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Affinity-Based Methods for the Separation of Parasite Proteins

C.R. Alves, F.S. Silva, F.O. Oliveira Jr, B.A.S. Pereira, F.A. Pires and M.C.S. Pereira Instituto Oswaldo Cruz – Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brasil

1. Introduction

Affinity chromatography-based techniques have been developed to purify parasite proteins and improve our understanding of the parasite life cycle. These advances can be translated into concrete proposals for new drugs, diagnostic methods and vaccines for parasite diseases and help to reduce social inequality.

Affinity chromatography has been demonstrated to be a powerful tool for the isolation and purification of parasite proteins and has potential applications for diagnosis and therapy. Many studies have focused on parasite proteins that modulate host cell defense, as gp63, a glycoprotein from *Leishmania spp.*, that is involved in the cleavage of the complement factor C3b to iC3b, which promotes adhesion of promastigotes to macrophages via complement receptor 3 (Brittgham et al., 1995). This route of internalization does not lead to production of oxygen radicals or NO and favors parasite subsistence within the host cell. Another example is the cysteine protease B (CPB), an important virulence factor of the *Leishmania (L.) mexicana* complex, that inhibits lymphocytes Th1 and/or promotes the Th2 response either through proteolytic activity or through epitopes derived from its COOH-terminal extension (Pereira et al., 2011).

Due to the important role of these molecules, many researchers seek to develop specific and potent inhibitors for therapeutic strategies. Aspartic protease, a potential target for antiparasitic therapies, has been isolated from *Trypanosoma cruzi* by affinity chromatography using a specific inhibitor of this enzyme (Pinho et al., 2009); this enzyme is target for treatment of infections caused by HIV (Wlodawer & Vondrasek, 1998) and Candida (Hoegl et al., 1999). This enzyme has also been reported in *Plasmodium* spp. and *Schistosoma mansoni*, where it plays an important role in host hemoglobin degradation (Klemba & Goldberg, 2002). Additionally, specific inhibitors of plasmepsins and renin are viable drugs for the treatment of patients with malaria and high blood pressure.

These parasite proteins, along with others, have been tested as new targets for chemo- and immunotherapies for parasite diseases. They have been assessed by lectins or protease inhibitor affinity chromatography. The separation of sugars based on lectin affinity is one of main procedure that has been used. This technique is based on the ability of lectins to bind

specifically to certain oligosaccharide structures on glycoconjugates isolated from parasites. Parasite proteins are processed through a multi-lectin affinity column, and they bind to the immobilized lectins through their sugar chains. Certain glycoconjugates are important for the parasite life cycle, and lectin affinity chromatography can help to reveal their roles (Guha-Niyogi et al., 2001).

The use of protease inhibitors in affinity chromatography is another important approach for assessing parasite proteins. Proteases hydrolyze peptide bonds and can therefore degrade proteins and peptides that influence a broad range of biological functions, including the process of parasite infection (Mackeron et al., 2006). The specificity of the protease inhibitor used is an important aspect of this methodology; L- trans-epoxy-succinylleucylamido-(4-guanidino) butane (specific to cysteine-protease), pepstatin A (to aspartyl-protease) and aprotinin (to serine-protease) are frequently immobilized on a solid matrix for this technique.

Glycosaminoglycan (GAG) affinity is the only affinity chromatography method that is based on the sugar chains of lectin-like proteins. Some of these molecules (such as heparin sulfate, heparan sulfate, dermatan sulfate, keratan sulfate and chondroitin sulfate) contain complex oligosaccharide structures, which may be displayed on cell surfaces, incorporated into the extracellular matrix or attached to secreted glycoproteins, suggesting that they play structural roles (Dreyfuss et al., 2010). GAGs have been reported as potential candidates for therapeutic intervention against parasitic infections, such as leishmaniasis and Chagas diseases (Azevedo-Pereira et al., 2007; Oliveira-Jr et al., 2008).

According to the general principle of affinity chromatography (Fig. 1), a protein of interest is recovered based on its capacity to bind a specific functional group (ligand) that is immobilized on a bead material (matrix) that has been packed into a solid support (column). Although many ligands (enzymatic substrates, inhibitors of an enzyme, lectin, sugar residues, vitamins, enzyme cofactors, monoclonal antibodies) have been used to isolate proteins based on affinity, only lectin, an enzyme inhibitor and glycosaminoglycans have been used to obtain parasite proteins. The most commonly used matrix materials for the attachment of the ligand are polysaccharide derivatives (cellulose, dextran and agarose) and polyacrylamide.



Fig. 1. The principle of affinity chromatography. The ligand is covalently bound to a matrix (A). The functionalized matrix is then able to bind to a target protein aided by a binding buffer (B). Afterwards, the bound proteins are eluted with a different buffer (C).

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In these procedures, the soluble proteins are prepared from crude parasite lysates (or subcellular fractions) and loaded onto a column under chemical (buffer) and physical (temperature and pressure) conditions that promote the specific binding of the protein to the immobilized ligand (affinity) in what is known as the binding phase. Proteins that do not bind to the immobilized ligand under these conditions are removed from the solid phase by application of a constant liquid phase, which is referred to as the wash phase. Then, the bound protein can be recovered by changing the buffer conditions to favor desorption during the elution phase.

In this chapter, we describe the use of affinity chromatography to assess parasite proteins and the importance of these methods for public health. Several affinity chromatography protocols are considered. Additionally, we discuss our experience using affinity chromatography to obtain parasite proteins, and we include some unpublished results related to *Dermatobia hominis* third (L3) instar larvae proteases.

2. The use of affinity chromatography in parasite protein studies

2.1 Lectin affinity-based separation of parasite proteins

There are relatively few studies available in the current literature describing the use of lectins to affinity-purify glycosylated proteins from parasites. However, the reports on this subject demonstrate that this technique is useful for the retrieval of putative virulence factors or potential protective immunogens from a large array of parasites, including apicomplexan, trypanosomatids and nematodes (e.g., Fauquenoy et al., 2008, Gardiner et al., 1996, Smith et al., 2000). In addition to its utility in the isolation of parasite factors, lectin-based affinity chromatography is also a valuable resource for characterization of the structure of carbohydrates bound to proteins from these organisms due to the distinct specificities of the lectins that are available for this type of analysis.

Lectins are proteins that specifically bind to sugars, and they have been used for many types of studies, ranging from blood typing to immune regulation analysis (Rüdiger & Gabius, 2001). These proteins are generally isolated from plants (mostly legume seeds), where they can be found in abundance. Their usage is determined by the particular sugar structures that they are able to bind (Rüdiger & Gabius, 2001). The surveyed literature the use of six plant lectins [concanavalin A (Con A), ricin, jacalin, peanut agglutinin (PNA), wheat germ agglutinin (WGA) and Wisteria floribunda agglutinin (WFA)] in studies of parasites glycoproteins. Furthermore, one report described the use of Biomphalaria alexandrina lectin (BaSII), which in contrast to the others is a lectin obtained from an animal.

