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Targeting deeper the human serum fucome by a liquid-phase multicolumn platform in combination with combinatorial peptide ligand libraries

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ABSTRACT

Combinatorial peptide ligand library (CPLL) was evaluated as an off line step to narrow the differences of protein concentration in human serum prior to the capturing of human fucome from disease-free and breast cancer sera by a multicolumn platform *via* lectin affinity chromatography (LAC) followed by the fractionation of the captured glycoproteins by reversed phase chromatography (RPC). Two monolithic lectin columns specific to fucose, namely *Aleuria aurantia* lectin (AAL) and *Lotus tetragonolobus* agglutinin (LTA) columns were utilized to capture the fucome, which was subsequently fractionated by RPC yielding desalted fractions in volatile acetonitrile-rich mobile phase, which after vacuum evaporation were subjected to tryptic digestion prior to LC-MS/MS analysis. AAL has a strong affinity towards core fucosylated N-glycans and has a weak binding towards fucose in the outer arm while LTA can bind to glycans having fucose present in the outer arm. The combined strategy consisting of the CPLL, multicolumn platform and LC-MS/MS analysis permitted the identification of the differentially expressed proteins (DEPs) in breast cancer serum yielding 58 DEPs in both the LTA and AAL fractions with 6 DEPs common to both lectins. 17 DEPs were of the low abundance type, 16 DEPs of the borderline abundance type, 4 DEPs of the medium abundance type and 15 DEPs of the high abundance type. The remaining 6 DEPs are of unknown concentration. Only proteins exhibiting 99.9% protein identification probability, 95% peptide identification probability, and a minimum of 5 unique peptides were considered in finding the DEPs *via* scatterplots.

1. Introduction

Protein glycosylation represents the most abundant post-translational modification (PTM) causing over 70% of all human proteins to bear this important and complex PTM [1]. One of the most important aspects of this PTM is its alteration in diseases and especially in cancers. Thus, the urgent need for tools and strategies for isolating and assessing disease induced alteration in glycosylation. Fucosylation of glycoproteins (or the so-called fucome) has been recognized as one of the most common aberration in cancerous cells [2–6]. This research article is aimed at introducing the use of combinatorial peptide ligand libraries (CPLL) to narrow the protein concentration range in human serum prior to capturing and fractionating the human fucome followed by comparative analysis of fucome in cancer serum with respect to disease free serum *via* liquid chromatography–tandem mass spectrometry (LC-MS/MS). Thus, this investigation describes a novel approach incorporating the following: (i) narrowing serum proteins dynamic concentration range by CPLL beads, a solid-phase extraction technique based on peptide affinity, (ii) capturing the human fucome by lectin affinity chromatography (LAC) and (iii) fractionation of the captured fucome by reversed phase chromatography (RPC). Steps (ii) and (iii) are integrated in a liquid-phase multicolumn platform that is a slightly modified version of the previously reported one [7] in the sense that the depletion columns that were previously used online to remove albumin and immunoglobulins (Ig's) have been replaced by an off line protein equalization *via* the CPLL approach, which has been shown to be very effective in narrowing the protein concentration range in many biological fluids and extracts [8–11], thus allowing an in-depth proteomics profiling.

To capture a given sub-glycoproteomics, e.g., the fucome, LAC has been shown recently to offer the potential to achieve this goal [7,12–17]. Two fucose specific lectins namely, *Aleuria aurantia* lectin (AAL) and *Lotus tetragonolobus* agglutinin (LTA) were immobilized onto the surface of glyceryl methacrylate

Abbreviations: AAL, *Aleuria aurantia* lectin; AIBN, 2,2′-azobis(isobutyronitrile); CPLL, combinatorial peptide ligand library; DEPs, differentially expressed proteins; Fuc, fucose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; GMM, glycerylmethacrylate; Ig's, immunoglobulins; LAC, lectin affinity chromatography; LTA, *Lotus tetragonolobus* agglutinin; PETA, pentaerythritol triacrylate; RPC, reversed phase chromatography; TFA, trifluoroacetic acid.

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(GMM)/pentaerythritol triacrylate (PETA) monolith, which was very recently introduced by Gunasena and El Rassi for performing immuno affinity chromatography at reduced nonspecific interactions [18]. AAL has a strong affinity towards N-glycans possessing a fucose residue attached to the innermost GlcNAc (represented as Fucα1→6GlcNAc→R) of the *N*-linked-core structure (i.e., core fucosylated N-glycans) and has weak binding towards fucose in the outer arm such as Fucα1→2Galβ1→4GlcNAcβ1→R, Galβ1→4(Fucα1→3)GlcNAc→R and Galβ1→3(Fucα1→4)GlcNAc→R, where R = H or sugar [19]. On the other hand, immobilized LTA can bind to glycans having fucose present in the outer arm including Fucα1→3/1→4GlcNAc and Fucα1→2 Gal. LTA also has an affinity for glycans containing the Le^x determinant represented as Galβ1→4(Fucα1→3)GlcNAcβ1→R [20]. The haptenic sugar for AAL and LTA is α-L-fucose.

The importance and significance of the combination of the CPLL technology with the multicolumn platform stem from facilitating the capturing and fractionating the human fucome at all level of protein abundance prior to LC-MS/MS analysis. The CPLL technology, also called ProteoMiner™, should allow in principle the processing of human serum prior to the multicolumn platform to yield representative serum protein samples that have much narrower concentration range than the originally existing range that usually spans over 10–12 orders of magnitude. This wide dynamic concentration range represents a real challenge for existing instrumentation and methods including LC-MS/MS. It is expected that upon narrowing the concentration range differences between the serum proteins, the lectin column sites in the multicolumn platform will be available to all fucosylated proteins on a more or less an equitable ground, a fact that should in principle facilitate the capturing of these proteins at all level of original abundance. In other words, the lectin column binding sites will not be primarily occupied by the high abundance fucosylated proteins and to lesser extent by the medium abundance fucosylated proteins but they will be available to all fucosylated proteins at equitable level from the serum treated by the CPLL beads. This should facilitate the capturing of more fucosylated proteins by the multicolumn platform. Thus, a wider range of DEPs should be readily detected by LC-MS/MS, a fact that may lead to approaching the next generation DEPs; meaning low abundance DEPs.

