Using Single Lectins to Enrich Glycoproteins in Conditioned Media

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Lectins are sugar-binding proteins that can recognize and bind to carbohydrates conjugated to proteins and lipids. Coupled with mass spectrometry technologies, lectin affinity chromatography is becoming a popular approach for identification and quantification of glycoproteins in complex samples such as blood, tumor tissues, and cell lines. Given the commercial availability of a large number of lectins that recognize diverse sugar structures, it is now possible to isolate and study glycoproteins for biological and medical research. This unit provides a general guide to single-lectin-based enrichment of glycoproteins from serum-free conditioned media. Due to the unique carbohydrate specificity of most lectins and the complexity of the samples, optimization steps may be required to evaluate different elution buffers and methods as well as binding conditions, for each lectin, for optimal recovery of bound glycoproteins. © 2015 by John Wiley & Sons, Inc.

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INTRODUCTION

Lectins are multivalent sugar-binding proteins that reversibly and non-enzymatically recognize a broad range of glycan motifs without displaying catalytic activity (De Hoff et al., 2009). In recent years, lectins have been extensively used for targeted enrichment of glycoproteins from complex samples due to their selective affinities for specific carbohydrate epitopes. Their unique ability to reversibly bind to the limited range of specific glycan structures on glycoproteins has made them one of the most exploited tools for isolation and investigation of glycoproteins for biological and medical research (Fanayan et al., 2012). Lectins with different specificities (Table 24.6.1) can be used in different platforms either singly, in serial combinations or as mixtures of lectins.

Lectin-based affinity chromatography can be performed in single-, serial-, or multi-lectin approaches using various biological samples. This unit describes the single-lectin-based affinity chromatography, using a single agarose-bound lectin to capture and enrich glycoproteins from serum-free conditioned media. The Basic Protocol provides stepwise instructions for single-lectin-based affinity chromatography, and the Support Protocol describes the preparation of concentrated serum-free conditioned media. As illustrated in Figure 24.6.1, the workflow includes preparation of concentrated conditioned medium and the agarose-bound lectin affinity matrix, combining the conditioned medium and lectin affinity matrix and packing the column, collection of unbound fraction and elution

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Table 24.6.1 Lectins Commonly Used for Enrichment of Glycoproteins and Glycopeptides, Glycan Binding Specificities, and Respective Elution Buffers

Lectin[a]	Specificity	Elution buffers
Concanavalin A (Con A)	High-mannose type, branched α-mannosidic structures	Mixture of 200 mM α-methylmannoside/200 mM α-methylglucoside
Wheat germ agglutinin (WGA)	*N*-acetylglucosamine; chitobiose (sialic acid)	200 mM N-acetylgalactosamine
Jacalin (JAC)	Galactosyl (β-1,3) *N*-acetyl-galactosamine (*O*-glycoproteins)	800 mM galactose or 100 mM melibiose
Sambucus Nigra lectin (SNA, EBL)	Sialic acid attached to terminal galactose in (α-2,6)	500 mM lactose in buffered saline followed by 500 mM lactose in acetic acid
Peanut agglutinin (PNA)	Galactosyl (β-1,3) *N*-acetylgalactosamin (T-Antigen)	200 mM galactose in 10 mM HEPES buffering solution, ph 7.5
Lens Culinaris agglutinin (LCA)	α -linked mannose residues	Mixture of 200 mM α-methyl mannoside in base Buffer + 200 mM α-methyl glucopyranoside in base buffer
Phaseolus vulgaris leucoagglutinin (PHA-L)	Tri/tetra-antennary complex-type *N*-glycan	100mM acetic acid
Aleuria Aurantia lectin (AAL)	Fucose linked (α -1,6) to *N*-acetylglucosamine; Fucose linked (α -1,3) to *N*-acetyllactosamine	100 mM L-fucose

[a]Lectins available from Vector laboratories, Sigma Aldrich, and Life Technologies.

of bound glycoprotein fraction, and concentration of bound and unbound fractions for downstream analysis.