Con A is a lectin that can be extracted from jack beans of the species *Canavalia ensiformis* (family Fabaceae). It binds to mannose or glucose residues and is thus characterized as a mannose-binding lectin. This lectin presents a high affinity for the oligosaccharide GlcNAcβ2Mana6(GlcNAcβ2Mana3)-Manβ4GlcNAc. It is also known to be a potent mitogen (Beckert & Sharkey, 1970; Rüdiger & Gabius, 2001).

Ricin, along with jacalin and PNA, is a lectin that binds to galactose. Specifically, it binds with high affinity to the motif Gal β 4GlcNAc β 2Man α 6 (Gal β 4-GlcNAc β 2Man α 3) Man β 4GlcNAc. Ricin is highly toxic because it can impair ribosome activity through cleavage of the nucleobases of ribosomal RNA, and it has potential to be used as a biological

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weapon. This lectin is extracted from *Ricinus communis* (Family Euphorbiaceae) (Rüdiger & Gabius, 2001; Lord et al., 2003).

Jacalin binds to galactose and N-acetylgalactosamine, and presents a high affinity for the motif Gal β 3GalNAc α . It is obtained from *Artocarpus integrifolia* (Family Moraceae). It is commonly used to isolate IgA from human plasma (Kabir, 1998, André et al., 2007).

Like Con A, PNA is a legume lectin and is isolated from plants that belong to the family Fabaceae. It is extracted from *Arachis hypogea* and binds specifically to the monosaccharide galactose and to the motif Gal β 3GalNAc α , similarly to the binding motif of jacalin. PNA is used as a marker of T-cell subpopulations and to differentiate between the stages of the Leishmania parasites life cycle (Dumont & Nardelli, 1979, Wilson & Pearson, 1984, Rüdiger & Gabius, 2001).

WGA is obtained from the species *Triticum vulgare*. It presents a low affinity for N-acetylgalactosamine, but it binds to the sialic acid N-acetylneuraminic and to the motif GlcNAcβ4GlcNAc

WFA is isolated from *Wisteria floribunda*, a woody liana of the family Fabaceae. Although some uncertainty regarding its binding specificity remains, it seems that this agglutinin binds preferentially to the monosaccharide N-acetylgalactosamine and to the motif GalNAca6GalNAc. WFA is used to fractionate lymphocyte populations, and although it is not mitogenic like Con A, it can induce lymphokine production in murine splenocytes (Jacobs & Poretz, 1980; Rüdiger & Gabius, 2001).

BaSII is a lectin that can be isolated from the snail *B. alexandrina*, an intermediate host of the trematoda parasite *Schistosoma mansoni*, the causative agent of schistosomiasis. It specifically binds to the motif Fuc α 1,2Gal β 1,4Glc (Mansour, 1996).

2.1.1 General procedures for the isolation of parasite proteins by lectin affinity

The rational for lectin-based affinity chromatography is the same as for other types of affinity-based fractionation: a sample is exposed to a solid phase that has been coupled to an affinity separation molecule (a lectin, in this case) under conditions that are adequate for binding (Fig. 2A). The unbound material from the sample is washed away (generally using the same buffer applied to equilibrate the solid-phase), and in the final step, the affinity-bound fraction is recovered by altering the equilibrium conditions of the solid phase (by changing the system pH or salt concentration) or by adding a molecules that competes for the binding site of the ligand.

To provide several practical examples, a collection of lectin affinity-based methodologies used to isolate and/or characterize glycoproteins from distinct parasites is listed in the Table 1.

It is important to note that some techniques, such as metabolic radioactive labeling (by [3H]myristic acid or [3H]-glucosamine, for example) and cell disruption (by Triton X-100, dioxane or hypotonic solution), must be applied prior to lectin chromatography to allow for the identification of molecules eluted from the column or the preparation of suitable samples for the chromatography column, respectively.

		Couto et al., 1990		Brickman & Balber, 1993		Mansour, 1996	:	Gardiner et al., 1996		Gerold <i>et</i> al., 1996	Gerold et	al., 1996	Nolan <i>et</i> al., 1997		
Second phase	Methods	None		None		HPLC		Dylasis		Gel filtration (Biogel P 30)	None		Ion exchange chromatograp	hy (DEAE-52)	
	Elution	0.1 M N-acetyl-D-glucosamine in 10mM Tris-Hcl (pH 7.2), 150 mM	NaCl	0.2 M acetic acid in 10 mM Mops buffer (pH 6.9), 1mM MgSO ₄ , 1 mM ECTA, 0.2% (v/v) Triton X-100, 10 µg/ml DNase I, 0.05 mM leupeptin, 2.5 mM PMSF, 5 mM iodoacetamide, 0.05 mM TPCK		300 mM L-fucose in 20 mM Tris-HCl (pH 7.3), 0.1% (v/v) Triton X-100, 150 mM NaCl, 1mM CaCl ₂ , 1mM MgCl ₂		0.5 M alpha-methyl mannoside (Con A) or 0.5 M galactose (ricin) in 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% NP-40, and 50 µg/ml antipain, leupeptin and E-64		Isoelectric focusing	50 mM Tris-HCI (nH 7 4) 0.02%	sodium azide, 100 mM GalNAc	0.5 M α-methylmannoside in 10mM Tris buffer (pH 6.5) containing 0.1%	(w/v) Triton X-100	1
	Wash	10mM Tris-Hcl (pH 7.2), 150 mM	NaCl	10 mM Mops buffer (pH 6.9), 1mM MgSO ₄ , 1 mM EGTA, 0.2% (v/v) Triton X-100, 10 µg/ml DNase I, 0.05 mM leupeptin, 2.5	mM PMSF, 5 mM iodoacetamide, 0.05 mM TPCK	20 mM Tris-HCl (pH 7.3), 0.1% (v/v) Triton X-100, 150 mM NaCl, 1mM CaCl ₂	1mM MgCl ₂	10 mM l'ris-HCl (pH 7.4), 150 mM NaCl, 1 mM MgCl ₂ , 5 mM CaCl ₂ , 2% NP-40, 100 µg/ml of antipain,	leupeptin and E-64	10mM Tris-HCl (pH 7.5), 150 mMNaCl, 1mM CaCl ₂	1mM MnCl ₂ 50 mM Tris-HCl	(pH 7.4), 0.02% sodium azide	10mM Tris buffer (pH 6.5), 0.1% (w/v)	Triton X-100	
	Bind	10mM Tris-Hcl (pH 7.2), 150 mM NaCl	:	10 mM Mops buffer (pH 6.9), 1mM MgSO ₄ , 1 mM EGTA, 0.2% (v/v) Triton X-100, 10 µg/ml DNase I, 0.05 mM	mM iodoacetamide, U.U5 mM TPCK	20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM PMSF, 1% (v/v) Triton X- 100, 1 mM PCMB, 1 mM o-	phen, 1 mM iodoacetamide	10 mM Tris-HC (pH 7.4) ,150 mM NaCl, 1 mM MgCl ₂ , 5 mM CaCl ₂ , 2% NP-40, 100 μg/ ml of antipain, leupeptin and E-	64 2 2	10mM Tris-HCl (pH 7.5), 150 mMNaCl, 1mM CaCl ₂ , 1mM MnCl ₂	50 mM Tris-HCl (nH 7 4)	0.02% sodium azide	50 mM Tris buffer (pH 7.5), 150 mM NaCl, 1% (w/v)	Triton X-100	
First phase	Matrix	Sepharose		Agarose		Sepharose		Sepharose (Con A) or agarose (ricin)		Sepharose	Aoarose	0	Sepharose		
Isolated	protein	85 kDa surface	glycoprotein	Membrane ? Glycoprotein		37 kDa glycoprotein	;	Small Variable Surface Glycoprotein		Variant Surface Glycoprotein	Variant	Surface Glycoprotein	Invariant surface	glycoprotein heavily N- glycosylated	
Organism		Trypanosoma cruzi	ł	Trypanosoma brucci rhodesiense		Schistosoma mansoni	E	n A 1 rypanosoma brucei		n A Trypanosoma congolense	Trunanosoma	congolense	n A <i>Trypanosoma</i> brucei		
Ligand		WGA	·	Ricin		BaSII	;	Concanavali or ricin		Concanavali	WFA		Concanavali		



