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Protein Purification by Affinity Chromatography

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1. Introduction

Affinity chromatography is a method which depends essentially on the interaction between the molecule to be purified and a solid phase that will allow the separation of contaminants. Lectins are carbohydrate-binding proteins which can be purified by affinity chromatography; also, the presence of multiple molecular forms of lectins in a preparation can be separated. Immobilized lectins have been useful to affinity protein purification. In immunoaffinity chromatography an antibody or an antigen is immobilized on a support so as to purify the protein against which the antibody was developed. Monoclonal antibodies are extremely useful as immunosorbents for purification of antigen. Immobilization of monoclonal antibody on a suitable material to the column produces a support that will bind with high selectivity to protein against which the antibody was developed. Affinity chromatography containing DNA is a highly specific and important technique for the purification of DNA-binding proteins involved in the transcription, replication and recombination. The success of affinity chromatography depends on the conditions used in each chromatographic step. So, the optimization of protocol is essential to achieve optimal protein purification with maximum recovery.

2. Nomenclature and basic concepts

The term "affinity chromatography", first used by Cuatrecasas et al. (1968), refers to a purification technique which depends essentially on the highly specific interaction between the molecule to be purified and the solid phase that will allow the separation of contaminants. This method has several other terms such as "bioselective adsorption", which was appropriately used to denominate an adsorption chromatography that uses a very special kind of affinity between the desired biological product and a biomolecule (Porath, 1973). For example, the biological affinity between an enzyme (protein with catalytic activity) and its substrate and/or other small ligand – usually in the active or allosteric site of the enzyme – results from a selective interaction.

The adsorption corresponds to the fixation of the molecules of a substance (the adsorbate) on the surface of another substance (the adsorbent), which may be immobilized to an insoluble support. The adsorbate and the adsorbent can be referred as bioligands. The bioligands may be specific or may not have absolute specificity of interaction. Many bioligands (e.g. NAD^+, ATP, coenzyme A) may bind different enzymes, being then called group-specific ligands. In the same manner, chitin (a polysaccharide composed by *N*-acetyl-D-glucosamine units) may be an adsorbent for several different molecules if they possess a group (e.g. a binding or catalytic site) able to interact with chitin.

Affinity chromatography is a powerful tool for the purification of substances in a complex biological mixture. It can also serve to separate denatured and native forms of the same substance. Thus, biomolecules which are difficult to purify have been obtained using bioselective adsorbents, e.g. immobilized metal ions (Ni^{2+} and Zn^{2+}) used to purify proteins containing zinc finger domains with natural affinity to divalent ions (Voráčková et al., 2011). The relative specificity degree of the affinity chromatography is due to the exploitation of biochemical properties inherent in certain molecules, instead of using small differences in physicochemical properties (such as size, form and ionic charge, which are employed by other chromatographic methods).

Affinity chromatography may be used with different final objectives. If the aim is a rapid purification of a macromolecule with high yield, many controls and careful attention are necessary to establish the best conditions for a high bioselectivity of the system; the researcher must be prepared to adjust the chromatographic conditions and to circumvent possible absence of bioselectivity or low yields. If the objective is to first demonstrate a bioselectivity for further purification, the choice of the bioselective adsorbent is dependent on the physiological interaction between the bioselective component and the macromolecule to be purified. In this case, the researcher must spend a lot of time establishing the bioselectivity before starting the isolation experiments.

A good bioselectivity means that the affinity of the molecule by the ligand exceeds all factors of non-specific adsorption that are present in the system. Also, the affinity should not be so strong, since the biomolecule must be removed from the column. A well-designed affinity method should consider the selection of the ligand molecule or the insoluble support to be used; they must have specific and reversible binding affinity for the molecule being purified.

After defining the protocol, purification by affinity chromatography is a rapid method, compared with others less specific. The technique also enables the concentration of the molecule of interest resulting in a small volume of a concentrated product.

Standard procedures of protein purification result in obtainment of homogeneous protein. However, a considerable cost of supplies and hours of work is often required and a low yield is obtained after several steps. The power of affinity chromatography is often larger than other chromatographic techniques, resulting in several hundred or thousand-fold purification factors in a single step.

3. Supports for affinity chromatography

A good support for affinity chromatography should be chemically inert or have minimal interaction with other molecules, having high porosity and large number of functional

groups capable of forming covalent bonds with the molecule to be immobilized. Many materials are available (Table 1). A variety of supports with immobilized ligands, or stable media for the immobilization of ligands through different functional groups are commercially available. The ligand molecule to be used should contain a group capable of being chemically modified, often an amino group, which will allow connection with the matrix without destroying its capacity to bind to the molecule of interest.