BASIC PROTOCOL

ENRICHMENT OF GLYCOPROTEINS USING AGAROSE BOUND LECTIN AFFINITY CHROMATOGRAPHY

This protocol describes lectin-based affinity chromatography of glycoproteins. Agarose-bound lectin slurry is combined with concentrated conditioned media and the mixture is loaded onto an empty polypropylene gravity column. Unbound proteins and glycoproteins are collected in the flow through, and bound glycoproteins are eluted using either a competitive sugar or a low pH buffer. The bound and unbound fractions are concentrated and eluted proteins in the bound fraction are identified by liquid chromatography–tandem mass spectrometry (LC–MS/MS). The proteins in the bound and unbound fractions are quantified using an appropriate protein assay, and each fraction is resolved by one-dimensional polyacrylamide gel electrophoresis to visualize differences in the bound and unbound fractions from each lectin as well as differences between each lectin.

Materials

Agarose-bound lectin (Vector Laboratories, Sigma Aldrich, or Life Technologies)
Binding buffer (see recipe)
Concentrated conditioned serum-free medium (see Support Protocol)
CHAPS detergent (Sigma-Aldrich, cat. no. C3023)

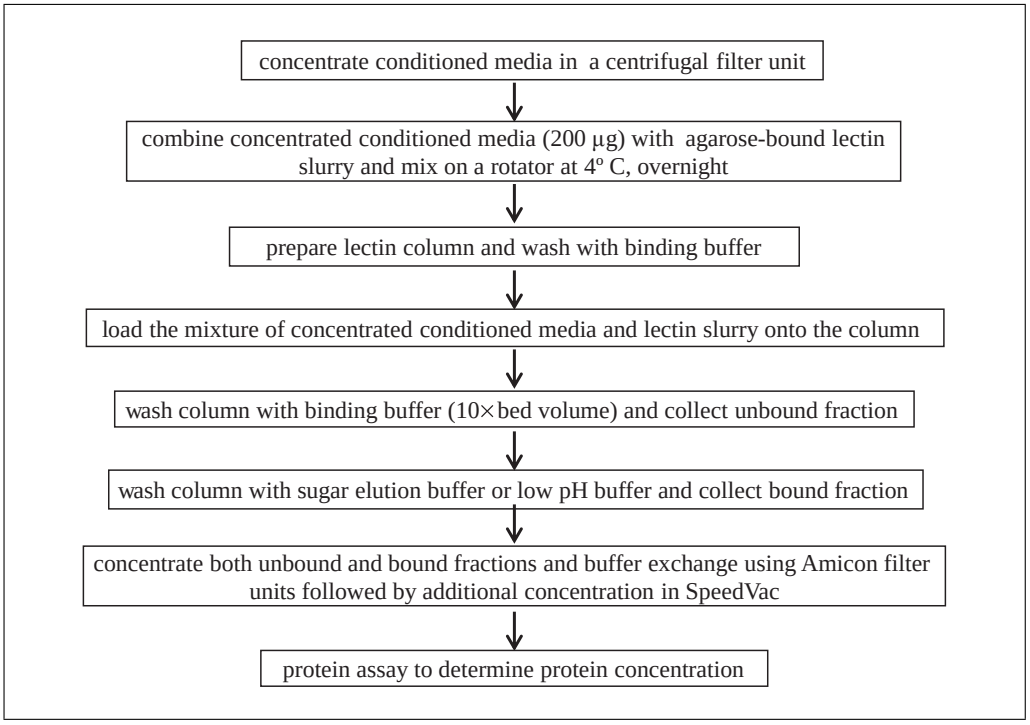

Figure 24.6.1 Overview of the workflow in single lectin affinity chromatography of conditioned media.

Elution buffer, lectin-specific (see Table 24.6.1)
25 mM Tris·Cl, pH 7.5 (*APPENDIX 2E*)

Poly-prep chromatography columns with porous discs (Bio-Rad Laboratories, cat. no. 731-1550), empty polypropylene gravity flow columns with 2-ml bed volume and 10-ml sample reservoir
15-ml conical tubes
Amicon Ultra-15 10K centrifugal filter units (Millipore, cat. no. UFC901008)
SpeedVac

Additional reagents and equipment for determination of protein concentration (*UNIT 3.4*; Olson and Markwell, 2007)

Prepare agarose-coupled lectin slurry and bind glycoproteins

1. Thoroughly mix the agarose-bound lectin slurry prior to taking an aliquot.

   In our experience, Vector Laboratories is the best global supplier of a wide range of lectins in different formats, including agarose-bound and biotinylated lectins.