2. Materials and methods

2.1. Materials

The unconjugated lectins namely, AAL and LTA were purchased from Vector Laboratories (Burlingame, CA, USA). Pooled breast cancer serum from six donors (stages 2, 3 and 4) and pooled disease-free human serum from six donors (same age group and race as the cancer serum) were purchased from Bioreclamation (Jericho, NY, USA). Stainless steel tubing of 4.6 mm ID was obtained from Alltech Associates (Deerfield, IL, USA). The ProteoMiner™ bulk beads were purchased from Bio-Rad (Hercules, CA, USA). Glycerylmethacrylate (GMM) was purchased from Monomer-polymers & Dajac Labs (Feaster-Ville, PA, USA). The AcroSep™ SDR columns were purchased from Pall Life Sciences (Port Washington, NY, USA). Pentaerythritol triacrylate (PETA), 2,2′-azobis(isobutyronitrile) (AIBN) and 1-dodecanol were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Sodium periodate, sodium cyanoborohydride, trifluoroacetic acid (TFA) and L-(-)-fucose were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cyclohexanol and HPLC grade acetonitrile were obtained from Fisher Scientific (Fair Lawn, NJ, USA). The reversed phase chromatography column (ProSwift™ RP-1S) was purchased from Dionex Corporation (Sunnyvale, CA, USA).

2.2. Methods

2.2.1. Monolithic affinity columns with surface immobilized lectins

A polymerization mixture weighing 5 g and containing 7.6% (w/w) GMM, 7% (w/w) PETA, 59.1% (w/w) cyclohexanol 22.9% (w/w) dodecanol and 3.4% (w/w) water containing 1.0% (w/w) AIBN with respect to the monomers [21] was sonicated for 15 min, purged with nitrogen for 5 min and introduced into a stainless steel columns of 25 cm × 4.6 mm ID each that function as molds for the monoliths and were heated at 60 °C for 15 h in a gas chromatography oven. The resulting monolithic columns were washed extensively with acetonitrile followed by water. The monolithic support was transferred from the 25 cm column to a shorter column (3 cm × 4.6 mm ID) by connecting the two columns with a 1/4″-union and running water through the columns at flow rate of 3.0 mL/min until the modified monolithic support is transferred. Two 3 cm × 4.6 mm ID columns were prepared by this procedure.

The 3 cm monolithic columns were perfused with a freshly prepared 0.1 M $NaIO_4$ solution for 2 h at room temperature to convert the diol into aldehyde groups followed by a 5 min water wash at 1 mL/min using a reciprocating HPLC pump. Then, the lectin immobilization was done on column by recycling 2 times a 0.5 mL immobilization solution of the lectin of interest through the column at 0.5 mL/h with a micro-syringe pump at 4 °C. This was followed by an additional treatment for overnight of the column with the same lectin immobilization solution at 0.05 mL/h with the same micro-syringe at 4 °C. The lectin immobilization solution was made of 1 mg of AAL or LTA in 0.5 mL of 0.1 M sodium acetate pH 6.4, containing 0.1 M fucose and 50 mM sodium cyanoborohydride. After this lectin immobilization step, a 10 mL solution containing 0.4 M Tris/HCl, pH 7.2, and 50 mM sodium cyanoborohydride was pumped with a micro-syringe through the columns at 3 mL/h for 3 h at room temperature. The immobilized lectin columns thus obtained were stored in 20 mM Tris-HCl, pH 7.4, and 0.08% NaN_3 at 4 °C until use. More information on lectin immobilization on various supports can be found in a recent review article [22].

2.2.2. Sample treatment with the combinatorial peptide ligand libraries

The bulk ProteoMiner™ beads were swelled in 20% (v/v) aqueous ethanol (the storage solution) at 4 °C for 12 h. 500 μL of the final slurry, which have 100 μL of beads, were introduced into a single spin column. Six spin columns were prepared in this way. Out of these six spin columns, 3 columns were used to treat the disease-free serum and 3 columns were used for the breast cancer serum treatment. For each spin column, the storage solution was removed by centrifuging at 1000 × *g* for 60 s. Then, 600 μL of 10 mM NaH_2PO_4, 150 mM NaCl, pH 7.4 (wash buffer) were added, and the spin column was rotated end-to-end several times for 5 min, followed by centrifugation at 1000 × *g* for 60 s. This washing step was repeated once again. The serum samples were centrifuged at 3650 × *g* for 10 min. Thereafter, 1 mL of the centrifuged serum was treated with 100 μL of the settled beads (i.e., a spin column) by rotating each of the spin columns end-to-end at room temperature for 2 h. The unbound proteins were centrifuged at 1000 × *g* for 60 s. To each of the spin columns, 600 μL of wash buffer were added and rotated end-to-end for 5 min followed by centrifugation at 1000 × *g* for 60 s. This wash was repeated for another three times. The same procedure was followed with 600 μL of deionized water. This was followed by elution with 3 × 100 μL of 9 M urea, 2% (w/v) CHAPS in 5% (v/v) acetic acid (elution reagent). The elution reagent was added to the column and incubated at room temperature for 15 min with light vortexing in between. The three elution volumes