Supports	References
Affi-gel blue gel	Wong et al., 2006
α-casein-Agarose	Kocabiyik & Ozdemir, 2006
Chitin	Sá et al., 2008; Coelho et al., 2009; Santana et al., 2009; Napoleão et al., 2011a
Fetuin-fractogel	Guzmán-Partida et al., 2004
Fetuin-Sepharose CL-4B	Bhowal et al., 2005
Ferromagnetic levan composite	Angeli et al., 2009
GalNac-Sepharose CL-4B	Gade et al., 1981
Galactosyl-Sepharose	Franco- Fraguas et al., 2003
Glutathione reduced (GSH)-Sepharose	Hamed et al., 2011
Guar gel	Coelho & Silva, 2000; Santos et al., 2009; Nunes et al., 2011; Souza et al., 2011
IMAC (immobilized metal ion affinity chromatography)-Sepharose	Voráčková et al., 2011
Sephadex G25	Santana et al., 2009
Sephadex G50	Fenton-Navarro et al., 2003
Sephadex G75	Correia & Coelho, 1995
Sepharose-manose gel	Latha et al., 2006
Lectin-Sepharose CL-4B	Paiva et al., 2003; Silva et al., 2011
Trypsin-Agarose	Leite et al., 2011

Table 1. Supports for affinity chromatography.

One example is the agarose, a polysaccharide obtained from agar, which provides numerous free hydroxyl groups and is the most widely used (Chung et al., 2009). The ligand may be covalently bound to it through a two step process. In the first step, the agarose reacts with cyanogen bromide to form an "activated" intermediate which is stable and commercially available. In the second step, the molecule to be immobilized reacts with agarose to form the covalently bound product (Voet & Voet, 1995). A support containing trypsin immobilized on agarose was used to purify trypsin inhibitor from liver of *Oreochromis niloticus* (Leite et al., 2011). Chromatography on α-casein-Agarose was useful for purification of an intracellular chymotrypsin-like serine protease from *Thermoplasma volcanium* (Kocabiyik & Ozdemir, 2006).

Sepharose (a tradename of a registered product of GE Healthcare) is a beaded form of agarose cross-linked through lysine side chains. It is a common support for

chromatographic separations of biomolecules and can also be activated with cyanogen bromide. For example, glutathione S-transferases from Down syndrome and normal children erythrocytes were purified by chromatography on matrix containing glutathione reduced (GSH) immobilized on Sepharose (Hamed et al., 2011).

Insoluble polysaccharide matrices – such as chitin, guar gel and Sephadex – have been used to purify lectins (carbohydrate-binding proteins) and will be discussed later.

4. Extraction and purification of proteins by affinity chromatography

To obtain a pure protein is essential for structural characterization and exploration of its function in nature. These proteins should be free of contaminants if they will be used for biotechnological purposes, such as the evaluation of their potentiality to purify and characterize other molecules, as well as for studies on the ability to recognize receptors and induce different cellular responses.

Proteins are dependent of environmental conditions to maintain their stability and for this reason some parameters are crucial in all steps of the purification protocol: pH, ionic strength, temperature and dielectric constant. The balance of these parameters, characteristic for each protein, is essential for obtainment of the pure molecule in its native form. The protein activity is due to the maintenance of protein structure that may be stabilized by strong bonds, like disulfide bridges, and weak bonds, like hydrophobic interactions and hydrogen, electrostatic or saline bonds.

In the purification processes of a protein, the following parameters should be considered: the selection of the procedure for protein extraction from the biological source, the assays for monitoring protein concentration in each step, the methods of solubilization, and the environmental conditions for stabilization. The prior separation is based on differences in solubility and usually corresponds to the preparation of a homogenate or extract. After extraction and centrifugation, the separation can be based on molecular mass, electric charge and protein affinity for other molecules.

Many proteins have the ability to bind strongly (but not covalently) to specific molecules and thus can be purified by affinity chromatography. Figure 1 shows the steps of an affinity chromatography for isolation of a protein. Initially, the affinity support must be equilibrated with a binding buffer to achieve adequate conditions for affinity interaction between the protein and the immobilized molecule (step 1). When an impure solution (crude extract or a partially purified preparation) is passed through the affinity support, the protein of interest interacts with the ligand (adsorption) and the other contaminants (other proteins or molecules) are washed from the column with the binding buffer (step 2). The desired molecule can be obtained highly purified by changing the elution conditions to release the protein from the support (step 3). For example, the elution may be performed changing the conditions of pH, ionic strength or temperature (non-bioselective desorption), or with a solution containing a high concentration of free ligand that will compete for the binding-sites of the protein (a bioselective desorption).

A crude extract can be directly applied in an affinity chromatography column. The application of crude extract has the advantage of avoiding other steps that lengthen the process. However, substances that may interfere in this process, like other proteins, nucleic

acids and lipids are present in higher concentrations in crude extracts. In general, before the chromatography, one or more steps for partial separation of undesirable constituents are incorporated into the purification protocol.