2. For each sample, transfer 500 µl lectin slurry to a microcentrifuge tube, and gently invert the tube a few times.

   500 µl of lectin slurry will result in a 250 µl bed volume.

3. Centrifuge the tube 15 min at 1000 × *g*, room temperature.
4. Carefully remove supernatant (~250 µl), and discard.
5. Add an equal volume (250 µl) of binding buffer to the lectin, and gently invert the tube a few times to mix.
6. Centrifuge 15 min at 1000 × *g*, room temperature and remove supernatant.
7. Repeat steps 5 and 6 twice more for a total of three washes with binding buffer.

8. Add 200 μg concentrated conditioned media to the agarose-lectin slurry and mix thoroughly.

   Acceptable sample concentration range could vary depending on the type of lectin or source of conditioned media. We typically use 200 μg of concentrated conditioned media per 250 μl of agarose-lectin bed volume.

9. Seal the tube with parafilm (to avoid loss of sample through leaking) and mix on a rotator at low speed overnight at 4°C.

   Performing this step at 4°C is important for stability of both the lectin and proteins. Lectins are susceptible to leaching at room temperature.

   Overnight incubation allows more time for lectin-glycoprotein interaction, resulting in higher binding compared to shorter incubation times.

Pack column with agarose-lectin–conditioned medium mixture and collect bound and unbound fractions

10. Place an empty gravity flow column on a column rack or clamp stand, and wash with 5 ml binding buffer to ensure the column flows smoothly.

11. Using a pipette, load the mixture of agarose lectin and conditioned culture medium into the bed volume of the gravity column.

    Gently rest the tip of the pipette on the inside of the column, just above the bed volume, and dispense the sample making sure not to splash it on the sides of the column.

12. Place a porous disc on top of the gel bed without introducing bubbles between the disc and the gel bed.

    The porous disc helps retain the packing in the column while allowing the flow of liquid.

13. Wash the column with 2.5 ml (10 bed volumes) of binding buffer containing 0.1% (v/v) CHAPS, and collect the unbound fraction in a 15-ml conical tube.

    CHAPS is added to the binding buffer immediately prior to use. Buffers with detergents should not be stored for long periods of time.

14. Add 2.5 ml of elution buffer containing 0.1% (v/v) CHAPS to the column, and elute the bound fraction into a 15-ml conical tube.

    CHAPS is added to the elution buffer immediately prior to use. Buffers with detergents should not be stored for long periods of time.

    Glycoproteins can be eluted from lectins using an appropriate competitive sugar or a low pH (e.g., pH 3.0) buffer. Elution buffers for commonly used lectins are listed in Table 24.6.1.

Exchange the buffer and concentrate the proteins in the bound and unbound fractions

15. Transfer the bound and unbound fractions to Amicon Ultra-15 centrifugal filter units.

16. Concentrate the samples by centrifugation for 30 min at 3000 × *g*, ?°C.

    The initial volume of each bound and unbound fraction is ~2.5 ml. This step concentrates each sample to approximately 200 μl.

17. Dilute each concentrated sample by adding 3 ml of 25 mM Tris, pH 7.2, and concentrate each sample by centrifugation for 30 min at 3000 × *g*, 4°C.

18. Repeat step 17 two more times.

    The buffer exchange washes away traces of salt, detergent, or other small molecules.

19. Add 100 μl of 25 mM Tris, pH 7.2, to each buffer exchanged sample, and transfer each sample to a correspondingly labeled microcentrifuge tube.

    The total volume should be 300 to 400 μl.

20. Concentrate the buffer-exchanged samples to dryness in a SpeedVac without heating.

    Make sure the tubes are uncapped in the SpeedVac.