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J J L L L L	totein	Matrix	Bind	Wash	Elution	Methods	
	lycoprotein actions	Agarose	10mM Tris-HCl (pH7.4, 0.5 M NaCl, 0.02% NaN3, 100 mM Ca ²⁺ , 10 mM Mg ²⁺	10mM Tris-HCl (pH7.4, 0.5 M NaCl, 0.02% NaN3, 100 mM Ca ₂ , 10 mM Mg ²⁺	0.5 M galactose (PNA) or 0.8 M galactose (jacalin)	Gel filtration (Sephadex G-25); Anion Exchange chromatography (Mono Q - jacalin- binding material	Smith et al., 2000
	lycoproteins	Agarose	10 mM Tris (pH 7.4) 0.5 M NaCl, 10 mM CaCl ₂ , 100 mM MnCl ₂ , 0.5% Triton X-100	10 mM Tris (pH 7.4) 0.5 M NaCl, 10 mM CaCl ₂ 100 mM MnCl ₂ 0.25% CHAPS	0.2M methylmannopyranoside and 0.2 M methylglucopyranoside in 10 mM Tris (pH 7.4) 0.5 M NaCl, 10 mM CaCl ₂ , 100 mM MnCl ₂ 0.25% CHAPS	None	Redmond et al., 2004
ch a th a di d	lycoproteins eesenting ant poly-N- etyllactosam e trbohydrate lains	Agarose	50 mM Tris-HCl (pH 6.8), 400 mM NaCl, 0.8% Triton X-100, 0.1 M TLCK, 1 µg/ml leupeptin, 0.1% sodium azide	50 mM Tris-HCl (pH 6.8), 400 mM NaCl, 0.8% Triton X-100, 0.1 M TLCK, 1 μg/ml leupeptin, 0.1% sodium azide	30 mg/ml actose and 30 mg/ml galactose in 12,5 mM Tris-HCl (pH 6.8), 100 mM NaCl, 0.2% Triton X- 100, 25 mM TLCK, 0.25 µg/ml leupeptin, 0.025% sodium azide	None	Atrih <i>et al.,</i> 2005
N 100	-linked ycoproteins	Agarose	10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM CaCl2, 1 mM MnCl2, 1% (v/v) Triton X-100, protease inhibitor mixture	10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM CaCl2, 1 mM MnCl2, 1% (v/v) Triton X-100	1% (w/v) SDS in 100 mM Tris-HCl (pH 7.4) or 0.5 M α-methyl-	None	Fauquenoy et al., 2008
Ú. 10.	oteinases	Sheparose	20 mM Tris-HCl (pH 7.2), 5% (v/v) glycerol, 0.5% (v/v) CHAPS	20 mM Tris-HCl (pH 7.2), 5% (v/v) glycerol, 0.5% (v/v) CHAPS	50 mM a-Dmannose in 20 mM Tris-HCl (pH 7.2), 5% (v/v) glycerol, 0.5% (v/v) CHAPS	Anion exchange chromatography (DEAE-Sepahcel)	Rebello et al., 2009



During the affinity chromatography procedure, other methods, such as isoelectric focusing, may be used instead of the application of competing carbohydrates to elude the columnbound material. Furthermore, distinct affinity columns can be used in sequence to purify fractions with specific characteristics from a single sample.

As for the handling of the material that is eluted from an affinity column, many options for further purification are available, depending on the analysis method chosen for the study. Some of these options include: anion exchange chromatography, size exclusion chromatography and dialysis.

The combination of these accessible approaches allows for a vast array of study possibilities. Several examples of the results obtained by applying lectin-affinity chromatography in association with other techniques are described in the following paragraphs.

2.1.2 Parasite proteins isolated by lectin affinity chromatography

The structure of an N-linked oligosaccharide from a surface glycoprotein of *Trypanosoma cruzi*, an important human parasite that causes Chagas disease, was defined in a study using lectin chromatography (Couto et al., 1990). It was determined that the structure of this oligosaccharide is comprised of complex carbohydrate chains that possess a terminal sialic acid, α -L-fucose and a galactosyl(α 1,3)galactose unit.

The cellular localization of glycoproteins of *Trypanosoma brucei rodhesiense*, a subspecies of the parasite responsible for the African sleeping sickness, was analyzed using ricin-based chromatrography (Brickman & Balber, 1993). It was observed that the ricin-binding proteins were primarily located in the vesicles of the lysosomal / endosomal system.

Gardiner et al., (1996) characterized small glycoproteins isolated from the surface of *Trypanosoma vivax*, which causes bovine trypanosomiasis. That study was the first to detail the characteristics of a *T. vivax* Variable Surface Glycoprotein (VSG). The isolated protein, designated ILDat 2.1 VSG, presented a molecular mass of 40 kDa and contained mannose (or a derivative sugar) in small quantities, and it was poorly retained by the lectin affinity column. It is possible that carbohydrates comprise only the C-terminal anchoring structure of this protein.

The characteristics of a fucosyllactose determinant of a *S. mansoni* glycoprotein were identified using affinity chromatography based on a lectin that was isolated from a host of this parasite, *B. alexandrina*. This determinant is expressed in the outer chain of a single unit of complex type N-linked oligosaccharides (Mansour, 1996).

Additionally, the VSG glycosyl-phosphatidylinositol membrane anchors of *Trypanosoma congolense*, another trypanosomatide species that causes bovine trypanosomiasis, were studied by lectin affinity (Gerold et al., 1996) using a modification of the technique in which the bound proteins are electrophoretically desorbed (Reinwald et al., 1981). This analysis allowed for description of the VSG GPI-anchor in this parasite: it contains a β 1,6-linked galactose as the terminal hexose of the branch and an N-acetyl-glucosamine residue. Also, it was observed that *T. congolense* synthesizes two potential GPI-anchor precursors, one of which is insensitive to phospholipase C activity.