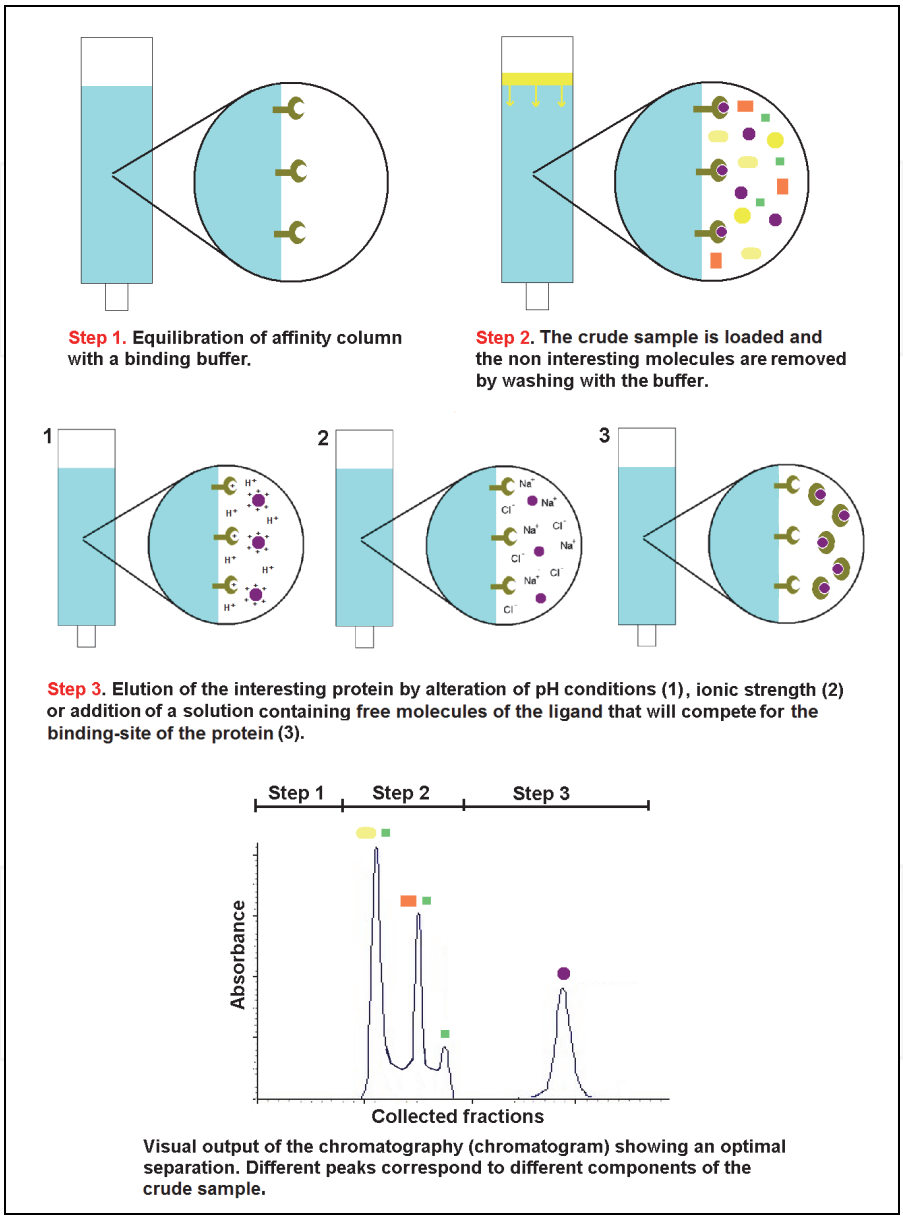

Fig. 1. Schematic representation of the equilibration (1), adsorption/washing (2) and desorption (3) steps of an affinity chromatography for protein purification.

Among the parameters used to evaluate if a preparation is pure can be cited electrophoresis, immunological and chromatographic methods. The homogeneity of a protein preparation should not be judged by isolated parameters. The indication of protein purity is obtained by analysis of various speculations.

Affinity chromatography is a useful tool in proteomics studies; this method plays an essential role in the isolation of protein complexes and in the identification of protein–protein interaction networks. In glycoproteomics, serial lectin affinity chromatography was applied in the process for identification of over thirty proteins from the human blood with *O*-glycosylation sites (Durham & Regnier, 2006). Affinity chromatography is also required for quantification of protein expression by using isotope-coded affinity tags (Azarkan et al., 2007).

5. Forces that stabilize proteins and affinity interactions

The protein structures are maintained by hydrophobic effects and interactions between polar residues and other types of connections (Voet et al., 2008). For enzymes, the active sites are constituted by amino acid residues in direct contact with the substrate and those amino acid residues indirectly involved in substrate binding through a water molecule as intermediate or by the side chain of an amino acid. Many of the mentioned residues may be in contact with a single substrate; the connection can occur through various combinations of hydrophobic interactions, ionic bonds, hydrogen bonds and charge transfer. The enzyme specificity for a particular substrate depends mainly on the steric positioning of each amino acid in the active site. Substrates or inhibitors can be accommodated in the active site; some are adjusted better than others.

The ideal conditions for affinity chromatography correspond to those in which the adsorbate-adsorbent interaction resembles an enzyme-substrate binding. However, in general, the adsorbent support can interact with proteins applied to the column by ionic interactions, hydrogen bonds, hydrophobic interactions, or other binding sites present on the surface of the protein.

In affinity chromatography occur bioselective and non-bioselective interactions; the contribution of these interactions is dependent of the medium used and the physico-chemical characteristics of the preparation containing the protein to be purified. The bioselective adsorption constitutes one of the most effective and complex methods of protein separation.

