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Affinity Chromatography of Lectins

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1. Introduction

Affinity chromatography is a technique used to purify compounds, such as proteins, that have the ability to non-covalently and reversibly bind specific molecules, known as ligands. This method differs from the classical chromatography techniques in that the protein is purified on the basis of a unique biochemical property. In affinity chromatography, the ligand is covalently attached to a matrix, which must be chemically inert, porous, and have a variety of functional groups suitable for coupling with diverse ligands. Various matrices and ligands are used in affinity chromatography, depending on the protein to be purified (Voet & Voet, 1995). A particular affinity chromatography technique, which uses carbohydrate adsorbents (ligands or matrices) for purification of glycan-binding proteins or lectins, is called carbohydrate affinity chromatography.

Lectins are a diverse group of proteins that bind specifically various carbohydrates. They are present in every organism, suggesting their role in basic biological functions, including regulatory, adhesive, defence against pathogens, and many others (Varki et al., 2009). In an organism, a number of different lectins are usually present in various isoforms, called isolectins (Van Damme et al., 1998). These differ only in slight variations in primary structure and carbohydrate-binding specificity, but can show differences in their biological activities (Leavitt et al., 1977). Therefore, when isolating a lectin it is important to purify the individual isolectins.

Different types of lectin are distinguished according to their overall structure – merolectins contain a single carbohydrate-binding domain, hololectins are composed of two or more such domains and chimerolectins contain additional, non-lectin domains, usually catalytic in function (Peumans & Van Damme, 1995). The majority of lectins contain multiple binding sites, and can thus, by binding and cross-linking of specific glycoreceptors on cell surfaces, agglutinate cells such as erythrocytes. Lectins also act as recognition molecules in cell–molecule and cell–cell interactions, and are involved in cellular processes, including cell adhesion, migration, differentiation, proliferation and apoptosis. Furthermore, many lectins elicit diverse physiological responses in various organisms and possess immunomodulatory properties and thus have potential roles in cancer and metastasis (Perillo et al., 1998; Sharon & Lis, 1989), which makes them potentially useful in biotechnological and biomedical applications.

The binding of lectins to carbohydrates is noncovalent and reversible, involving hydrogen bonds, hydrophobic, electrostatic and van der Waals interactions and dipole attraction. The

binding of sugars (mono- and disaccharides) to a lectin is relatively weak, with dissociation constants in the millimolar or micromolar range. On the other hand, interactions of multivalent lectins with complex, branched carbohydrates containing multiple epitopes, result in high-avidity binding with nanomolar or even picomolar dissociation constants (Varki et al., 2009).

In general, lectins possess biochemical and binding properties which are very convenient for their purification by carbohydrate affinity chromatography. They do not react catalytically with carbohydrates, modifying the ligand, unless the lectin is a subdomain of a modular protein that contains another catalytic (glycosidase) domain. As noted earlier, lectins bind carbohydrates noncovalently and reversibly and at least the most widely used ligands, mono- and disaccharides, are usually bound relatively weakly, so that the lectin is readily released from an affinity column by competitive elution using specific free carbohydrates. Moreover, lectins and carbohydrates are both usually stable compounds, therefore elution techniques using extreme conditions of pH and/or ionic strength can also be applied to release a lectin from the carbohydrate affinity column.

In this chapter, current carbohydrate affinity chromatography methods for purification of lectins exhibiting various carbohydrate-binding specificities are presented. The affinity ligands, matrices, ligand coupling methods and elution techniques used are described in detail. Throughout the chapter, all sugars are of the D-configuration, unless otherwise stated.

2. Lectin isolation using carbohydrate affinity chromatography

Any source of interest can be selected for isolation of lectins, from humans, animals, plants, fungi to microorganisms, including viruses. First, a lectin must be released from the source into solution, usually by liberating it from the cells. Several methods can be used, depending on the mechanical characteristics of the source tissue, such as homogenization by mechanical disruption or by lysis (Voet & Voet, 1995). Lectins are water-soluble proteins, therefore are extracted with aqueous buffers and then centrifuged and filtered if necessary to remove the cell debris. Extraction should be carried out at 4 °C to prevent denaturation and degradation, by proteolytic enzymes, of the lectin of interest. Moreover, addition of protease inhibitors to the extraction buffer is recommended if proteolytic activity is observed in the extract (Kvennefors et al., 2008; Matsumoto et al., 2011; Watanabe et al., 2007), although some lectins have been found to be resistant to proteolytic degradation (Pohleven et al., 2009). In addition, glycosidase inhibitors, such as glucosidase inhibitor 1-deoxynojirimycin (0.2 mM) and the mannosidase inhibitor 1-deoxymannojirimycin (0.2 mM), have also been added to the extract to prevent cleavage of immobilized carbohydrate ligands in the affinity column (Watanabe et al., 2007). Before the extract is applied to the carbohydrate affinity column, some of the impurities can be removed using fractional precipitation with ammonium sulphate and/or other classical chromatography techniques. To follow the lectin fractions throughout the purification procedure, agglutinating activity can be monitored using erythrocytes specific for the lectin; a given lectin does not agglutinate all types of red blood cells, only those exhibiting the lectin-specific glycans.