Nolan et al., (1997) identified a new invariant surface glycoprotein that is heavily N-glycosylated in the bloodstream forms of *Trypanosoma brucei* and designated it as ISG₁₀₀. This glycoprotein presents a large internal domain composed of a serine-rich repetitive motif, which was previously undescribed, and N-glycosylation sites on the N-terminal domain. Additionally, ISG₁₀₀ is encoded by a single gene, whereas the trypanosomal plasma membrane proteins are commonly encoded by tandemly repeated genes that are part of a multigene family.

Potentially protective glycoprotein fractions from *Haemonchus contortus*, a parasitic nematode in ruminants, were also obtained by lectin chromatography (Smith *et al.*, 2000). The findings from that study confirmed the potential of the *H. contortus* PNA-binding glycoprotein fraction as an efficacious antigen against this parasite infection in sheep. Furthermore, this study identified another highly protective fraction that binds to jacalin. This second protective fraction presents sialyted versions of the same oligosaccharides that bound to the PNA column.

Another study on the protective properties of the glycoproteins of *H. contortus* was performed by the same group (Smith et al., 2003). The results showed that the four purified glycosylated zinc metalloproteinases from this parasite were such an efficacious antigen that, to an extent, they could account for most of the protection conferred by the ureadissociated whole glycoproteins fraction. However, the role for the glycan moieties of these enzymes in the protection process was not clear.

The capacity of glycoproteins from *Caenorhabditis elegans*, a free living nematode, to induce protection from a challenge with *H. contortus* in sheep was assayed by Redmond et al. (2004). The lectin affinity methodology was able to identify glycoproteins with molecular masses between 25 and 200 kDa in extracts prepared from *C. elegans*, but the fractionated glycoproteins were not able to confer protection against an *H. contortus* challenge. These findings suggest that the conserved glycan moieties between these two species of worm are not solely responsible for the protections levels observed when native *H. contortus* antigens are used.

Trypanosoma brucei glycoproteins were shown to present distinctive structural features, such as the presence of giant poly-N-acetyllactosamine carbohydrate chains (Atrih et al., 2005). The recovered affinity-bound molecules were predominantly, but not exclusively, from the flagellar pocket. These glycoproteins carry massive glycans, representing the largest poly-LacNAc structures reported to that date, and they may produce a gel-like matrix in the lumen of the flagellar pocket and/or the endosomal/lysosomal system. Despite their remarkable size, these glycans present a very simple neutral structure, containing only mannose, galactose and N-acetylglycosamine.

Important glycoproteins from the apicomplexan parasite *Toxoplasma gondii* have also been analyzed by lectin affinity methods. It was shown that these components are pivotal factors for host invasion and intracellular development of parasites (Fauquenoy et al., 2008).

Cysteine proteinases from promastigostes of *Leishmania (Viannia) braziliensis* were shown to be anchored to the membrane by glysoylphosphatidylinositol structures in an analysis of the hydrophobic fraction of promastigote forms. These enzymes are suggested to play a role in the process of parasite survival inside its hosts (Rebello et al., 2009).

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2.1.3 Remarks on the isolation of proteins by lectin affinity chromatography

These reports provide examples of the uses of lectin affinity chromatography to identify potentially antigenic fractions of parasites that could be used for vaccine development. Also, they point to the potential of this method to characterize glyconjugates, such as the glycoproteins that are present on the parasite surface or secreted by these organisms. However, apart from these purely structural or clinically oriented applications, this method may also be relevant in other investigations, including studies of host-parasite interactions. This hypothesis is reinforced by reports indicating that lectin-glycan binding is important for the infection and virulence processes of some parasites, *e.g. Acanthamoeba castellanii* (Garate et al., 2006), *H. contortus* (Turner et al., 2008), *L. (V.) braziliensis* (Rebello et al., 2009) and *T. gondii* (Fauquenoy et al., 2008)



Fig. 2. Illustration of the affinity chromatography methodologies. The target molecules are bound to their ligands immobilized on a solid phase matrix. (A) Lectin affinity chromatography, (B) Protease inhibitor affinity chromatography and (C) Glycosaminoglycan affinity chromatography. Proteins = blue circle; carbohydrates = red pentagon and hexagon; protease inhibitors = green drop-like form; ions =yellow circles; and solid phase matrix beads = gray circle.

2.2 Protease inhibitors affinity-based separation of parasite proteins

Methodologies for the purification of parasite proteases have been applied in studies investigating the biological roles of these enzymes in parasite, including their participation in the infection process and in the survival of the parasites inside their hosts (McKerrow et al, 2006). Inhibitor affinity chromatography consists of the fractionation of parasite samples based on the reversible interactions between proteases and their specific inhibitors while the latter are covalently attached to a matrix (Fig. 2B). This technique can also be performed using irreversible inhibitors under particular conditions that will be described further in this section.

It is also interesting to note that, based on the specificity of the inhibitor used in the affinity chromatography, it is possible to suggest the enzyme class of the isolated protein. However, complementary analyses, such as characterization of the proteolytic activity, are often necessary to confirm these findings. Nevertheless, this purification strategy presents an initial advantage when compared to other methodologies.

In this section, fractionation approaches for serine-, aspartic acid- and cysteine proteases in specific parasites will be described. These approaches must take the class of the studied enzyme into consideration, as well as the inhibitor to be used and the characteristics of the mobile phase used for chromatography.