21. Suspend each pellet in 50 μl of 25 mM Tris, pH 7.2.

22. Determine protein concentration using a BCA protein assay (*UNIT 3.4*; Olson and Markwell, 2007).

    Samples can be stored up to several months at –20°C.

    The bound and unbound fractions can be analyzed by different approaches:

    For LC-MS/MS analysis of the lectin fractions, the samples are first fractionated by SDS-PAGE and proteins visualized by staining. It may be possible to visualize the differences in protein levels between the bound and unbound fractions on the SDS-PAGE gel. Additionally, individual lanes can be excised, cut into ~10 slices, and digested with trypsin, generating peptide mixtures of optimal molecular weight for analysis by mass spectrometry (see UNIT 16.10 or 23.1; Moore et al., 2001; Link and Washburn, 2014)

    Alternatively, each bound and unbound fraction can be dot blotted to nitrocellulose or polyvinylidene fluoride (PVDF) membranes, treated with PNGFase to release N-glycans (O-glycans are released by β-elimination), digested with trypsin, and analyzed by LC-MS/MS. Keep in mind, however, that this approach is only feasible after N-glycan release.

PREPARATION OF CONCENTRATED SERUM-FREE CONDITIONED MEDIA

SUPPORT PROTOCOL

This protocol must be completed prior to the Basic Protocol to obtain the concentrated, serum-free conditioned media from the cultured cells. Cells are normally grown in serum-containing medium until 80% to 90% confluent, followed by incubation in serum-free medium for 48 hr. The resulting conditioned medium is then collected and concentrated by centrifugation in a centrifugal filter unit. The protein concentration is then determined using an appropriate protein assay. Concentrated media can be stored at −20°C for up to a few months.

Materials

Cells (e.g., LIM2405 colon cancer cells)
Cell culture medium (e.g., RPMI 1640 available from Invitrogen or Sigma-Aldrich)
Fetal bovine serum (FBS; available from Invitrogen or Sigma-Aldrich)
Media supplements (e.g., human recombinant insulin and glutamine; available from Invitrogen or Sigma-Aldrich)
Phosphate-buffered saline (PBS), pH 7.2 (*APPENDIX 2E*)

100-mm culture dish
Amicon Ultra-15 10K centrifugal filter units (Millipore, cat. no. UFC901008)

Additional reagents and equipment for determination of protein concentration (*UNIT 3.4*; Olson and Markwell, 2007)

1. Grow cells, to 80% to 90% confluence in a 100-mm culture dish in 30 ml medium containing 10% FBS and media supplements.

2. Remove media and wash cells three times with cold (4°C) 1× PBS.

   Remove all buffer between washes to ensure minimal carryover of FBS.

3. Add 30 ml serum-free medium, and incubate cells 48 hr at 37°C, 5% CO_2.

4. Collect the conditioned medium (~30 ml) and transfer 15 ml to each of two Amicon Ultra-15 centrifugal filter units.

5. Concentrate the conditioned medium by centrifugation for 30 min at 3,000 × *g*, 4°C.

6. Add 10 ml 1× PBS to the concentrated sample and concentrate by centrifugation for 30 min at 3,000 × *g*, 4°C.

7. Repeat step 6 three more times for a total of four washes.

   The sample in each Amicon centrifugal filter unit will be concentrated to approximately 1 ml in 30 min. The combined volume of concentrated medium after the washes will be ~500 to 1000 μl.

8. After the fourth wash, remove the filter from the unit and transfer the concentrated medium to a fresh microcentrifuge tube.

9. Measure concentration of secreted proteins in each concentrated sample using a suitable protein assay method, such as the BCA protein assay (*UNIT 3.4*; Olson and Markwell, 2007).

   There is no protein assay that is either specific to proteins (i.e., not affected by any non-protein components) or uniformly sensitive to all protein types (i.e., not affected by differences in protein composition). Choosing a suitable protein assay involves selecting the method that is most compatible with the samples and choosing an appropriate assay standard.