In affinity chromatography the bioselective elution (desorption) should be attempted not only to prove that a particular purification was possible due to a bioselective adsorption, but also because the bioselective elution often provides high levels of purification. Large numbers of reversible interactions (hydrophobic attraction and hydrogen or electrostatic bonds) are involved in recognition of the free ligand (in elution solution) by the protein which was adsorbed on the matrix (Scouten, 1981).

6. Lectins: Prototypes in protein purification by affinity chromatography

The term lectin (from Latin *lectus*, past participle of *legere*, which means "to select") was introduced by Boyd (1954) and describes a protein heterogeneous group of non-immune

origin, containing two or more binding sites for mono or oligosaccharides. These molecules have the ability to agglutinate cells such as erythrocytes (hemagglutination), lymphocytes, fibroblasts and bacteria, being also able to precipitate glycoconjugates (Goldstein et al., 1980; Barondes, 1988; Kennedy et al., 1995; Correia et al., 2008; Sá et al., 2009a).

To be considered a lectin, the hemagglutinating activity should be inhibited by a carbohydrate; when addition of mono or oligosaccharides neutralizes the agglutination phenomenon, the protein is considered a potential lectin.

The lectin purification may be performed by conventional or high resolution techniques. However, in most of the purification processes, the affinity chromatography is used. The lectins are real models of protein purification exploring affinity interactions.

The lectin extracted from *Canavalia ensiformis* seeds (jack bean) named Concanavalin A (Con A) was the first lectin to be crystallized. Since then, an increasing number of lectins with similar or different specificities have been obtained.

Lectins have been purified from *Cratylia mollis* seeds using Sephadex (cross-linked dextran gel) matrices allying the gel filtration property of this support and the ability of the lectins to bind glucose (Paiva & Coelho, 1992; Correia & Coelho, 1995).

Guar gel beads produced by cross-linking of refined guar gum (a polysaccharide composed of glucose and mannose) with epichlorohydrin in a mixture of water and 2-propanol (Gupta et al., 1979) have been used to purify galactose-specific lectins (Santos et al., 2009; Nunes et al., 2011).

Chitin-binding lectins can be isolated by affinity chromatography on columns containing powder of chitin from crab shells hydrated with the equilibrating solution. This is a cheap, efficient, and rapid technique to purify these lectins, which have great potential as insecticidal and antimicrobial agents (Sá et al., 2009a; Sá et al; 2009b; Santana et al., 2009; Coelho et al., 2009; Ferreira et al., 2011; Napoleão et al., 2011a; Napoleão et al., 2011b).

A ferromagnetic levan (a homopolysaccharide composed of D-fructofuranosyl) composite was developed and efficiently used in purification of *C. mollis* lectin (Angeli et al., 2009). Egg glycoproteins were immobilized and the affinity matrix was efficient to purify lectins from extracts of *Phaseolus vulgaris*, *Lens culinaris*, and *Triticum vulgaris* (Zocatelli et al., 2003).

Lectins can be used for observation of the most diverse phenomena and the study of these proteins allows the evaluation of different cell surfaces. It is known that all cells have a membrane containing carbohydrates, consisting mainly of glycoproteins and glycolipids, that are different for each cell and which may constitute the lectin receptors. In the same cell, the surface structure can change characteristically due to normal development course or cases of illness. The lectins have been used very successfully in histochemistry (Beltrão et al., 1998, Lima et al., 2010) and electrochemistry (Souza et al., 2003, Oliveira et al., 2008, 2011b) with diagnostic purposes.

6.1 Purification of lectins from autochthonous and introduced species at Northeastern Brazil by affinity chromatography

The motivation to search lectins in autochthonous and introduced species from a particular region of a country is primordially due to the perspectives to develop a biotechnological

leading edge. In the Laboratory of Glycoproteins from the Department of Biochemistry of the *Universidade Federal de Pernambuco* (Brazil), the first plant tissues evaluated in order to identify hemagglutinating activity (indicating the presence of lectins) were the seeds of *C. mollis* (Paiva & Coelho, 1992; Correia & Coelho, 1995). This legume, also known as camaratu bean, is important as human food and as native forage in the Semi-Arid Region from the State of Pernambuco, northeastern Brazil. Since then, many other lectins have been purified. Examples of lectins purified by affinity chromatography in the Laboratory of Glycoproteins are shown in Table 2.

Lectin	Plant (tissue)	Affinity support used	References
BmoLL	*Bauhinia monandra* (leaf)	Guar gel	Coelho & Silva (2000)
BmoRoL	*B. monandra* (root)	Guar gel	Souza et al. (2011b)
Cramoll	*Cratylia mollis* (seeds)	Sephadex	Paiva & Coelho (1992); Correia & Coelho (1995)
cMoL	*Moringa oleifera* (seeds)	Guar gel	Santos et al. (2009)
WSMoL	*M. oleifera* (seeds)	Chitin	Coelho et al. (2009)
MuBL	*Myracrodruon urundeuva* (bark)	Chitin	Sá et al. (2009b)
MuHL	*M. urundeuva* (heartwood)	Chitin	Sá et al. (2008)
MuLL	*M. urundeuva* (leaf)	Chitin	Napoleão et al. (2011)
PpeL	*Parkia pendula* (seed)	Sephadex	Lombardi et al. (1998)
PpyLL	*Phthirusa pyrifolia* (leaf)	Sephadex	Costa et al. (2010)
OfiL	*Opuntia ficus indica* (cladodes)	Chitin	Santana et al. (2009)

Table 2. Lectins purified by affinity chromatography from different tissues of autochthonous and introduced plants from northeastern Brazil.