pr	Organism	Isolated	First phase				Second phase	References
)	protein	Matrix	Bind	Wash	Elution	Methods	
or jacalin	Haemonchus contortus	Glycoprotein fractions	Agarose	10mM Tris-HCl (pH7.4, 0.5 M NaCl, 0.02% NaN3, 100 mM Ca ²⁺ , 10 mM Mg ²⁺	10mM Tris-HCI (pH7.4, 0.5 M NaCl, 0.02% NaN3, 100 mM Ca ₂ , 10 mM Mg ²⁺	0.5 M galactose (PNA) or 0.8 M galactose (jacalin)	Gel filtration (Sephadex G-25); Anion Exchange chromatography (Mono Q - jacalin- binding material only)	Smith <i>et al.</i> , 2000
anavalin A	. Caenorhabditis elegans	Glycoproteins	Agarose	10 mM Tris (pH 7.4) 0.5 M NaCl, 10 mM CaCl ₂ , 100 mM MnCl ₂ , 0.5% Triton X-100	10 mM Tris (pH 7.4) 0.5 M NaCl, 10 mM CaCl ₂ 100 mM MnCl ₂ 0.25% CHAPS	0.2M methylmannopyranoside and 0.2 M methylglucopyranoside in 10 mM Tris (pH 7.4) 0.5 M NaCl, 10 mM CaCl ₂ , 100 mM MnCl ₂ , 0.25% CHAPS	None	Redmond et al., 2004
	Trypanosoma brucei	Glycoproteins presenting giant poly-N- acetyllactosam ine carbohydrate chains	Agarose	50 mM Tris-HCl (pH 6.8), 400 mM NaCl, 0.8% Triton X-100, 0.1 M TLCK, 1 μg/ml leupeptin, 0.1% sodium azide	50 mM Tris-HCI (pH 6.8), 400 mM NaCl, 0.8% Triton X-100, 0.1 M TLCK, 1 μg/ml leupeptin, 0.1% sodium azide	30 mg/ml lactose and 30 mg/ml galactose in 12,5 mM Tris-HCl (pH 6.8), 100 mM NaCl, 0.2% Triton X- 100, 25 mM TLCK, 0.25 µg/ml leupeptin, 0.025% sodium azide	None	Atrih <i>et al.</i> , 2005
anavalin A	Toxoplasma gondii	N-linked glycoproteins	Agarose	10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM CaCl2, 1 mM MnCl2, 1% (v/v) Triton X-100, protease inhibitor mixture	10 mM Tris-HCI (pH 7.5), 150 mM NaCl, 1 mM CaCl2, 1 mM MnCl2, 1% (v/v) Triton X-100	1% (w/v) SDS in 100 mM Tris-HCl (pH 7.4) or 0.5 M a-methyl-	None	Fauquenoy et al., 2008
anavalin A	Leishmania (V.) braziliensis	Cysteine proteinases	Sheparose	20 mM Tris-HCl (pH 7.2), 5% (v/v) glycerol, 0.5% (v/v) CHAPS	20 mM Tris-HCl (pH 7.2), 5% (v/v) glycerol, 0.5% (v/v) CHAPS	50 mM a-Dmannose in 20 mM Tris-HCl (pH 7.2), 5% (v/v) glycerol, 0.5% (v/v) CHAPS	Anion exchange chromatography (DEAE-Sepahcel)	Rebello et al., 2009

Table 2. Protease Inhibitors affinity-based

Ligand	Organism	Isolated protein			First phase		Second phase	References
			Matrix	Bind	Wash	Elution	Methods	
Pepstatin A	Neospora caninum	52 kDa	Agarose	5 mM NaOAc, (pH 5.5)	5 mM NaOAc, (pH 5.5)	0.1 M Tris-HCl, 0.5 M NaCl, (pH 8.5)	None	Naguleswaran <i>et</i> al, 2005
Aprotinin	Leishmania amazonensis	115 and 56 kDa	Agarose	10 mM Tris-HCl, 5 mM CaCl ₂ ,	10 mM Tris-HCl, 5 mM CaCl ₂ , (pH7,5)	10 mM Tris-HCl, 1,5 mM NaCl, (pH7,5)	None	Silva-Lopez et al; 2005
Bacitracin	Trichomonas vaginalis	60 and 30 kDa	Agarose	20 mM NaOAc, (pH 4.0)	20 mM NaOAc, (pH 4.0)	0.1 M Tris-HCl, 1.0 M NaCl 25% 2- propanol. (nH 7 0)	Bio-Gel P-60	Sommer <i>et al;</i> 2005
Aprotinin	Leishmania braziliensis	60 and45 kDa	Agarose	10 mM Tris-HCl, 5 mM CaCl ₂ , (pH7,5)	10 mM Tris-HCl, 5 mM CaCl ₂ , (pH7,5)	10 mM Tris-HCl, 1,5 mM NaCl, (pH7,5)	None	Guedes HL <i>et al</i> ; 2007
Aprotinin	Trypanosoma cruzi	75 kDa	Agarose	10 mM Tris-HCl, 5 mM CaCl ₂ , (pH7,5)	10 mM Tris-HCl, 5 mM CaCl ₂ , (pH7,5)	10 mM Tris-HCl, 1,5 mM NaCl, (pH7,5)	None	Silva-Lopez <i>et al;</i> 2008
Aprotinin	Leishmania donovani	115 kDa	Agarose	10 mM Tris-HCl, 5 mM CaCl ₂ , (pH 7.5)	10 mM Tris-HCl, 5 mM CaCl ₂ , (pH7,5)	10 mM Tris-HCl, 1.5 M NaCl, (pH7,5)	None	Choudhury <i>et al;</i> 2009
Pepstatin A	Trypanosoma cruzi	120, 48 and 56 kDa	Agarose	0.1 M NaOAc, 1.0 M NaCl, (pH 3.5)	0.1 M NaOAc, 1.0 M NaCl, (pH 3.5)	0.1 M Tris-HCl, 1.0 M NaCl, (pH 8.6)	HPLC gel filtration	Pinho <i>et al</i> ; 2009
Aprotinin	Leishmania donovani	58 kDa	Agarose	10 mM Tris-HCl, 5 mM CaCl ₂ , (pH7,4)	10 mM Tris-HCl, 5 mM CaCl ₂ , (pH7,4)	10 mM Tris-HCl, 1 M NaCl, (pH7,5)	DEAE	Choudhury et al; 2010
Pepstatin A Benzamidine	Rhipicephalus (B.) microplus Dlasmodium	42 kDa 37 kDa	Agarose	20 mM NaOAc, 1 M NaCl, (pH 5.3) 0.1% Triton X-100	20 mM NaOAc, 1 M NaCl, (pH 5.3) 0.1% Triton X-100 in	100 mM Tris-HCl, 1 M NaCl, (pH 8.6)	Mono-Q	Cruz et al; 2010 1 i et al: 2010
	ookinete		Jephilatuse	in PBS	PBS			отот <i>и</i> , тото
Aprotun	Letshmanta chagasi	LCSII (105, 66, 60 kDa); LCSI (60, 58 kDa) and LCSIII (76, 68 kDa)	Agarose	10 mM Tris-HCI, 5 mM CaCl ₂ , (pH7,5)	10 mM 1ris-HCl, 5 mM CaCl ₂ , (pH7,5)	10 mM 1rrs-HCl, 1,5 mM NaCl, (pH7,5)		Silva-Lopez <i>et al;</i> 2010
E-64	Trypanosoma cruzi	60 kDa	Sepharose	20 mM Tris-HCl, 150 mM NaCl, 100 M PMSF, (pH 7.4)	20 mM Tris-HCl, 150 mM NaCl, 100 M PMSF, (pH 7.4)	20 mM Tris-HCl, 150 mM NaCl, 100 M PMSF, 2.0 M NaCl, (pH 7.4)	None	Bourguignon <i>et</i> <i>al</i> 2011

Table 2. (continued)

Aprotinin and pepstatin A are examples of inhibitors that are frequently used in the isolation of serine- and aspartic acid proteases, respectively, from many parasite species (Bond & Beynon). Other inhibitors that have been previously described in the isolation of serine proteases include soybean trypsin inhibitor (SBTI) and chloromethylketone (CMK). As for the purification of cysteine proteases, the use of three other inhibitors has been reported: L-transepoxysuccinyl-leucylamido-[4-guanidino]butane (E-64), bacitracin and glycyl-phenylalanyl-glycyl-semicarbazone (Table 2). It must be emphasized that these inhibitors cannot be used to isolate all of the proteases classes from parasites, as they present distinct affinities for members of different groups and families within these enzyme classes. Therefore, investigation of the possible variations present in the active site of these enzymes may prove useful.