Once the extract exhibiting agglutinating activity is prepared, the carbohydrate affinity chromatography method appropriate for purifying the lectin has to be selected. This involves the adsorbent that can bind the lectin and separate it from the extract, and an elution technique

that can release the bound lectin from the column. It is therefore important to know the specificity and stability of the lectin and also to consider the stability of the adsorbent, since extremes of pH and temperature may be used for ligand coupling and elution. Carbohydrates and, in general, lectins are relatively stable molecules. The specificity of the desired lectin in the extract can be determined by examining its agglutinating activity, using various classes of erythrocytes that expose diverse glycans on their surfaces. For example human blood group A expresses non-reducing terminal α-*N*-acetylgalactosamine, group B, terminal α-galactose and group O, fucosyl-galactose (Schenkel-Brunner, 2007). In addition, hapten inhibition of agglutination, using various carbohydrates or glycoproteins, can be used to determine the specificity of the lectin, enabling the lectin-specific adsorbent to be selected. The stability of the lectin can be assessed by examining agglutinating activity of the extract, pretreated under various conditions of pH. However, observations suggest that the lectin in an extract is more stable than the isolated one, probably because it is stabilized by bound carbohydrates.

A wide variety of adsorbents have been described for lectin isolation and can be divided into the following groups: carbohydrate (mono-, di, and polysaccharide) ligands and glycoprotein ligands, both immobilized to the carrier matrix, and adsorbent (polysaccharide) matrices (Sections 3 and 4). In the latter case, no ligand is required, since the polysaccharide matrices themselves bind lectins, while in the former cases, ligand is coupled to a previously activated matrix. Depending on the ligand, one of several methods can be applied to activate the matrix with various reagents and subsequently immobilize carbohydrate or glycoprotein ligands. After the coupling, the amount of carbohydrate or glycoprotein ligand is determined (Section 5).

After the extract containing the lectin has been applied to the carbohydrate affinity adsorbent and appropriately incubated (for example for several hours at 4 °C or 1 hour at room temperature), the impurities are washed off the column and the bound lectin then eluted. This is usually performed using gravity-flow column chromatography or fast protein liquid chromatography (FPLC). Depending on the specificity and stability of the lectin and adsorbent, various elution techniques can be used, such as competitive elution with specific carbohydrates, desorption by changing the pH and/or ionic strength of the eluent, or elution with urea solutions. When extremes of pH are used for elution, the protein fractions and column have to be neutralized by buffer as soon as possible (Section 6). The eluted protein fractions can be detected by measuring their absorbance at 280 nm and the affinity column capacity readily estimated by following the agglutinating activity of the flow-through.

Carbohydrate affinity chromatography is a simple, one-step method for purifying lectins. However, in some cases additional separation techniques have to be used subsequently to purify the lectin to homogeneity, such as ion-exchange chromatography and/or gel filtration. For example, isolectins cannot be separated by carbohydrate affinity chromatography, since they show very similar carbohydrate-binding specificities; ion-exchange chromatography has been used successfully to separate individual isolectins (Guzmán-Partida et al., 2004; Horibe et al., 2010; Leavitt et al., 1977; Mishra et al., 2004; Ren et al., 2008; Sultan et al., 2009). Moreover, different lectins can sometimes be co-isolated from the extract, especially when the ligand is immobilized to polysaccharide matrices, which also bind lectins. In that case, serial carbohydrate affinity chromatography can be applied using two or more adsorbents in series (Chen et al., 1999; Kato et al., 2011; Lavanya Latha et al., 2006; Moreira et al., 1998; Ooi et al., 2002; Pohleven et al., 2011; Trindade et al., 2006).