Saline extract (0.15 M NaCl) from *C. mollis* seeds showed hemagglutinating activity on erythrocytes from humans and other animals. The lectin activity was inhibited by glucose and mannose. The extract was treated with ammonium sulfate (0-40% and 40-60%), producing three fractions (F): 0-40F and 40-60F (precipitate fractions) and 40-60SF (supernatant fraction) with hemagglutinating activity. The hemagglutinating activity was concentrated (94%) in 40-60F, and a lectin (Cramoll 1) was purified by affinity chromatography on Sephadex G-75 followed by ion exchange chromatography on CM-cellulose (Correia & Coelho, 1995). Additionally, two other molecular forms were obtained from 0-40F (Cramoll 3) and 40-60FS (Cramoll 2) through affinity chromatography on Sephadex G-75, ion exchange using CM-Cellulose column, and molecular exclusion using Bio-Gel P (Paiva & Coelho, 1992). The characterization of the isoforms was performed by electrophoresis and immunological methods. Cramoll 1 was crystallized by Tavares et al. (1996). *C. mollis* lectins showed several biological activities such as mitogenic effect on human lymphocytes (Maciel et al., 2004), antitumor activity on Sarcoma 180 when encapsulated into liposomes (Andrade et al., 2004), potential anti-helminthic against *Schistosoma mansoni* (Melo et al., 2011a), healing activity on cutaneous wounds in healthy and immunocompromised mices (Melo et al., 2011b), and induction of death on epimastigotes of *Trypanosoma cruzi* (Fernandes et al., 2010).

Moringa oleifera is a multipurpose tree with great importance in industry and medicine. Lectins have been found in extracts from distinct tissues of *M. oleifera* (Santos et al., 2009). Seeds from moringa are used to treat water for human consumption and different lectins were detected in this tissue (Santos et al., 2005; Katre et al., 2008; Santos et al., 2009; Coelho et al., 2009). Santos et al. (2005) found a water-soluble *M. oleifera* lectin (WSMoL) that is the unique *M. oleifera* lectin inhibited by fructose. WSMoL was isolated through affinity chromatography on chitin column and showed larvicidal activity against fourth-stage larvae of *Aedes aegypti* (Coelho et al., 2009). This lectin is also a potential natural biocoagulant for water, reducing turbidity, suspended solids and bacteria (Ferreira et al., 2011). Genotoxicity assessment of WSMoL showed that it was not mutagenic and was not able to promote breaks in DNA structure (Rolim et al., 2011).

Santos et al. (2009) purified a lectin with coagulant properties from *M. oleifera* seeds (cMoL) by affinity chromatography on guar gel. cMoL agglutinated erythrocytes from rabbit and human, was insecticidal for *Anagasta kuehniella* and, when immobilized, served as an affinity support able to interact with humic acids (Oliveira et al., 2011a; Santos et al., 2011).

Coelho & Silva (2000) purified a galactose-specific lectin (BmoLL) from the fresh leaves of *Bauhinia monandra*. Also, other galactose-specific lectin was purified from *B. monandra* secondary roots, BmoRoL (Souza et al., 2011). These lectins were purified in milligram quantities by affinity chromatography on guar gel. BmoLL showed insecticidal activity on *Callosobruchus maculatus*, *Anagasta kuehniella* and *Zabrotes subfasciatus* (Macedo et al., 2007) while BmoRoL showed antifungal and termiticidal activities (Souza et al., 2011); thus, these lectins have biotechnological potential for application in control of agricultural pests.

In our studies, the presence of lectin isoforms has been revealed. The exploration and knowledgement of multiple molecular forms of lectins in extracts or in early stages of fractionation is very important. A substantial proportion of proteins have been described with multiple molecular forms having or not defined genetic origin.

The *Parkia pendula* (visgueiro) is a majestic tree from the Brazilian Atlantic Forest that stands out by their generous production of vegetables. Extracts of its seeds showed hemagglutinating activity with erythrocytes from humans and various animal species. The best monosaccharide inhibitors of the hemagglutinating activity from *P. pendula* were α-methyl D-mannoside, D (+)-mannose and D (+)-glucose, in descending order. To purify the lectin, a seed extract in 0.15 M NaCl, was fractionated with ammonium sulfate (40%). The 0-40F recovered 97% of total hemagglutinating activity. The dialyzed preparation was chromatographed by affinity on Sephadex G-75, and eluted with 0.3 M glucose. The purity of the obtained preparation allowed the crystallization of the lectin (Lombardi et al., 1998).

Other supports for purification of *P. pendula* lectin by affinity chromatography were also exploited for its purification. Although the lectin was not inhibited by *N*-acetyl-D-glucosamine, the support chitin was used to purify two molecular forms of lectin (Souza, 1989). The absence of inhibitory effect of carbohydrate on hemagglutinating activity does not imply in an inability of lectin to adsorb on an affinity support containing this carbohydrate (Lis & Sharon, 1981).