   Some important criteria to consider when choosing a suitable protein assay:

   -Compatibility of the assay with the sample type
   -Assay range and the sample volume required
   -Speed and convenience of the assay for a given number of samples
   -Availability of a spectrophotometer or a plate reader required to measure the color produced (absorbance) by the assay

10. Store the concentrated media for up to several months at –20°C.

    Samples with volumes larger than the loading capacity of the centrifugal device may need to be divided in smaller aliquots and split across several tubes.

    Since these centrifugal units are single-use devices, once one has been used for a particular sample, it cannot be "washed" to be used with another sample. Aliquots of the same sample, however, may be passed through the column up to an additional three times to increase purity.

    To avoid filter unit fracture, never exceed the maximum centrifugation speed or the recommended number of spins (usually four).

    High amounts of debris in conditioned media can block the membrane. To avoid this, pellet cell debris before passing the sample through the device. If the media is not centrifuged, dislodged cells or cell debris could become trapped in the chromatography column and lead to non-glycosylated protein contaminants in the eluted glycoprotein fraction.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent for the preparation of all buffers. For common stock solutions, see APPENDIX 2E.

All reagents/buffers are stored at room temperature unless otherwise stated.

Binding buffer

25 mM Tris, pH 7.4
150 mM NaCl
1 mM Mn^{2+}
1 mM Ca^{2+}
0.05% (w/v) NaN_3
store at 4°C up to several months

COMMENTARY

Background Information

Protein glycosylation is one of the most abundant post-translational modifications found on proteins, and is estimated to occur on more than half of the plasma proteome (Anderson and Anderson, 1998; Apweiler et al., 1999). Altered glycosylation are associated with many diseases, including atherosclerosis, diabetes, immunological, and inflammatory disorders, neurological degeneration as well as cancer (Botella-Lopez et al., 2006; Raghav et al., 2006; Carlsson et al., 2008; Blixt et al., 2011; Chacko et al., 2011).

A major challenge in glycoproteomic research is the enormous complexity and the large dynamic range in protein concentrations in biological samples. In biological materials such as serum, plasma, cell lysates, tissue extracts, and conditioned media, biologically relevant glycoproteins are often present in small quantities, masked by the higher abundance proteins. There is general agreement that suitable sample fractionation or enrichment steps are required to detect glycoproteins, including potential disease biomarkers. Detection of glycoproteins can generally be achieved by selective enrichment of the glycoproteome subset of the sample, based on the unique properties of their glycans, prior to a more in-depth analysis (Domon, 2009).

Several methods for enrichment of glycoproteins from complex biological samples are available. These include the use of lectins, hydrazide chemistry (UNIT 4.3; Zhang, 2007), hydrophilic interaction liquid chromatography, and immobilized titanium dioxide or boronic acid (Tousi et al., 2011). There are advantages and limitations to each method.

Lectins, with selective affinities for specific carbohydrate epitopes, are finding extensive applications as tools of choice for selective enrichment of glycoprotein fractions. Lectin-based enrichment of glycoproteins can greatly enhance the identification of the glycoproteome when coupled with modern mass spectrometry. Lectin affinity chromatography has been easily applied to a number of biological samples, including cell lines (Lee et al., 2012), conditioned media, tumor tissues (Cho et al., 2008) and serum or plasma (Nilsson et al., 2009). A large number of lectins with diverse sugar specificities, some with overlapping selectivity for particular carbohydrate structures, are now commercially available (Table 24.6.1).

Lectins are commonly employed in the form of lectin affinity chromatography to enrich for glycoproteins and to detect changes in glycosylation patterns between normal and diseased samples. Lectin affinity chromatography to capture glycoproteins or glycopeptides involves loading the protein sample or trypsin-digested peptides onto a lectin affinity column. Glycoproteins with a specific sugar sequence bind the immobilized lectin, while non-specific material remains unbound and passes through the column or is washed off prior to elution. Glycoproteins bound to lectins can be eluted using a low pH buffer or a competitive inhibitory sugar (Fanayan et al., 2012). Table 24.6.1 provides some details on specificities and elution buffers for some commonly used lectins. Figure 24.6.1 provides a summary of the above steps for single lectin affinity chromatography of conditioned media.