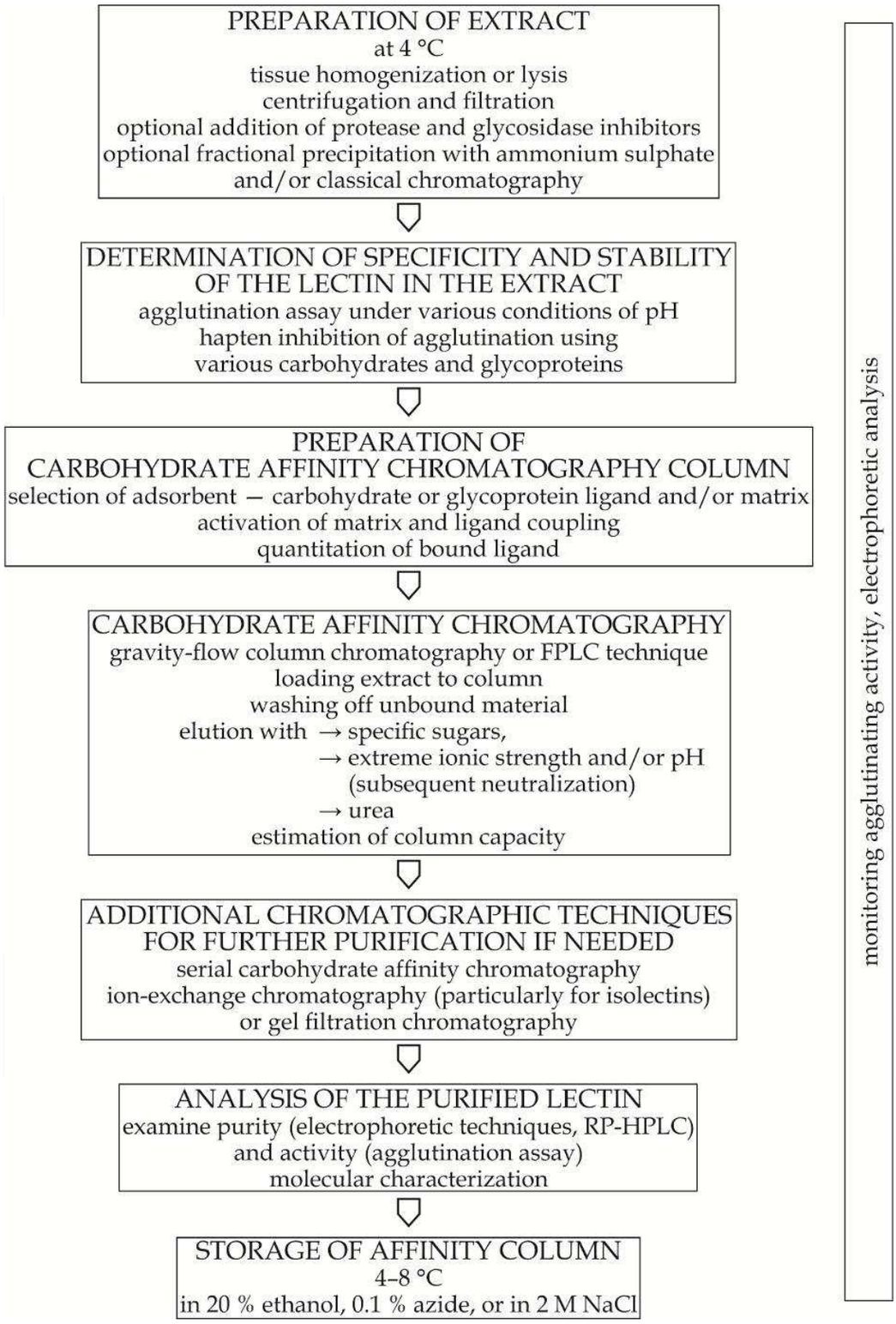

Fig. 1. Protocol for separation and purification of lectins using carbohydrate affinity chromatography

For example, the extract is applied to the affinity column that binds, besides the lectin of interest, non-desired lectins. Eluted lectins are then separated on a column that only binds non-desired lectins, and the lectin of interest is purified by simply washing the column.

Whatever the purification method used, the protein fractions are analyzed for purity using biochemical methods, such as electrophoretic techniques or reversed-phase high-performance liquid chromatography (RP-HPLC), and lectin activity is assessed by agglutination assay. After use, affinity chromatography columns should be stored at 4–8 °C, in the presence of a bacteriostat e.g. 20 % ethanol or 0.1 % azide (w/v) or in 2 M NaCl. The protocol for lectin purification using carbohydrate affinity chromatography is presented in Figure 1.

3. Ligands

In carbohydrate affinity chromatography, mono-, di-, and polysaccharides, as well as glycoproteins, can be used as matrix-immobilized ligands.

3.1 Mono- and disaccharides

Of all the ligands, mono- and disaccharides coupled to a Sepharose matrix are the most widely used adsorbents. Recently, isolation of lectins from various sources has been described using numerous sugars, such as lactose (Almanza et al., 2004; Braga et al., 2006; Fujii et al., 2011; Hamako et al., 2007; Kawsar et al., 2009; Mendonça-Franqueiro et al., 2011; Naeem et al., 2007b; Pohleven et al., 2009; Rocha et al., 2009; Silva et al., 2007), sucrose and glucose (Pohleven et al., 2011), galactose (Konami et al., 1991; Lavanya Latha et al., 2006; Mo et al., 2000; Moreira et al., 1998; Naeem et al., 2007b; Nagata, 2005), mannose (Andon et al., 2003; Ooi et al., 2010; Suseelan et al., 2002), fucose (Cammarata et al., 2007; Mansour & Abdul-Salam, 2009), L-rhamnose (Jimbo et al., 2007; Watanabe et al., 2008), and sugars with 2′-acetamido groups *N*-acetylgalactosamine (Gerlach et al., 2005; Perçin et al., 2009; Qureshi et al., 2006), *N*-acetylglucosamine (Kaur et al., 2005; Kim et al., 2006; Maheswari et al., 2002; Wang & Ng, 2003), and *N,N*′-diacetylchitobiose (Konami et al., 1991; Koyama et al., 2002).

Direct coupling of small ligand molecules, such as mono- and disaccharides, to the matrix can often result in binding of the lectin to sugar ligands being prevented, due to steric hindrance by the matrix. Spacer groups can minimize such interference and, furthermore, enable a variety of functional groups to be introduced, allowing the use of alternative techniques for ligand coupling to the matrix. A spacer group can be attached to the carrier (Section 4.2), or alternatively, sugar derivatives can be synthesized by attaching spacers, such as 6-amino-hexyl and ε-aminocaproyl groups (Lis & Sharon, 1981). Several carbohydrate derivatives have recently been synthesized and utilized in affinity chromatography for lectin isolations, such as the Galβ1–4Fuc derivative containing a hydrophilic spacer modified with a free amino group (Takeuchi et al., 2011), an iodoacetamidyl derivative of $Glc_1Man_9GlcNAc_2$ (Watanabe et al., 2007), and a derivative of L-fucose with a BSA spacer (Argayosa & Lee, 2009). In addition, a novel method for covalent immobilization of a range of carbohydrate derivatives onto polymeric resin beads has been described (Chen et al., 2007).

3.2 Polysaccharides

Recently, several polysaccharides have been immobilized to various matrices in order to isolate lectins. These include chitin, a polymer of a *N*-acetylglucosamine (Bovi et al., 2011; Narahari & Swamy, 2010; Santi-Gadelha et al., 2006; Trindade et al., 2006), mannan, a mannose polymer (Argayosa et al., 2011; Kvennefors et al., 2008; Naeem et al., 2007a; Ourth et al., 2005), alginate, a copolymer of β-mannuronate and α-L-guluronate (Roy et al., 2005), chitosan, a polysaccharide composed of glucosamine and *N*-acetylglucosamine (Chen & Xu, 2005), glucan laminarin (Chen et al., 1999) and exopolysaccharide extracted from *Azospirillum brasiliense* (Mora et al., 2008).