The features of the buffer (temperature, pH and ionic strength) to be used may vary according to the ligand's physicochemical characteristics, the chemical environment of the parasite enzyme and the analyzed species of parasite. For example, distinct buffers were used for the purification of serine proteases from *S. mansoni* and *Trichinella spiralis* using benzamidine. It is also noteworthy that for each organism, a different matrix was used to immobilize the inhibitor, sepharose for *S. mansoni* and celite for *T. spiralis*. The use of distinct buffers in studies that are based on the same inhibitor is also noted in reports of SBTI, E-64, bacitracin and glycyl-phenyalanyl-glycyl-semicarbazone, all of which are cysteine protease inhibitors.

Affinity chromatography with an irreversible inhibitor has also been described previously; the cysteine-protease inhibitor is an example of this strong binding. In the interaction between E-64 and cysteine-protease, a covalent bond is established (Matsumoto, 1989). Therefore, a reaction between the epoxy groups of the inhibitor and the thiopropyl group of the sepharose matrix is necessary to bind E-64 to a solid support. This reaction prevents the reaction of E-64 with the cysteine residue at the protease catalytic center. However, this does not affect the bond between the inhibitor and cysteine-protease; instead, it only results in inhibition of the proteolytic activity (Govrin, 1999).

2.2.1 Parasite proteins isolated by cysteine-protease inhibitors affinity chromatography

There is only one published example of the use of E-64 affinity chromatography to assess cysteine-protease isolated from a parasite, and this study was conducted with the *T. cruzi* epimastigote. In this study, chromatography was useful for assessing the effects of β -Lapachone naphthoquinones on a 60 kDa cysteine-protease activity present in *T. cruzi*. The results demonstrated the potential of this protease inhibitor as a new antichagasic compound (Bourguignon et al., 2011). Another example of a cysteine-protease isolated by inhibitor affinity chromatography in parasites was described for *Plasmodium falciparum*. In this case, a glycyl-phenyalanyl-glycyl-semicarbazone-based column was used to isolate a protease with a molecular weight of 27 kDa, as determined by SDS-PAGE (Shenai et al, 2000).

2.2.2 Parasite proteins isolated by serine-protease inhibitors affinity chromatography

Aprotinin affinity-based chromatography was useful for the isolation of a serine-protease of 115 kDa (Silva-Lopez et al., 2005), a 68 kDa (Morgado- Diaz et al., 2004; Silva-Lopez et al., 2004) and a 56kDa (Silva-Lopez et al., 2004) from *L*.(*L*.) amazonensis compared to other

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purification procedures that were used to isolate parasite serine peptidase enzymes (Kong et al., 2000; Ribeiro de Andrade et al., 1998). In *Leishmania (V) braziliensis* promastigotes, 60 kDa and 45 kDa enzymes were purified using the aprotinin affinity-based and activity esterase assessed against N-alpha-benzoyl-L-arginine ethyl ester hydrochloride and Nalpha-p-tosyl-L-arginine methyl ester hydrochloride (Guedes et al., 2007). Furthermore, three protein profiles were isolated from *Leishmania chagasi* promastigotes, including LCSI (58 and 60 kDa), LCSII (60, 66, 105 and kDa) and LCSIII (68 and 76 kDa), which were characterized as serine-protease enzymes based on their activity toward α-N-ρ-tosyl-L-arginine methyl ester substrate (Silva-Lopez et al., 2010). Furthermore, serine proteases with molecular weights of 75 kDa (Silva-Lopez et al., 2008) and 115 kDa (Choudhury et al., 2009) were identified as excretory products of *T. cruzi* and components of the sub-cellular environment in *Leishmania donovani*, respectively, although the chromatography step was not able to produce a homogeneous fraction. Furthermore, a intracellular serine protease of 58 kDa was were purified from *Leishmania donovani* (Choudhury et al., 2010).

In addition, the aprotinin affinity-based chromatography was useful for the isolation of serine-proteases of 35 kDa and 26 kDa from *Anisakis simpZex* (Morris et al, 1994), 43 kDa from *Candida albicans* (Morrison et al, 1993), 15 kDa from *Schistosoma mansoni* (Salter et al, 2000), 42 kDa from *Rhipicephalus* (B.) *microplus* (Cruz et al, 2010), 60 kDa and 30 kDa from *Trichomonas vaginalis* (Sommer *et al*; 2005) and 35 to 52 from *Caenorhabditis elegans* (Geier et al; 1999).

Benzamidine-celite was applied in the isolation of serine proteases among the excreted or secreted proteins of *T. spirali*. The recovered proteases were not purified to homogeneity, and they showed molecular masses of 18 kDa, 40 kDa and 50 kDa (Todorova & Stoyanov). A similar finding was reported for the serine-proteases of *Chrysomya bezziana* larvae by using an SBTI-based column to purify four proteins with molecular masses of 13 kDa, 16 kDa, 26 kDa and 28 kDa (Muharsini et al., 2000).

Because it is possible to isolate heterogeneous products using inhibitors for affinity-based chromatography, we assessed a serine-protease from the third (L3) instar larvae of *D. hominis*. This ectoparasite causes dermatobiose in vertebrates, including humans, and it is particularly relevant in cattle, where it can cause a drop in production of meat and milk, leather as well depreciation (Maia & Guimarães, 1985).

Due to the association of DEAE-Sephacel with aprotinin agarose, it was possible to assess a serine protease from L3 larvae (Fig. 3). The fractions obtained by ion change chromatography containing estearasic activity were pooled and then fractionated on an aprotinin-agarose column. This fraction showed a profile with multiple bands by SDS-PAGE and silver staining, and only one band of enzyme activity (50 kDa) was detected by gelatin-SDS-PAGE at pH 7.5 (Fig. 3). Interestingly, this band of 50 kDa was not initially detected in the extracts from L3 by gelatin-SDS-PAGE. The expression of this enzyme is likely low in these larvae, and it can only be detected after concentration by chromatographic methods. The proposed strategy to isolate a serine protease allowed for the detection of a band of 50 kDa in extracts of L3 larvae, and this band had not been previously detected in the direct analysis of the total extract by gelatin-SDS-PAGE. Additionally, this fraction was found to have esterase activity (data not shown).



Fig. 3. Electrophoresis of proteins from L3 instar larvae of *Dermatobia hominis* eluted from a column of aprotinin-agarose. A total of 20 µg of protein from each fraction was resolved by SDS-PAGE (A) and gelatin-SDS-PAGE (B) and the bands were detected by silver staining and negative coloration, respectively. The arrow indicates a serine protease of 50 kDa. The molecular mass markers are indicated (kDa). These results are representative of two independent assays

2.2.3 Parasite proteins isolated by aspartyl-protease inhibitors affinity chromatography

Affinity-based chromatography based on pepstatine A was used to isolate a 52 kDa aspartyl protease from *Neospora caninum* tachyzoites (Naguleswaran et al., 2005) and a 45 kDa enzyme from *S.mansoni* (Valdivieso et al., 2003). In *Trypanosoma cruzi* epimastigotes, two aspartyl proteases were isolated (cruzipsin-I and cruzipsin-II). The molecular mass was estimated to be 120kDa by high performance liquid chromatography gel filtration, and the activities of these enzymes were detected in a doublet of bands (56 kDa and 48 kDa). These findings demonstrate that both proteases are novel *T. cruzi* acidic proteases. The physiological function of these enzymes in *T. cruzi* is not completely defined (Pinho et al., 2009).

