged) can be purified on immobilised chloramphenicol (e.g. chloramphenicol caproate agarose) and eluted with chloramphenicol solution. Another example from the same category would be the β-galactosidase tag/APTG pair (APTG: p-aminophenyl-1-thio-β-D-galactoside) (Kimple, 2013).

Examples for Affinity Purifications in Industrial and Medical Applications

Protein A

Protein A from *Staphylococcus aureus* (SpA) is a bacterial surface protein that contains several Ig-binding domains followed by other variable and conserved regions. The ability of Protein A to bind to the Fab and Fc regions of antibodies have led its widespread use in protein immunoprecipitation of antibodies or Fc-tagged proteins, in biosensors and for the immobilisation of antibodies for affinity chromatography (Rigi, 2019).
With the increasing importance of therapeutic antibodies, the efficient production of Protein A as the matrix of choice for antibody purification has increasingly come into focus too. At the same time, the interaction of Protein A with the immune system, through B-cell and monocyte binding, has opened therapeutic options for autoimmune diseases, such as, rheumatoid arthritis and idiopathic thrombocytopenic purpura (Navegantes, 2017).

Production and purification of Protein A via bivalent cation affinity chromatography (His-Tag)

S. aureus is a pathogen and therefore large-scale production of Protein A in homologous bacterial cultures is avoided. Consequently, Protein A production has been recommended for heterologous bacterial hosts and yeasts. In fact, the commercially available Protein A is expressed in *E. coli*.

For affinity chromatography purposes, Protein A needs to be first immobilised on a matrix (essential steps shown in figure 2, on the following page) (Kangwa, 2015). Recombinant Protein A, containing an N-terminal His-tag and C-terminal Cys-His sets, is expressed in *E. coli.* Following cell lysis, the recombinant Protein A is purified from the crude extract on a Ni^{2+}-NTA (NTA: nitrilotriacetic acid) column. Then, the purified Protein A can be covalently attached to a matrix via the Cys-residues, for instance, to epoxy-activated sepharose to give a Protein A-sepharose matrix. The latter can be used for the isolation of immunoglobulin G from complex mixtures.

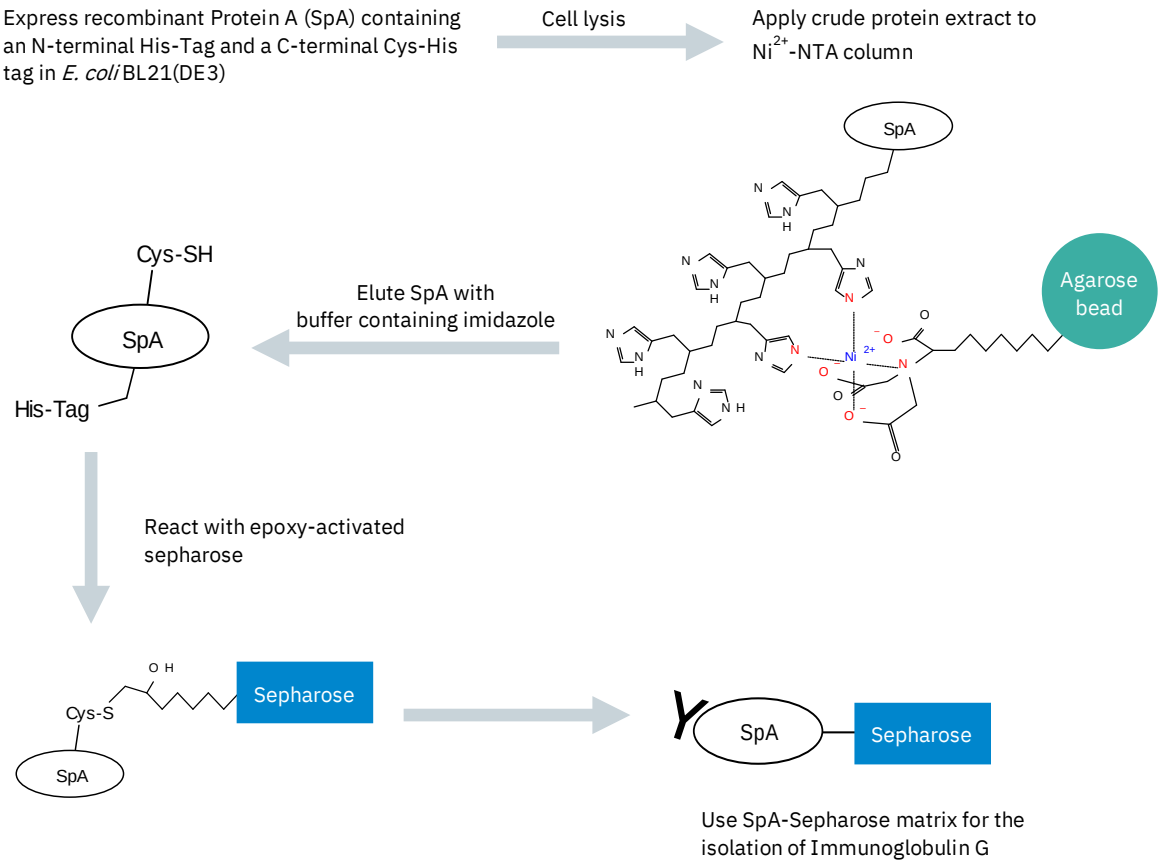

Figure 2. A) Generation of a Protein A-Sepharose affinity matrix for the purification of immunoglobulins, involving metal affinity chromatography.