3.3 Glycoproteins

Carbohydrate moieties of glycoproteins, called glycans, can also be used as ligands for lectin isolation. Usually, glycans contain a variety of carbohydrates and therefore can be used for the isolation of lectins with various specificities (Lis & Sharon, 1981). Recently, numerous glycoproteins have been immobilized, usually to cyanogen bromide activated Sepharose. These include fetuin (Bhowal et al., 2005; Guzmán-Partida et al., 2004; Matsumoto et al., 2011; Naeem et al., 2007b; Ooi et al., 2002; Rittidach et al., 2007; Sun et al., 2007; Valadez-Vega et al., 2011; Yang et al., 2007), mucin from porcine (*Sus scrofa*) stomach (Chumkhunthod et al., 2006; Souza et al., 2010; Takahashi et al., 2008) and bovine (Bos taurus) submaxillary mucin (Imamichi & Yokoyama, 2010; Kawagishi et al., 1994; Naganuma et al., 2006), thyroglobulin (Ren et al., 2008; Wang et al., 2004) and ovalbumin (Mo et al., 1999). Besides, desialylated glycoproteins treated with mild acid hydrolysis or neuraminidases, which removes sialic acid, thus exposing other saccharides, have been used as ligands, such as asialofetuin (Bhat et al., 2010; Kaur et al., 2006; Naeem et al., 2007b; Nagre et al., 2010; Shangary et al., 1995) and asialomucin (Vega & Pérez, 2006).

Another method used for lectin isolation, based on immobilized glycoprotein ligands, is affinity chromatography on agarose-immobilized porcine (*Sus scrofa*) blood plasma proteins (Kajiya et al., 2003) or on stroma (Alpuche et al., 2010; Dresch et al., 2008; Vazquez et al., 1993; Veau et al., 1999) or membranes (Castillo-Villanueva et al., 2007) of various erythrocytes, fixed with 1 % glutaraldehyde and physically entrapped in various matrices.

4. Matrices

In carbohydrate affinity chromatography, polysaccharide matrices can be used in two ways – as adsorbents for binding lectins, or as carriers to which ligands can be attached.

4.1 Adsorbent matrices

A number of matrices are polysaccharide-based, and can thus be used as adsorbents for isolation of lectins with appropriate specificities, without prior immobilization of carbohydrate ligands. The most widely used is Sepharose, but Sephadex is also used. Both are commercially available (GE Healthcare). Sepharose is an agarose-based, bead-formed matrix, containing 2, 4, or 6 % agarose, hence designated Sepharoses 2B, 4B, or 6B. Furthermore, Sepharose CL gels are cross-linked derivatives of Sepharose, and thus chemically and physically more resistant, i.e. stable over a wider pH range, and can be applied when extreme conditions are used for

coupling or elution. Agarose is a galactose-anhydrogalactose copolymer (Angal & Dean, 1977), so Sepharose has recently been used extensively as an adsorbent for isolation of lectins specific for galactosides and related carbohydrates (Cao et al., 2010; De-Simone et al., 2006; Kato et al., 2011; Moura et al., 2006; Pohleven et al., 2011). In contrast, Sephadex is a beaded gel prepared by cross-linking dextran, a polysaccharide composed of glucose. It has been used for purifying glucose/mannose-specific lectins from various sources (Biswas et al., 2009; Rangel et al., 2011; Roh & Park, 2005).

Many galactose-specific lectins recognize terminal non-reducing galactosyl residues, rather than internal ones, in galactan polysaccharides (Lis & Sharon, 1981). Therefore, partial, mild hydrolysis of Sepharose by acid treatment – for example incubation in 1 M HCl for three hours at 50 °C (Voss et al., 2006) – can be used to cleave the polysaccharide chains, thus exposing additional terminal galactosyl residues, without completely degrading the Sepharose (Ersson et al., 1973; Lis & Sharon, 1981). Recently, numerous lectins have been isolated on acid treated Sepharose (Jimbo et al., 2000; Kawagishi et al., 1994; Mishra et al., 2004; Stirpe et al., 2007; Voss et al., 2006).

In addition to Sepharose and Sephadex, commercially available Affi-Gel blue gel (Bio-Rad), composed of beaded, cross-linked agarose, has been used as an adsorbent matrix (Lin & Ng, 2008; Shao et al., 2011; Sharma et al., 2010; Wong et al., 2010). Further, naturally occurring cross-linked polysaccharides, which are inexpensive and simple to prepare with epichlorhydrin or divinyl sulphone, have also been extensively used as adsorbent matrices for isolating galactose-specific lectins (Lis & Sharon, 1981). These include guar gum or guaran (Sigma-Aldrich), a galactomannan polysaccharide that binds lectins with anomeric preference for α-galactose (Alencar et al., 2010; Santos et al., 2009; Souza et al., 2011; Sultan et al., 2009), a polysaccharide isolated from *Spondias purpurea* (Teixeira et al., 2007), a cross-linked seed gum matrix prepared from plant *Leucaena leucocephala* (Seshagirirao et al., 2005), a galactomannan from *Adenanthera pavonina* (Moreira et al., 1998; Teixeira-Sá et al., 2009; Trindade et al., 2006), and a cross-linked galactoxyloglucan from *Mucuna sloanei* (Teixeira-Sá et al., 2009). Moreover, adsorbent matrices from yeast glucan or curdlan, a polymer of glucose, were prepared to isolate glucose-specific lectins (Mikes & Man, 2003).