Myracrodruon urundeuva (aroeira-do-sertão) is a plant with importance in traditional medicine and its heartwood is resistant to fungi and termite attack. Lectins were isolated

from *M. urundeuva* bark (MuBL), heartwood (MuHL) and leaf (MuLL) by affinity chromatography on chitin columns. Similarly to *P. pendula* lectin, the hemagglutinating activity of MuLL is not inhibited by *N*-acetyl-D-glucosamine but the lectin bind to chitin. The affinity interaction between MuLL and this monosaccharide was demonstrated by affinity chromatography on *N*-acetyl-D-glucosamine-Agarose column (Napoleão et al., 2011a).

MuHL showed antimicrobial activity inhibiting the growth of bacteria and fungi (Sá et al., 2009a). The three lectins showed termiticidal activity against *Nasutitermes corniger* and insecticidal effect on fourth-stage larvae of *A. aegypti* (Sá et al., 2008; Sá et al., 2009b; Napoleão et al., 2011a; Napoleão et al., 2011b).

6.2 Applications: Immobilized lectins as affinity supports for protein purification

Various applications of lectins have been developed from the binding of these versatile molecules with free carbohydrates or glycoconjugates present in cell surfaces. The lectin applications have emerged in parallel to their discovery in 1888, with the description of the hemagglutination phenomenon, previously mentioned. Lectins have been applied for different purposes.

An immobilized lectin, covalently attached to a support, can separate glycoproteins or proteoglycans containing specific carbohydrate groups from a crude preparation. The elution of adsorbed material can be performed by treatment of support with a solution containing a competitive glycoside. The elution is usually performed near neutral pH, with minimal deleterious effects to the glycoprotein.

The interaction of a glycoprotein with an immobilized lectin can be used as a suitable technique to obtain preliminary information about the covalently linked carbohydrates to the glycoconjugate in the study. Lectins with different carbohydrate specificities, immobilized on Sepharose, have been applied as an analytical tool to assess and compare the carbohydrate residues.

Coelho (1982), using columns containing lectins with different specificity, detected microheterogeneities in human liver glycosidases. Con A revealed microheterogeneity in type A and B isoenzymes of beta-N-acetylhexosaminidase purified from human placenta.

A preparation of lectin from *C. mollis* containing Cramoll 1,4 isoforms was immobilized on inert support and used as an affinity matrix for purification of glycoproteins from human plasma, including the lecithin cholesterol acyl transferase (Lima et al., 1997). *C. mollis* seed lectins immobilized on cyanogen bromide-activated Sepharose 4B were used to purify a trypsin inhibitor from *Echinodorus paniculatus* seeds (Paiva et al., 2003) and a soybean seed protein with platelet antiaggregation and anticoagulant activities (Silva et al., 2011).

Immobilized *Euonymus europaeus* lectin was an efficient affinity ligand used in the capture step for purification of human influenza A viruses derived from MDCK cells; the main targets were two viral glycoproteins (Opitz et al., 2007).

Lectin affinity chromatography is a powerful fractionation technique in the identification of glycobiomarkers. Immobilized Con A was successfully used in the glycoproteomic analysis of pluripotent murine embryonic stem cells; differential patterns of binding to lectin allowed the identification of stage-specific glycopeptides (Alvarez-Manilla et al., 2010).

7. Immunoaffinity chromatography

The immunoaffinity chromatography consists of an antibody (immunoglobulin) immobilized on a support to purify the protein against which the antibody was developed. Antibodies specific for the protein of interest are produced by the immune system when the exogenous protein is inoculated in the animal; after, polyclonal antibodies are extracted from the blood serum of the animal, isolated and immobilized to constitute a matrix for purification of the protein of interest (Figure 2). Since the polyclonal antibodies are products from many different cells of the immune system, they are heterogeneous, differing in binding affinity for the protein inoculated on the animal.