Critical Parameters and Troubleshooting

Each lectin has a different binding and elution condition. Many lectins can bind under neutral pH conditions, while some lectins (e.g., Con A) can bind more efficiently at lower pH (~5.5). Moreover, most lectins with specificity

Table 24.6.2 Top Colon Cancer-Associated Proteins Identified by LC-MS/MS of Bound Fractions from Chromatography of Serum-Free Medium Conditioned with Cultured LIM2405 Colon Cancer Cells Using Different Single Lectin Columns

	Lectin				
	AAL	Con A	LCA	PNA	SNA
Top Colon cancer-associated proteins (CCAPs) identified in by LC-MS/MS analysis	NME1, EZR, JUP, YWHAZ, YWHAE, YHWAG, YWHAQ, CYCS, CHD7, PCDH7, CD44, CD59, MMP15, MACC1, MCC, MSH2, MUC7, ENO1, LYZ, RAB11A, RAB5C, RAN	NME1, EZR, ERBB2, YWHAZ, CYCS, MLH3, EGF, RAN, LYZ, RAB6A, RAB21, MIF, MLH3, TNFSF9, TNFRSF21, CD163, CD44	EZR, SPTBN1, YWHAE, YWHAZ, ENO1, CYCS, NPM1, PCNA, CD59, XPO4, VDR, ST14,	NME1, APC, APC2, ENO1, CBL, ST14,	APC, ABL1, GPC1,