Since polysaccharide matrices like Sepharose and Sephadex bind lectins, when using such matrices as carriers for carbohydrate ligand immobilization a variety of lectins can be co-isolated; ones specific for the matrix and one for the ligand. To purify the latter lectin, additional methods, such as gel filtration or ion-exchange chromatography have to be used. Alternatively, serial carbohydrate affinity chromatography can be introduced, in which two or more affinity columns are used in tandem. For example, in the first step, the extract is applied to the affinity column (with matrix-immobilized ligand) which, besides the lectin of interest, also binds non-desired (matrix-specific) lectins. The eluted lectin mixture is then applied to a second column (with matrix alone) that only binds the non-desired lectins. The non-bound lectin of interest is washed off the column and thus purified. Several authors have purified lectins from a variety of sources using serial carbohydrate affinity chromatography (Chen et al., 1999; Kato et al., 2011; Lavanya Latha et al., 2006; Moreira et al., 1998; Ooi et al., 2002; Pohleven et al., 2011; Trindade et al., 2006) or Ultrogel-A4 agarose beads to remove non-specific agarose-binding proteins from the extract prior to subjecting it to carbohydrate affinity chromatography (Kvennefors et al., 2008). In contrast, non-polysaccharide matrices, such as silica carriers (Synsorb) (Kaur et al., 2006; Mo et al., 2000;

Naeem et al., 2007b; Shangary et al., 1995), poly(2-hydroxyethyl methacrylate) matrix of Spheron 300 (Nahálková et al., 2001), fractogel affinity matrix (Guzmán-Partida et al., 2004) or polyacrylamide beads (Dresch et al., 2008; Hořejší & Kocourek, 1978; Veau et al., 1999), can be used to avoid non-specific binding of lectins to the polysaccharide matrix.

4.2 Carrier matrices

Carrier matrices are used as supports to which various carbohydrate or glycoprotein ligands are coupled. These matrices have to be previously activated for the covalent coupling to take place. Various matrices are commercially available (Sepharose, Mini-leak agarose, Toyopearl, Synsorb, Seralose, and Spheron besides others), some of them being pre-activated and others already coupled with carbohydrate ligands.

Numerous matrices, to which diverse ligands were immobilized by various coupling methods, have been used recently to purify lectins. The most widely used have been differently activated Sepharoses – divinyl sulphone (Almanza et al., 2004; Lavanya Latha et al., 2006; Pohleven et al., 2011) and epoxy (epichlorohydrin) activated Sepharose have been used to immobilize carbohydrates (Chen et al., 1999; Kaur et al., 2005; Maheswari et al., 2002; Mora et al., 2008; Nagata, 2005), and cyanogen bromide activated Sepharose for glycoproteins (Bhat et al., 2010; Bhowal et al., 2005; Kajiya et al., 2003; Mo et al., 1999; Naganuma et al., 2006; Nagre et al., 2010; Vega & Pérez, 2006; Yang et al., 2007). *N*-hydroxysuccinimide Sepharose was used for coupling with sugar derivatives containing amino groups (Takeuchi et al., 2011), and thiopropyl Sepharose for immobilizing iodoacetamidyl glycan derivatives (Watanabe et al., 2007). Mini-Leak agarose (Kem-En-Tec), a divinyl sulphone activated matrix (Valadez-Vega et al., 2011), and Seralose (Konozy et al., 2002) have also been used. Moreover, several kinds of Toyopearl affinity resins (Tosoh) exist that use various coupling chemistries for the attachment of ligands to formyl, carboxy, amino, epoxy or tresyl groups. For lectin isolation, Toyopearl AF-Amino 650 was used to immobilize glycoproteins (Imamichi & Yokoyama, 2010; Kawagishi et al., 1994; Takahashi et al., 2008).

Commercially available agarose matrices pre-coupled with carbohydrates or glycoproteins have also been used, such as agarose beads with immobilized lactose (EY Laboratories) (Hamako et al., 2007), galactose (Pierce) (Trindade et al., 2006), mannose (Sigma-Aldrich) (Ooi et al., 2002; Suseelan et al., 2002), *N*-acetylgalactosamine (Gerlach et al., 2005; Qureshi et al., 2006), *N*-acetylglucosamine (Sigma-Aldrich) (Kim et al., 2006; Wang & Ng, 2003), *N,N'*-diacetylchitobiose (Sigma-Aldrich) (Koyama et al., 2002), mannan (Sigma-Aldrich) (Naeem et al., 2007a; Ourth et al., 2005), and fetuin (Sigma-Aldrich) (Ooi et al., 2002; Rittidach et al., 2007).

In contrast, non-polysaccharide-based matrices have also been used, such as silica carrier Synsorb with immobilized galactose (Mo et al., 2000; Naeem et al., 2007b), amino activated silica beads with immobilized asialofetuin (Clifmar) (Kaur et al., 2006; Shangary et al., 1995), 2-acetamido-2-deoxy-β-glucopyranoside-glycosylated Spheron 300 (Nahálková et al., 2001), azlactone-activated fractogel coupled with fetuin (Guzmán-Partida et al., 2004), polyacrylamide gel with immobilized carbohydrates (Hořejší & Kocourek, 1978), poly(hydroxypropyl methacrylate-glycidyl methacrylate) beads with *N*-acetylgalactosamine

attached through epoxy groups of glycidyl methacrylate (Perçin et al., 2009) and monolithic columns with various immobilized sugars (Kato et al., 2011; Tetala et al., 2007) and the polysaccharide mannan (Bedair & El Rassi, 2004).