An aspartyl protease with molecular mass of 37 kDa (plasmepsin) was isolated from the surface of *Plasmodium ookinete*, and its sequence was determined by mass spectrometry (Li et al., 2010). This protease was purified by using a benzamidine affinity-based column, which is typically used for the isolation of serine proteases. Structural similarity between the active site residues of the serine- and aspartyl proteases is possible, as some hydrogen-bonded residues can are arranged without any strain, such as in the formation of an oxyanion hole, in a manner that resembles the active site of a serine protease (Andreeva et al., 2004)

2.2.4 Remarks on the isolation of proteins by protease inhibitors affinity chromatography

Although the studies that have been conducted to isolate parasite proteases are of great medical interest, no parasiticide drug has been proposed thus far. In general, the chromatography methods involving inhibitor-based affinity-capture have been useful only to describe these enzymes in parasites and to establish their biochemical properties, their functions and their application in drugs tests.

Furthermore, the heterogeneous material obtained from affinity-based chromatography may require additional procedures for purification of the enzyme. Thus, other techniques must be applied to obtain proteases with greater purity, including molecular exclusion and ion exchange chromatography.

2.3 Glycosaminoglycans affinity-based separation of parasite proteins

Microbes have developed different strategies to gain access into mammalian cells (Bermúdez et al., 2010; Caradonna & Burleigh 2011; Soong et al., 2011). The first step involves the recognition of molecules at the surface of the target cell, which triggers the activation of signaling pathways that are implicated in the parasite internalization (Abban & Meneses 2010; Epting et al., 2011). Host cell surface sulfated proteoglycans have been implicated as key molecules at the host cell-parasite interface, mediating the adhesion and invasion of numerous parasitic microorganisms (Jacquet et al., 2001; Kobayashi et al., 2010; O'Donnell & Shukla 2010).

2.3.1 Structure of glycosaminoglycans

Proteoglycans (PGs) are composed of core proteins that are covalently linked to glycosaminoglycan (GAG) chains. As components of the extracellular matrix, the structural diversity of PGs depends on the identity of the core protein and the GAG composition. GAGs are linear polysaccharides comprised of disaccharide repeats containing uronic acid and hexosamine. GAGs vary in the type of hexosamine, hexose or hexuronic acid unit. The sulfated GAGs are classified as heparin [2-O-sulfo-β-D-glucuronic acid (GlcUA-2S) or 2-Osulfo-a-L-iduronic acid (IdoUA-2S) and N-acetylglucosamine (GlcNAc) or Nsulfoglucosamine (GlcNS)], heparan sulfate [GlcUA, IdoUA or IdoUA-2S and GlcNAc or GlcNS], chondroitin sulfate [GlcUA and N-acetylgalactosamine (GalNAc)], dermatan sulfate [GlcUA or IdoUA and GalNAc] and keratan sulfate [galactose (Gal) and GlcNAc]. In fact, the structural diversity of PGs may provide sites of affinity for different ligands and, therefore, function as co-receptors or receptors for GAG-binding proteins (Dreyfuss et al., 2009; Ly et al., 2010). Although heparin is not found on the cell surface, this GAG has being

commonly used as tool for pathogen-host cell interaction assays. Heparins are negativally charged structures and native heparin presents molecular weights in the range of 5 to 30 KDa, whereas commercial heparin preparations are in the range of 12 kDa to 15 kDa.

2.3.2 Role of heparin-binding proteins in pathogen-host cell

Many pathogens express surface proteins that interact with GAGs in different stages of their life cycle. Although some parasites can bind to multiple GAGs (Coppi et al., 2007; Fallgren et al., 2001), heparan sulfate proteoglycan (HSPG) has been implicated in the recognition and/or invasion process of a wide range of pathogens, including viruses, bacteria and protozoan parasites (Bambino-Medeiros et al., 2011; Dalrymple & Mackow 2011; Yan et al., 2006;). Despite the role of heparin-binding proteins in many physiological and pathological processes, the basis of the heparin-protein interaction at the molecular level is still unclear.

Thus, efforts have been concentrated to enhance methods for the isolation and characterization of heparin-binding proteins, and, in parallel, to determine the role of this GAG in pathogen-host cell interaction. Currently, heparin affinity chromatography has been applied to the purification of GAG-binding proteins from different pathogens (Table 3). In these chromatography assays, the heparin is covalently coupled to agarose or sepharose beads and its sulfates and carboxylates chains are able to bind many proteins by basic amino acids (Fig. 2C).

This technique has been used to isolate heparin-binding proteins without loss of their biological activity, leading to a better understanding of the mechanism involved in the parasite invasion process. For example, chlamydial outer membrane complex (OmcB), a 60 kDa cysteine-rich protein, displays a protein motif (50-70OmcB peptide) that acts as an acceptor molecule to bind heparan sulfate (HS) and promote Chlamydia invasion in eukaryotic cells (Stephens et al., 2001). Attachment of Helicobacter pylori to gastric epithelial cells also involves HS recognition. Two major proteins, one with a molecular mass of 71.5 kDa and pI 5.0 (HSBP50) and the other with a molecular mass of 66.2 kDa and pI 5.4 (HSBP54), have been identified on the surface of bacterial cells that are able to bind HS. The amino acid sequences of these proteins (HSBP50 - VPERAVRAHT; HSBP54 VHLPADKTNV) are not homologous with bacterial adhesins or other HS-binding proteins (Ruzi-Bustos et al., 2001). Other proteins with the ability to bind heparin (66 and 60 kDa) have been identified in Staphylococcus aureus. The partial characterization of the amino acid sequences, which consist of DWTGWLAAA for the 66 kDa protein and MLVT for the 60 kDa protein, revealed no identity with HBPs from Chlamydia or Helicobacter pylori. HBPs from S. aureus have been demonstrated to be sensitive to heat and proteases, such as pronase E, proteinase K, pepsin and chymotrypsin (Liang et al., 1992). Interestingly, a 17kDa heparin-binding protein with pI 4.6 has also been isolated from S. epidermis and S. haemolyticus, but the amino acid sequence similarity is low between these two organisms (MXTAHSYTXKYNGYTAN and MATQTKGYYYSYNGYV, respectively) and other bacterial HBPs (Fallgren et al., 2001).