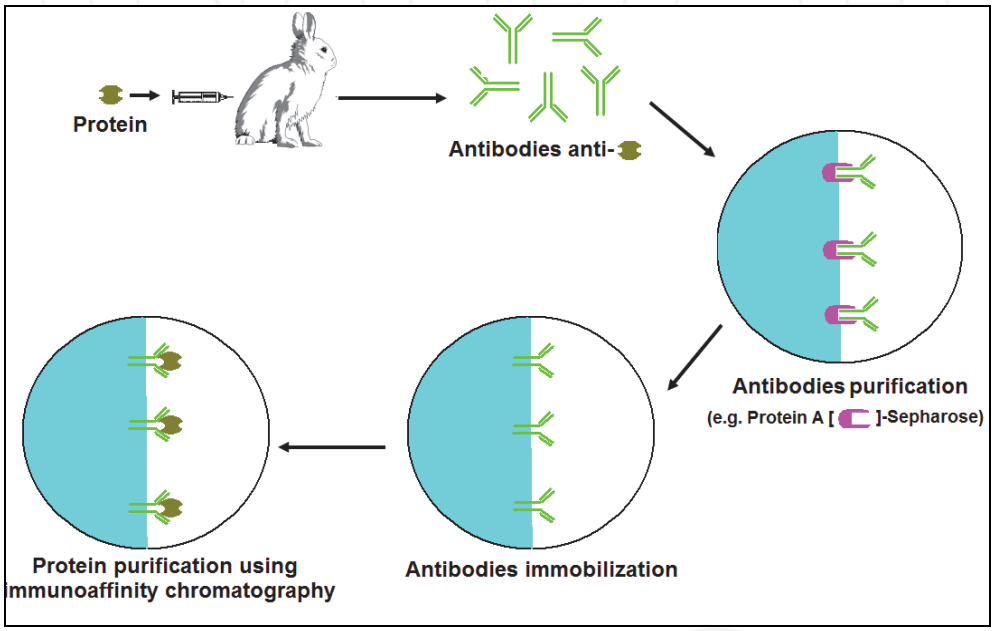

Fig. 2. Immunoaffinity chromatography for protein purification. First, the antibody for a specific protein is developed immunizing an animal. Next, the antibodies produced are purified and immobilized for use as immunoaffinity support.

Polyclonal antibodies (IgY) were developed against *Plasmodium falciparum* proteins and immobilized for use in immunoaffinity chromatography columns; the technique was more efficient than conventional chromatography (using two anion exchange columns) in purification of chimeric proteins expressed in *Escherichia coli* that are candidates for use as vaccine to prevent malaria (Qu et al., 2011).

The native Cramoll 1 was used to develop a serum anti-Cramoll 1 produced by rabbits. The anti-Cramoll 1 immunoglobulin (IgG anti-Cramoll 1) was obtained by affinity chromatography on Protein A-Sepharose. The antibody was conjugated to peroxidase (IgG anti-Cramoll 1-Per) and the conjugate was used to evaluate the structural assessment of the lectin (Correia & Coelho, 1995).

Monoclonal antibodies can be developed by collecting lymphocytes B (producing the desired antibody) from the spleen of the immunized animal and fusing them with a myeloma (a tumor of lymphocytes B). The resulting cells (hybridoma) have an unlimited capacity for division. When developed in culture they will produce large amounts of monoclonal antibodies.

Monoclonal antibodies are extremely useful as immunosorbents for purification of antigens. Immobilization of monoclonal antibody produces a support that can achieve a 10,000-fold purification in a single step. Polyol-responsive monoclonal antibody that recognize a highly conserved sequence in the β-subunit of bacterial RNA polymerase were used to purify RNA polymerase from five species of bacteria in one immunoaffinity chromatography step (Stalder et al., 2011). Nakamura et al. (2010) developed a sensitive and specific monoclonal antibody against a soluble lectin-like oxidized low-density lipoprotein receptor-1 (sLOX-1), expressed prominently in atherosclerotic lesions as a specific biomarker to diagnostic acute coronary syndrome at an early stage; this immunoassay was developed in order to establish a more sensitive assay that may also be useful in predicting cardiovascular disease risk in disease-free subjects.

The antigen-antibody complex often has a strong affinity and it is necessary that the release of the protein is performed in drastic conditions of pH and/or ionic strength. The elution is preferably performed in the reverse direction to sample application. To avoid denaturation of the molecule, the conditions are adjusted immediately after the elution.

8. Purification of DNA-binding proteins by affinity chromatography

DNA-binding proteins can be purified using different techniques of affinity chromatography. One of them is the affinity chromatography containing immobilized DNA, which is a highly specific technique for the purification of DNA-binding proteins involved in the transcription, replication and recombination. The affinity columns are usually generated by immobilization of synthetic oligonucleotides consisting of tandem repeated units or multiple copies of the same sequence (Gadgil et al., 2001). Purification factors can reach 10,000-fold. Figure 3 shows a schematic representation of DNA-affinity chromatography.

DNA-affinity chromatography is a powerful method with broad applicability; this technology has been extended for purifying transcription factors, polymerases, and nucleases (Chockalingam et al., 2001). Since affinity chromatography is based in the specific interaction between molecules, it is highly selective and offers high yield and purity (Gadgil et al., 2001). DNA affinity columns can be constructed depending on the DNA-binding properties of a protein (non-specific, specific, double- or single-stranded DNA). Apart from the purification of DNA-binding proteins, DNA affinity columns can also be used for the purification of nucleic acids, such as RNA and DNA. Golovina et al. (2010) described a fast and simple purification method for the 30S ribosomal subunits carrying lethal mutations using DNA-affinity chromatography. Kerrigan & Kadonaga (2001) developed a DNA affinity resin using agarose activated with cyanogen bromide.

Another technique used for isolation of DNA-binding proteins is ion metal affinity chromatography. The Mvo10b is a DNA-binding protein member of the Sac10b family from the mesophilic archaeon *Methanococcus voltae*, which may play an important role in the

organization and accessibility of genetic information in Archaea. This protein was purified by polyethyleneimine precipitation followed by nickel affinity chromatography; this protocol has potential application in the production of other thermophilic and mesophilic proteins in the Sac10b family (Xuan et al., 2009). A telomeric DNA-binding protein (Stn1p) from *Saccharomyces cerevisae* was purified by interaction with nickel-NTA resin followed by chromatography on Superdex 200 column (Qian et al., 2010).