For immobilization of small carbohydrate ligands, such as mono- or disaccharides, to carrier matrices, it is necessary to use spacer arms to separate the ligand from the support thus making immobilized small ligand molecules more accessible to the lectin binding. In addition, spacers can be used to introduce a variety of functional groups, which allows the use of alternative coupling techniques (Lis & Sharon, 1981). A spacer group can be attached either to the sugar derivative ligand (Section 3.1) or to the carrier matrix and then carbohydrate ligand is coupled to the immobilized spacer to obtain affinity column for lectin purification (Lis & Sharon, 1981). In addition, activated matrices, such as divinyl sulphone activated, epoxy activated, *N*-hydroxysuccinimide activated Sepharose, CH Sepharose, and Thiopropyl Sepharose, already contain spacer arms and are prepared for ligand coupling.

5. Coupling methods

There are various methods for covalently attaching a carbohydrate or glycoprotein ligand to a carrier matrix. First, the matrix is activated, which can be done in several ways using various reagents and conditions. Commercially available pre-activated matrices are also available. During the activation, spacer arms of various lengths can be introduced to the matrix. Ligands are then covalently coupled via their functional groups to differently activated matrices, resulting in links with different stabilities (Pepper, 1992). After coupling, the amount of carbohydrate ligand coupled to the beads can be determined by the phenol-sulphuric acid method (Dubois et al., 1956) or the 3,5-dinitrosalicylic acid method (Bailey et al., 1992). On the other hand, glycoprotein ligands can be determined using protein quantitation methods, such as the Bradford (Bradford, 1976) and BCA protein assay (Pierce) methods.

5.1 The divinyl sulphone method

This method of activation is appropriate for a wide variety of polymer matrices, such as Sepharose, Sephadex, Sephacryl, Fractogel, Ultrogel, cellulose, and dextran. Commercially available divinyl sulphone activated agarose can also be obtained. Such matrices are suitable for coupling with carbohydrate and (glyco)protein ligands via their hydroxyl or amino groups. With this method, a highly reactive vinyl group is introduced to the matrix simultaneously with a spacer. The activation using divinyl sulphone is fairly rapid (70 minutes at room temperature; (Levi & Teichberg, 1981)) and the activated material is stable for up to 12 months in aqueous suspension at 4 °C. The resulting vinyl groups are highly reactive with hydroxylic compounds, therefore coupling takes place at lower pH and temperatures than with other methods. The coupling with carbohydrates takes 15 hours at pH 10-11, at room temperature in the dark (Levi & Teichberg, 1981) while for proteins, at even lower pH values in the range of 6.5-10. However, the link formed is unstable in alkaline solutions (Lis & Sharon, 1981; Pepper, 1992), so acidic elution of bound lectins is advised. The method has recently been used for coupling sugars to Sepharose in order to isolate lectins from various sources (Almanza et al., 2004; Lavanya Latha et al., 2006; Pohleven et al., 2011).

5.2 The epoxide method

This method is widely used for coupling hydroxyl groups of carbohydrates to epoxy activated matrices. In addition, amino and sulfhydryl groups of ligands can also be used for (glyco)protein immobilization (Murthy & Moudgal, 1986). The activation of the matrix provides an active epoxy group for coupling and a flexible, extended (12-atom) hydrophilic spacer arm. However, these reactions require high pH values (12-13) and temperatures above 40 °C (Lis & Sharon, 1981); the reaction is also slow. The aggressiveness of the method does not allow the use of silica or glass beads that are destroyed under such conditions (Pepper, 1992). On the other hand, increasing ionic strength facilitates the ligand immobilization, therefore very high salt concentrations allow coupling at pHs as low as 8.5 (Murthy & Moudgal, 1986). The resulting ether bond is stable, which ensures low leakage of ligands from the column (Lis & Sharon, 1981; Pepper, 1992). This method has been used for the isolations of lectins by employing epichlorohydrin for carbohydrate coupling to Sepharose (Chen et al., 1999; Kaur et al., 2005; Maheswari et al., 2002; Mora et al., 2008; Nagata, 2005) or for cross-linking polysaccharides to prepare an adsorbent matrix (Teixeira et al., 2007). Commercially available matrices, such as epoxy activated Sepharose (GE Healthcare) can be obtained.

5.3 The cyanogen bromide method

A cyanogen bromide activated matrix can be used for immobilizing ligands containing an amino group. The coupling reaction is rapid (2 hours at room temperature) and takes place at pH values between 8 and 10, most frequently at pH 8.3 (Pepper, 1992), however coupling at pH 7.4 was also reported (Kajiya et al., 2003). In carbohydrate affinity chromatography, this method is often used to immobilize glycoproteins, since carbohydrates rarely contain amino groups (galactosamine, glucosamine). The method is not suitable for coupling small ligands, since cyanogen bromide activation does not introduce a spacer. Alternatively, sugar derivatives containing spacers with amino groups can be prepared and immobilized using this method (Lis & Sharon, 1981). For lectin isolation, numerous glycoproteins have been immobilized to cyanogen bromide activated Sepharose (Bhat et al., 2010; Bhowal et al., 2005; Kajiya et al., 2003; Mo et al., 1999; Naganuma et al., 2006; Nagre et al., 2010; Vega & Pérez, 2006; Yang et al., 2007).

5.4 The *N*-hydroxysuccinimide method

Matrices with immobilized *N*-hydroxysuccinimide, such as *N*-hydroxysuccinimide activated Sepharose and CH Sepharose, form stable amide bonds with ligands containing an amino group. They also provide an eight carbon spacer arm. In carbohydrate affinity chromatography, the method is appropriate for coupling glycoproteins and carbohydrates or, more often, carbohydrate derivatives containing an amino group (Takeuchi et al., 2011).