Trypanosomatidaes also exploit HS for successful parasite attachment to and/or invasion of the mammalian and vector hosts. The adhesion of Leishmania amastigotes to macrophages is mediated by HS, but not other sulfated polysaccharides (Love et al., 1993). Two heparin-

binding proteins, (65 and 54.5 kDa) from L. (V.) braziliensis promastigotes (HBP-Lb) recognize several molecules in the gut of Lutzomyia intermedia and Lutzomyia whitmani (Azevedo-Pereira et al., 2007). The biochemical characterization of these proteins revealed that only the 65-kDa HBP-Lb has metallo-proteinase activity, and this protein is primarily localized at the flagellar domain of the promastigotes. Surface plasmon resonance (spr) also demonstrated high-affinity binding at the flagellar domain, which forms a stable binding complex (Côrtes et al., 2011). In T. cruzi, HBPs also mediate parasite adhesion by recognition of PGHS on the surface of the target cells (Bambino-Medeiros et al., 2011; Calvet et al., 2003; Oliveira-Jr et al., 2008; Ortega-Barria & Pereira, 1991). Currently, three HBPs have been described in this parasite: a 60-kDa protein named penetrin (Ortega-Barria & Pereira, 1991) and two other proteins of 65.8 and 59 kDa that bind heparin, HS and chondroitin sulfate (CS). These proteins have been identified in both trypomastigotes and amastigotes (Oliveira-Jr et al., 2008). Interestingly, the HBP-HS binding is related to a specific region of the HS chain, the N-acetylated/N-sulfated HS domain, which promotes parasite attachment and invasion (Oliveira-Jr et al., 2008). Although only HS binding triggers T. cruzi invasion of mammalian cells (Ortega-Barria & Pereira, 1991; Calvet et al., 2003; Oliveira-Jr et al., 2008; Bambino-Medeiros et al., 2003), the multiple GAG recognition may provide an efficient association with other GAGs within the parasite life cycle. Recently, it has been demonstrated that sulfated proteoglycans are involved in the adhesion of epimastigotes to the luminal midgut epithelial cells of Rhodnius prolixus (Gonzalez et al., 2011).

2.3.3 Remarks on the isolation of proteins by glycosaminoglycans affinity chromatography

While the application of affinity chromatography has provided advances in our understanding of heparin-binding proteins, a large number of studies have focused on the parasite-host cell interface to improve our comprehension of the mechanisms that are activated by the receptor-ligand interaction (reviewed by Chen et al., 2008). The binding of Dengue virus to HS, for example, seems to result in the accumulation of virions at the surface of the human hepatoma cell line HuH-7 and elicit clathrin-dependent endocytosis (Hilgard & Stockert 2000). In addition to promote attachment and parasite invasion, HSPG also seems to be involved in the tropism of pathogen to specific tissues. The degree of HSPG sulfation guides the migration of Plasmodium sporozoites and the invasion of hepatocytes. Highly sulfated heparan sulfate at the surface of hepatocytes seems to regulate the proteolytic activity of the calcium-dependent protein kinase-6 on the CSP, which triggers the invasion of the parasite (Coppi et al., 2007).

Another interesting phenomenon is the release of syndecan-1, a transmembrane PGHS, as a mechanism of host defense inhibition. *Pseudomonas aeruginosa* induces syndecan-1 shedding through the enzymatic activity of LasA, leading to an enhancement of bacterial virulence (Park et al., 2001). A similar mechanism has been described for *Staphylococcus aureus* in which β -toxin, a secreted virulence factor, also induces syndecan-1 shedding by activating a metallo-proteinase involved in the host cell shedding mechanism, leading to enhancement of bacterial virulence due to the recruitment of inflammatory cells (Hayashida et al., 2009). Because heparan sulfate has been shown to be a receptor for a variety of pathogens, HS-binding polypeptides have been the subject of intense research and provide possibilities for drug intervention.

References		Ortega-	Barria and Pereira 1991	Liang et al., 1992		Ortega-	barrıa and Pereira 1991	Stephens et al., 2001	Ruzi-Bustos et al., 2001	Fallgren et al., 2001	Azevedo- Pereira et al., 2007	Oliveira-Jr et al., 2008		
Second	Elution	1.5M guanidine-HCl 2.0M None	potassium thiocyanate and NaCl gradient (0.25-3.0M)	NaCl gradient (0-1M), None followed by 2M NaCl in	200mM Tris-HCl pH 8.0	1.5M guanidine-HCl 2.0M None	potassium thiocyanate and NaCl gradient (0.25-3.0M)	2% SDS in PBS None	NaCl gradient (0-2M) None	NaCl gradient (0-2M) None	PBS containing 0.5% None glycerol, 0.5% Chaps and 2 2M NaCL DH7.2	NaCl gradient (0-1M) None		
First phase	Wash	PBS containing	0.05% Triton X- 100, pH7.2	PBS containing 100mM PMSF,	pH6.0	PBS containing	0.05% 1 riton X- 100, pH7.2	PBS	0.1M Sodium acetate, pH5.0	None	PBS containing, 0.5% glycerol and 2.0.5% Chaps, pH7.	PBS, pH 7.2		
	Bind	PBS, pH 7.2		PBS containing 100mM PMSF,	pH6.0	PBS, pH 7.2		50 mM DTT and 2% Triton X-100	0.05M sodium acetate pH5.0	PBS	PBS containing 0.5% glycerol and 0.5% Chaps, pH7.2	PBS and 0.5% glycerol, pH 7.2		
	Matrix	Sepharose		Sepharose		Sepharose		Agarose	Sepharose	Sepharose	Sepharose	Sepharose		
Isolated proteins		Penetrin - 60 kDa		<i>S. aureus</i> -HBP – 66 kDa and 60 kDa		Penetrin - 60 kDa		Outer membrane complex (COMC) - 60 kDa	Outer membrane protein (OMP) - 71.5 kDa and 66.2 kDa	HBP - 17 kDa	HBP-Lb - 65 kDa and 54.5 kDa	HBP-Tc - 65.8 kDa and 59 kDa		
Organism		Trypanosoma cruzi		Staphylococcus aureus		Trypanosoma cruzi		Chlamydial	Helicobacter pylori	Staphyloccocal	Leishmania (V.) braziliensis	Trypanosoma cruzi		



3. Conclusion

The chromatographic procedures described here maintain the minimal amount of native folding necessary for proteins to retain their biological and biochemical activities. Thus, the materials used as supports for packed affinity columns, including agarose, sepharose and celite (from diatomaceous earth), to immobilize ligands, such as lectins, protease inhibitors and glycosaminoglycans, do not interfere with the functional properties of these proteins.

Furthermore, proteins obtained by affinity-based procedure have been useful in understanding the biological processes related to the life cycles of parasites and in the interaction with hosts. These studies are essential to developing strategies, such as the use of vaccines and drugs, to control the parasite diseases.

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Most will agree that one major achievement in the bio-separation techniques is affinity chromatography. This coined terminology covers a myriad of separation approaches that relies mainly on reversible adsorption of biomolecules through biospecific interactions on the ligand. Within this book, the authors tried to deliver for you simplified fundamentals of affinity chromatography together with exemplarily applications of this versatile technique. We have always been endeavor to keep the contents of the book crisp and easily comprehensive, hoping that this book will receive an overwhelming interest, deliver benefits and valuable information to the readers.

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