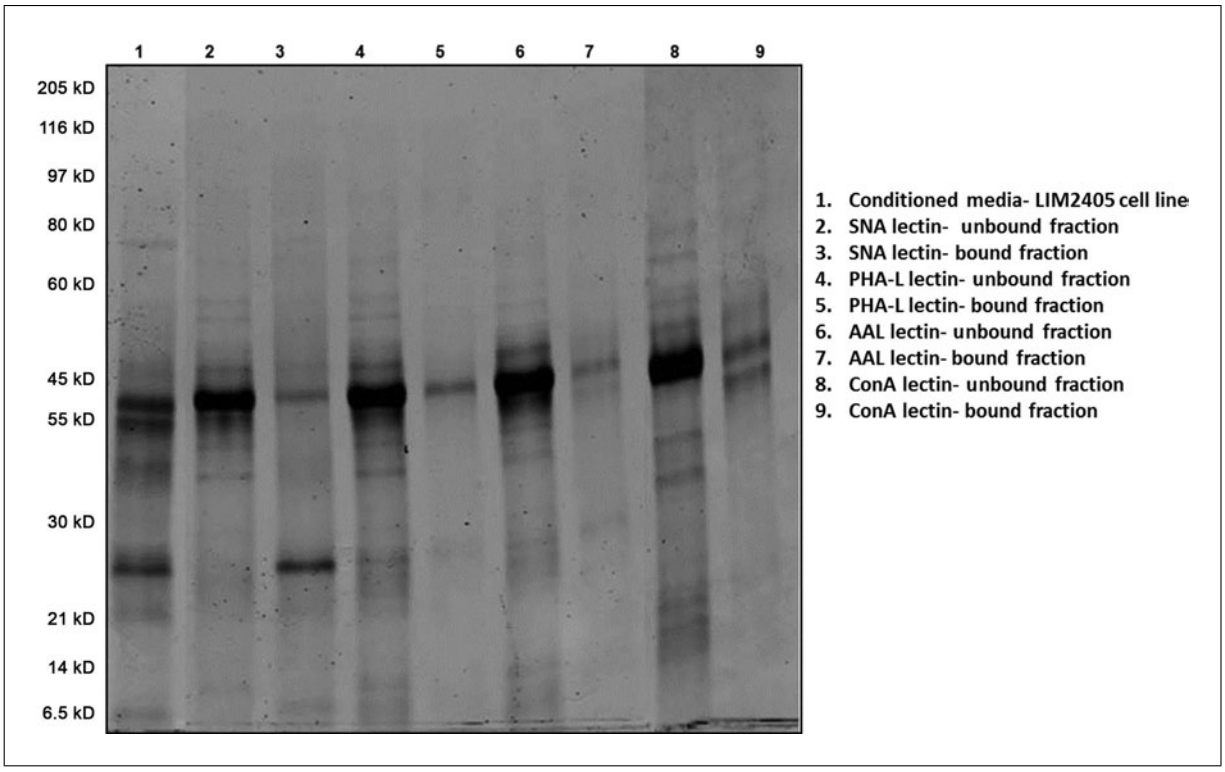

Figure 24.6.2 SDS-PAGE analysis of bound and unbound fractions from single lectin affinity chromatography of conditioned media from cultured LIM2405 colon cancer cells, using different lectins. For each fraction, 2 μg was loaded onto the gel. Glycan-binding specificities of the lectins used are listed in Table 24.6.1.

toward mannose-containing structures require the presence of metals, such as calcium or manganese ions.

Numerous workflows use commercially available immobilized lectins on agarose-based media in a centrifugal, gravity flow, or low-pressure liquid chromatography format. The major drawback of this matrix, however, is the severely constrained chromatography conditions due to flow rate and back pressure limitations, which can significantly increase analysis time and sample handling leading to sample losses and impacting the downstream proteomics analysis (Fanayan et al., 2012). When a high degree of reproducibility and speed is desired, lectins can be conjugated to HPLC-compatible matrices, consisting of small, rigid particles based on silica or synthetic polymers, which can withstand the flow rates or pressures characteristic of HPLC systems (Kullolli et al., 2010).

A notable drawback of using single lectin columns is that no lectin has sufficient selectivity to cover the whole glycoproteome being examined. An alternative platform may be using several lectin affinity

chromatography columns in tandem, known as serial lectin affinity chromatography, which can be used to purify different components of a mixture of glycoproteins (Satish and Surolia, 2001). In 2004, Yang and Hancock developed multiple lectin affinity chromatography, which uses physical admixtures of different immobilized lectins to capture the majority of glycoproteins present in a sample in one step (Yang and Hancock, 2004). It should, however, be noted that the environment and complexity of each matrix as well as the requirement of each platform can be different. Binding and elution conditions should therefore be empirically determined for optimal recovery of bound glycoproteins.

Anticipated Results

Results will vary based on numerous factors, including cell line, cell density, type, and amount of lectin. Figure 24.6.2 shows an example of a denaturing gel analysis of bound and unbound fractions from single-lectin affinity chromatography of conditioned media from a cell line, using different lectins. Table 24.6.2 provides a list of top cancer-associated proteins that were identified by LC-MS/MS of the bound fractions from chromatography of serum-free medium conditioned with cultured LIM2405 colon cancer cells using several common lectin columns (from the gel in Fig. 24.6.2). Protein recovery can be influenced by several factors such as the nature of the biological sample, lectin, lectin platform, or elution buffer used and can range from ~10% to 90% of starting material. In the example above, our observed protein recovery ranged from 10% to 40% of starting material.

Time Considerations

The assay can be separated into six stages: 1) preparation of concentrated serum-free conditioned media, 2) preparation of agarose-coupled lectin slurry and combining with conditioned medium, 3) packing the column with the lectin slurry–conditioned medium mixture, 4) collection of unbound fraction and elution of bound fraction, 5) concentration of bound and unbound fractions using Amicon centrifugal filters, and 6) measurement of protein concentration in bound and unbound fractions by protein assay.

The experimental stages will vary in time based on the number of cell lines and lectins used. The preparation of concentrated conditioned media is the longest stage of the protocol and can take 5 to 7 days depending on the growth rate of the cell line used: growth to 80% to 90% confluence (2 to 4 days); serum-free incubation (48 hr); and concentration of conditioned media (1 day). Lectin affinity chromatography will require additional 1 to 2 days, depending on the user. It should be noted that not all proteins recovered in the bound fraction will necessarily be glycosylated. When using LC-MS/MS to analyze bound fractions, it would not be possible to truly distinguish between glycosylated proteins and non-glycosylated proteins recovered in the bound fraction. Additional analyses are, therefore, needed to definitively distinguish glycosylated proteins from non-glycosylated contaminants, such as comparing the identified proteins to available glycoprotein databases.

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