5.5 The thiopropyl method

Thiopropyl matrices, such as Thiopropyl Sepharose, react reversibly under mild conditions with ligands containing thiol groups to form mixed disulphides and, in

addition, provide a spacer. Alternatively, iodoacetamidyl carbohydrate derivatives can be conjugated to thiopropyl matrices by alkylation of the thiol groups, as described (Watanabe et al., 2007).

5.6 The azlactone method

Azlactone-activated matrices, such as Fractogel, react with amine groups of (glyco)proteins, forming a stable amide bond under physiological conditions. In carbohydrate affinity chromatography, it has been used for immobilizing the glycoproteins to a matrix (Guzmán-Partida et al., 2004).

6. Elution techniques

Two main elution techniques, depending on the ligand and the adsorbent, can be applied to release a bound lectin from the affinity column, namely competitive elution and elution at different pH and/or ionic strength. When choosing the most appropriate technique to desorb a bound lectin, its specificity, its stability, and the type of adsorbent, namely sugar or complex polysaccharide, must be considered. When a mono- or disaccharide is used as a ligand, the lectin is readily released from the affinity column by competitive elution, using specific carbohydrates, if possible ones with higher affinity for the lectin than the adsorbent. Recently, the great majority of authors report the use of competitive elution of lectins using specific mono- or disaccharides. For example, when a lectin was isolated on Sepharose, galactose was used for desorption, while glucose was used with Sephadex. The concentrations of sugars used are usually 0.2 M (ranging from 0.01 – 0.5 M), while sugar gradients (from 0.1 to 0.3 or 1 M) have also been used (Table 1). Subsequently, these lectin-bound sugars must be removed to free the binding sites of the lectin, usually by extensive dialysis. Alternatively, to avoid the latter step, lectins can be desorbed from the column by changing the conditions to extremes of ionic strength or pH. The latter technique depends on the chemical stability of the matrix, ligand and adsorbed substances and is not suitable for lectins and adsorbents that are destroyed in such conditions. However, carbohydrates and lectins are usually stable molecules; nevertheless, care must be taken not to damage them irreversibly. Therefore, fractions containing the proteins should be neutralized immediately, usually with 2 M or 1 M Tris-HCl buffer, pH 7.5 (Table 1). The column also has to be equilibrated with the binding buffer. When complex, branched oligo- or polysaccharides with high avidity for lectins are used as adsorbents, the lectins cannot be readily eluted using monovalent sugars with lower affinity for lectins. In this case, extreme pH and/or ionic strength conditions should also be used. Several authors report the elution of lectins using acidic (20-100 mM glycine-HCl or β-alanine buffer, pH ~2.5, or 1-3 % acetic acid) or alkaline (10 mM NaOH, 0.1 M triethanolamine buffer, pH 11, or Tris-OH buffer, pH 11.4) solutions as well as buffers containing 1 M NaCl or 3 M $MgCl_2$ (gradient was also used) (Table 1). In some cases, lectins have been eluted using 6 M (Matsumoto et al., 2011) or 8 M urea (Suseelan et al., 2002). The carbohydrate binding activity of some lectins, such as C-type lectins, depends on divalent metal ions (Ca^{2+}), therefore can be eluted using buffers containing chelating agents, such as 2–100 mM ethylenediaminetetraacetic acid (EDTA) (Table 1).

Carbohydrate-specific lectin	Ligand	Matrix	Elution technique	Reference
Galactoside-specific lectins showing specificity for galactose, lactose, *N*-acetyl-galactosamine, *N*-acetyl-lactosamine, or *N,N'*-diacetyl-lactosediamine	Galactose	Synsorb	0.2 M lactose	Mo et al., 2000
	Lactose	Divinyl sulphone activated Sepharose	0.2 M lactose, 0.01 M NaOH neutralized with 2M Tris, pH 7.5, or 0.1 M glycine buffer, pH 2.6	Almanza et al., 2004; Pohleven et al., 2009; Rocha et al., 2009
	N-acetyl-galactosamine	Agarose	0.2 M galactose or 0.01 M *N*-acetylgalactosamine	Gerlach et al., 2005; Qureshi et al., 2006
	Fetuin	Agarose	6 M urea	Matsumoto et al., 2011
	Bovine submaxillary gland mucin	Cyanogen bromide activated Sepharose 4B	3 M $MgCl_2$	Naganuma et al., 2006
	Porcine stomach mucin	Toyopearl AF-Amino-650M	3 M sodium thiocyanate (NaSCN)	Takahashi et al., 2008
	Porcine stomach mucin	Sepharose 4B	20 mM 1, 3-diaminopropane neutralized with 1 M Tris–HCl, pH 7.0	Chumkhunthod et al., 2006
	Asialofetuin	Sepharose CL	0.2 M lactose	Naeem et al., 2007b
	Asialofetuin	Amino activated silica beads	0.1 M glycine-HCl buffer, pH 2.5, neutralized with 2 M Tris-HCl, pH 8.3	Kaur et al., 2006
	Asialomucin	Cyanogen bromide activated Sepharose 4B	0.05 M Tris-OH, pH 11.4, neutralized with 2 N HCl	Vega & Pérez, 2006
	/	Sepharose	0.2 M galactose, 0.01 M EDTA, 0.1 M isopropyl-β-D-thiogalactoside, or 1 mM HCl, pH 3.0	Cao et al., 2010; De-Simone et al., 2006
	/	Acid treated Sepharose	0.1 M or 0.2 M galactose, or 0.2 M lactose	Jimbo et al., 2000; Mishra et al., 2004; Stirpe et al., 2007; Voss et al., 2006
	/	Affi-gel blue gel	1 M NaCl	Lin & Ng, 2008
	Alginate	Guar gum	0.1 M galactose	Roy et al., 2005
	/	Guar gum	1 M NaCl, 0.1 M or 0.2 M lactose, or 0.1 M glycine buffer, pH 2.6	Alencar et al., 2010; Santos et al., 2009; Souza et al., 2011; Sultan et al., 2009
	/	Cross-linked *Leucaena leucocephala* seed gum	0.2 M lactose	Seshagirirao et al., 2005