B) Examples for chelator molecules used: BOC-AC-NTA and AB-NTA. Both molecules are able to chelate Ni^{2+} cations through provision of 4 of 6 ligands in a resulting octahedral complex, leaving the remaining positions for replacement of water through His-Tag binding.

Applications Involving Cellulose-Binding Domains (CBD) (Levy, 2002)

Cellulose-binding domains have been described as the part of enzymes with hydrolytic activity, such as, in cellulases and xylanases. Cellulose-binding domains help increase the concentration of the enzyme that would otherwise reside in the soluble fraction, on insoluble substrates, such as, cellulose or xylan. As these insoluble substrates are inexpensive, readily available affinity matrices for CBDs, the latter have become a popular affinity tag in a variety of separation, processing and production processes (Figure 3, below). A SciFinder™ reference search for the term, in 03/21, resulted in over 300 patent references involving CBDs.

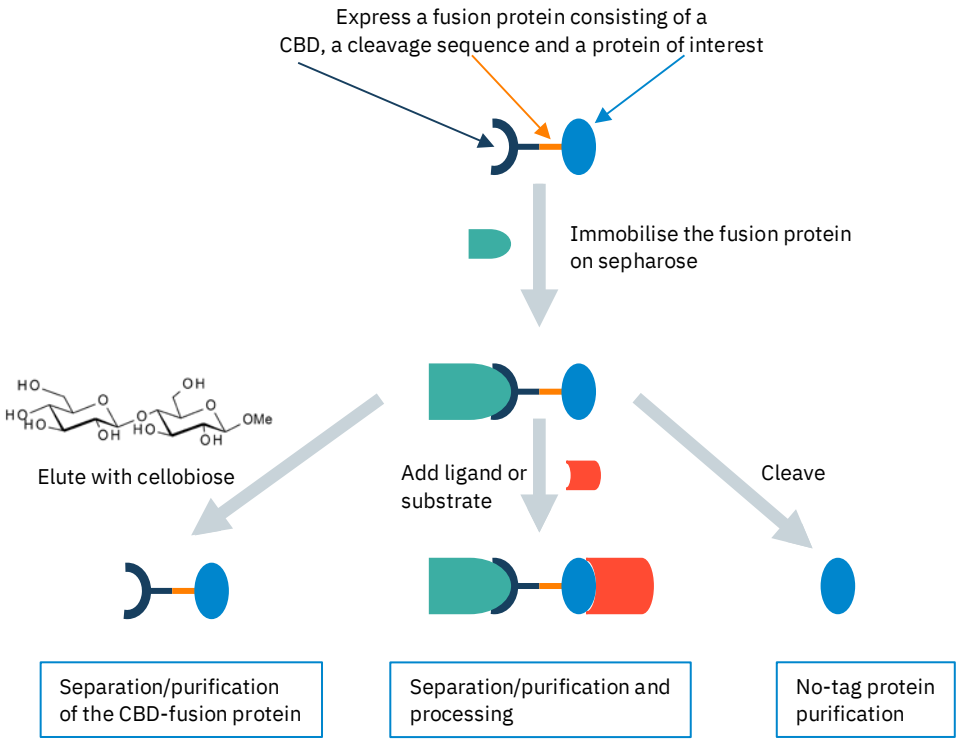

Figure 3. Schematic examples for applications of CBDs in manufacturing processes involving proteins. For the elution of the CBD-fusion protein, cellobiose derivative Methyl-β-D-cellobioside is frequently used.

Some examples of CBD-fused enzymes that retain their activity are include: α-amylase, lipase B or glucoamylase, and thus can be purified on cellulose as a high-capacity matrix, at a relatively low cost. An example of an industrial process of high relevance involving CBDs would be denim stonewashing; localised decolourisation creates a 'worn' effect and is achieved by targeting cellulases on the garment with the aid of CBDs. This replaced the original method based on abrasive stones.

Laundry powders often contain recombinant enzymes with little natural affinity to cellulose fabrics; fusion to CBDs can improve their performance. In addition to the affinity purification options they offer, CBDs can sometimes have a beneficial effect on the expression levels of recombinant proteins. One can find the CBD coding sequence in commercially available expression vectors, such as pET series and use them in the expression and purification process.

Lectin Affinity Chromatography (LAC)

Affinity chromatography based on a lectin matrix, is a particularly powerful method when it comes to the purification of glycoproteins (or indeed glycans), the resolution of mixtures of glycoproteins or even different glycoforms of one given glycoprotein.

Lectins are carbohydrate-binding proteins that bind their respective sugar ligand with high specificity. They are ubiquitous in nature where they play a variety of roles in molecular/cellular recognition events. Being particularly abundant in some plants, especially legumes, from which they can be extracted in high quantities, lectins have become useful candidates for the generation of affinity matrices, for glycoprotein purification (Sharon, 2004; Streicher, 2002).