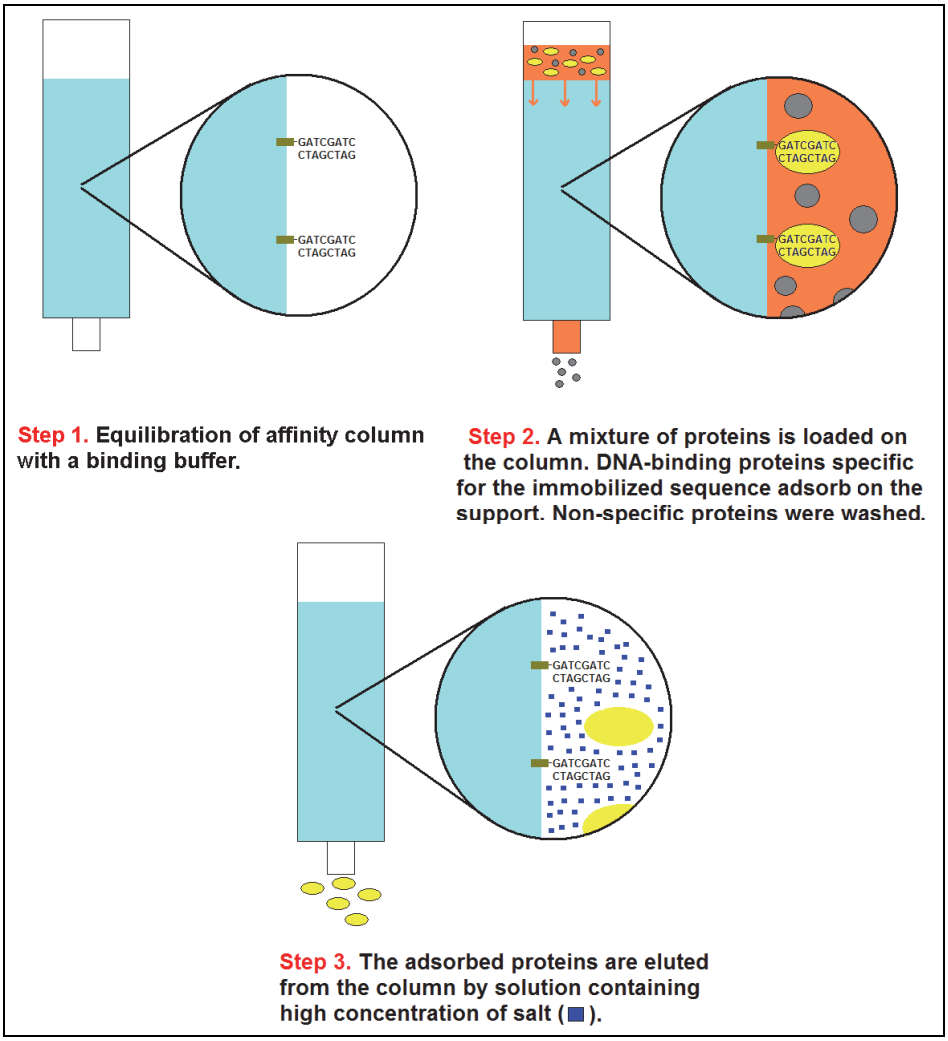

Fig. 3. Schematic representation of DNA-affinity chromatography. In the first step, the support containing a synthetic oligonucleotide consisting of a tandem repeated unit is washed equilibrated. Next, the mixture containing proteins is loaded and the DNA-binding protein that recognizes the oligonucleotide adsorb on the support. The elution is performed by solutions containing a very high concentration of salt.

9. Pitfalls in affinity chromatography

There are numerous problems in affinity chromatography, similar to any other techniques. One of the most common ways to immobilize ligands with free amino groups is by a reaction with Sepharose activated with cyanogen bromide. This method promotes the formation of ionic groups that may cause non-selective electrostatic adsorption. The immobilization of small ligands may create steric impediment problems that limit the functional capacity of the columns.

To minimize the steric interference between the support and substances interacting with the ligand, hydrocarbon spacer arms (such as in Sepharose CL-4B) are often interposed between the substrate and the ligand. However, these spacer arms may cause hydrophobic effects.

In affinity chromatography, the adsorption step is often performed with buffers of low ionic strength and the interference of non-selective electrostatic adsorption is inevitable. In addition, non-selective desorption of the desired molecule, increasing the pH or the ionic strength leads to at least a partial loss of activity, many times reversible but not always.

Special attention should be given to various affinity supports used under conditions of saturation of the protein to be applied to matrix; possible contaminations can result from non-selective interactions.

In immunoaffinity chromatography the disadvantages include the technical difficulty of producing monoclonal antibodies and the drastic conditions that are often required to elute the strongly adsorbed protein.

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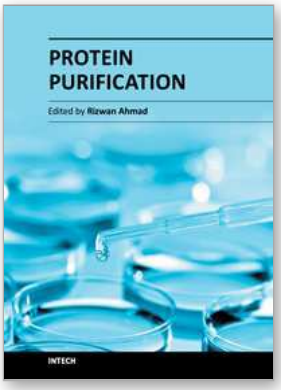

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