Carbohydrate-specific lectin	Ligand	Matrix	Elution technique	Reference
	/	Cross-linked *Adenanthera pavonina* galactomannan	0.2 M galactose or 0.1 M acetate buffer, pH 2.6	Moreira et al., 1998; Teixeira-Sá et al., 2009
	/	cross-linked *Spondias purpurea* polysaccharide	0.2 M galactose or 0.1 M β-alanine buffer, pH 2.6	Teixeira et al., 2007
Glucose/mannose-specific lectins showing specificity for *N*-acetyl-glucosamine, chitooligosaccharides, or chitin	Glucose	Divinyl sulphone activated Sepharose	0.2 M glucose or 0.01 M NaOH neutralized with 2M Tris, pH 7.5	Pohleven et al., 2011
	Mannose	Agarose	0.2 M mannose, 0.5 M mannopyranoside or 8 M urea	Andon et al., 2003; Ooi et al., 2010; Suseelan et al., 2002
	N-acetyl-glucosamine	Epoxy activated Sepharose 6B	0.1 or 0.2 M *N*-acetylglucosamine	Kaur et al., 2005; Maheswari et al., 2002
	Ovalbumin	Cyanogen bromide activated Sepharose 4B	0.2 M methyl α-D-mannoside	Mo et al., 1999
	/	Sephadex	0.2 M or 0.1 M glucose, 0.1 M glycine buffer, pH 2.6 or glucose	Biswas et al., 2009; Rangel et al., 2011; Roh & Park, 2005
	Mannan	Agarose	0.05 M mannose, 0.2 M methyl α-D-mannopyranoside or 2 mM EDTA	Argayosa et al., 2011; Naeem et al., 2007a; Ourth et al., 2005
	Chitin	/	0.1 M triethanol-amine, pH 11	Santi-Gadelha et al., 2006
Fucose-specific lectins	L-fucose	Agarose	0.1 – 1 M L-fucose gradient or 0.2 M L-fucose	Cammarata et al., 2007; Mansour & Abdul-Salam, 2009
L-rhamnose-specific lectin	L-rhamnose	Sepharose 4B	0.2 M L-rhamnose	Jimbo et al., 2007; Watanabe et al., 2008
Sialic acid-specific lectins showing specificity for neuraminyl oligosaccharides, or *N*-acetylneuraminic acid	Fetuin	Agarose	0.1 M *N*-acetyl-glucosamine	Rittidach et al., 2007; Sun et al., 2007
	Fetuin	Cyanogen bromide activated Sepharose 4B	0.05 M citrate buffer, pH 5.0, devoid of Ca^{2+} ions	Bhowal et al., 2005
	Rat erythrocyte stroma	Sephadex G-25	3 % acetic acid	Vazquez et al., 1993

Table 1. Carbohydrate affinity chromatography methods used to isolate lectins according to their carbohydrate-binding specificities.

7. Other methods for the purification of lectins

In addition to carbohydrate affinity chromatography, other methods have also been used to purify lectins. For example, some lectins have been purified using fractional precipitation with ammonium sulphate, followed by classical chromatographic methods, such as ion-exchange chromatography and gel filtration (Horibe et al., 2010; Pan et al., 2010). Lectins are usually glycoproteins, therefore some authors describe their isolation by affinity chromatography on the Sepharose-immobilized lectin Concanavalin A (Absar et al., 2005; Charungchitrak et al., 2011; Konkumnerd et al., 2010; Petnual et al., 2010; Yan et al., 2010). Moreover, ferromagnetic particles with immobilized polysaccharide levan have been prepared and used for isolation of lectins. The magnetic property of the particles favoured the washing of impurities using a magnetic field; the sugars were then used to release the lectins and recover the particles (Angeli et al., 2009). To purify ion-dependent lectins, which show affinity for metal ions, affinity precipitation using metal charged EGTA or affinity chromatography using iminodiacetic acid-Sepharose charged with metal ions was used. After adsorption, lectins were eluted using buffer containing EDTA (Naeem et al., 2006). A novel affinity chromatographic method for purifying lectins using glycosylated nanofibrous membrane has also been described. An affinity membrane with immobilized glucose ligands showed strong and reversible lectin binding capability (Che et al., 2011).

8. Conclusions

Carbohydrate affinity chromatography is by far the most widely used method for purifying lectins. It is a simple method that takes advantage of their specific properties. However, there is no generally applicable protocol. Selection of the most appropriate matrix, ligand and elution technique, apart from matrix activation and ligand coupling methods, can be difficult. These have to be selected according to the individual lectin. A variety of currently used methods are presented in Table 1, for isolating lectins according to their carbohydrate-binding specificity. To purify any lectin of interest, one of the described methods at least should be effective.

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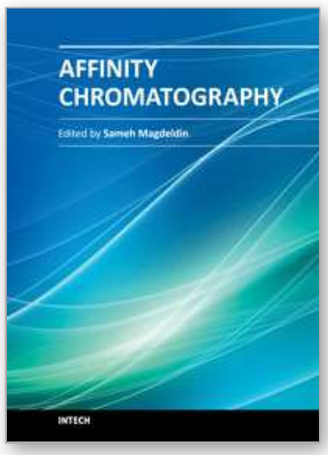

Affinity Chromatography

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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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