A typical sequence for the purification of a glycoprotein would look as follows:

1. Procurement or synthesis of an **affinity matrix containing a lectin** that selectively binds the terminal (mono-)saccharide in the target glycoprotein.

2. Apply **crude protein extract** in loading buffer to the column containing the affinity matrix.

3. **Wash** with buffer to remove all unbound components.

4. **Elute target protein** with an elution buffer containing the competing (mono-) saccharide (it will compete for and block the lectin binding sites).

5. **Dialyse** the eluted target glycoprotein/(mono-) saccharide solution against a buffer of choice to remove the latter.

An example of a target protein that can be purified this way would be the ectodomain (extracellular domain) of the notorious coronavirus, SARS-CoV-2 spike protein, which is the target of most coronavirus vaccines. The spike protein is heavily glycosylated with, among others, mannose-terminated oligosaccharides (Grant, 2020). Figure 4 shows the purification process of a recombinant SARS spike protein via LAC (McPherson, 2016):

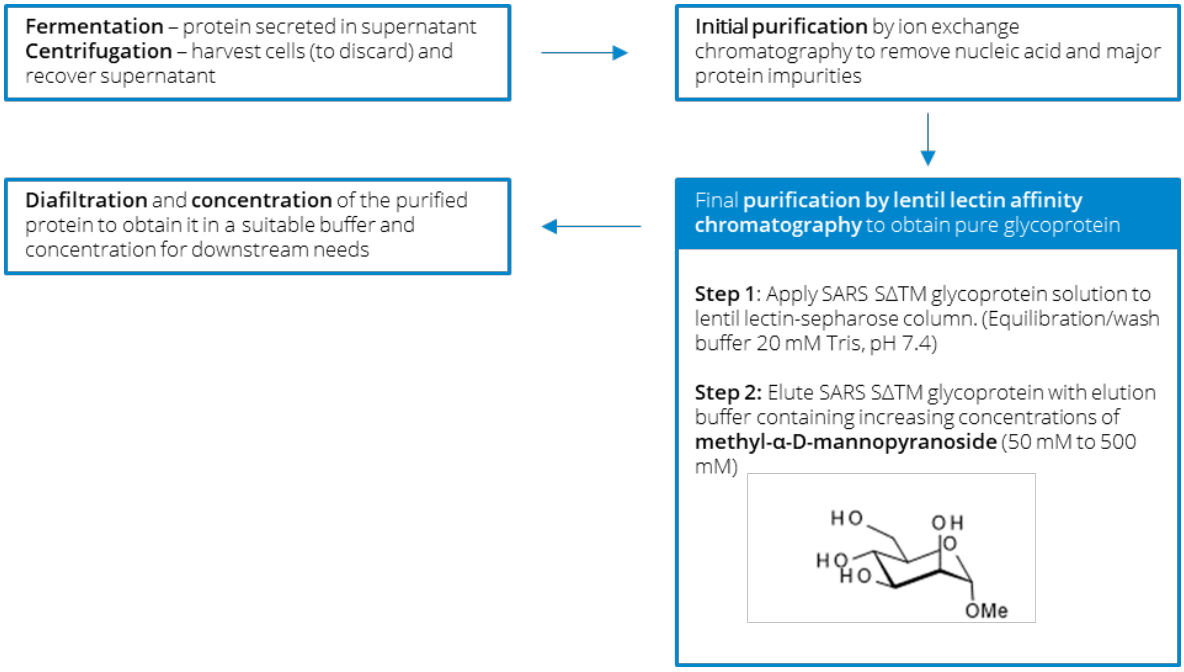

Figure 4. Purification of a SARS S protein via lentil lectin affinity chromatography, which is eluted with methyl-α-D-mannopyranoside.

Common Affinity Pairs in Lectin Affinity Chromatography

The table below lists some of the commercially available immobilised lectins and complementary saccharides used for elution.

Immobilised lectin	Lectin saccharide specificity	Saccharide for elution
Lens culinaris (lentil) lectin	Glucose/mannose	Methyl-α-D-mannopyranoside
Concanavalin A (ConA)	Mannose	
Galanthus nivalis lectin (GNA)	Manα3Man	
Wheat germ agglutinin (WGA)	N-acetylglucosamine including oligomers (chitin)	N-acetylglucosamine
Vicia villosa lectin	Terminal GalNAc residues	N-acetylgalactosamine
Peanut agglutinin (PNA)	T-antigen (Galα3GalNAc)	Galactose
Jacalin	T-antigen and sialylated T-antigen	
Ricin	Gal- and Lac-termini of oligosaccharides	Galactose, lactose
Sambucus nigra lectin (SNA)	Neu5Ac-α-2,3- and α-2,6-Gal	Lactose
Aleuria aurantia lectin (AAL)	Fucose-termini of oligosaccharides	L-fucose
Ulex europaeus lectin (UEA I)	α-linked fucose	

Table 2. Some commercially available immobilised lectins for lectin affinity chromatography.

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