Methods 56 (2012) 254–259

Contents lists available at SciVerse ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

Combining lectin affinity chromatography and immunodepletion – A novel method for the enrichment of disease-specific glycoproteins in human plasma

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ARTICLE INFO

Article history:
Available online 22 December 2011

Keywords:
Plasma proteomics
Lectin affinity chromatography
Immunoaffinity depletion
Llama heavy chain antibodies
MS^E mass spectrometry

ABSTRACT

Drastic enrichment of potential disease-specific glycoprotein markers in human plasma can be achieved by the combination of affinity- and immuno-depletion. In the affinity-fractionation step all glycoproteins carrying a certain glycostructure are isolated by lectin affinity chromatography, thus depleting other components. Against the respective glycoprotein fraction isolated from the plasma of healthy individuals antibodies are raised in llamas. The llama heavy chain antibodies (which are particularly stable) directed at the isolated plasma glycoprotein fraction are immobilized and the immunoaffinity column thus obtained is used to deplete the respective glycoprotein fraction of patient plasma samples. Depletion of proteins normally found in human plasma by 99.8–99.9% can be achieved, resulting in a 800–1000-fold enrichment of potential disease-specific proteins in the flow-through of the immunoaffinity column.

1. Introduction

1.1. The problem

The early detection of diseases as well as the follow-up of therapy can often be supported by the determination of molecular markers, which reflect pathological changes in intra- and intercellular processes. In particular changes in the concentration and the modification state of proteins in body fluids can serve as sensitive diagnostic indicators [1]. The identification of new protein biomarkers in addition to established marker proteins (e.g. the cancer markers PSA, CA-125 and CEA) is therefore a task of high priority in clinical research [2]. Rapid improvements in protein analytics, in particular in mass spectrometric techniques, allow the quantitative measurements of protein amounts in the low femtomole range. However, the complexity of the human plasma proteome [3,4] and especially the detection of minor protein components in the presence of a large excess of other proteins poses a formidable problem, both with respect to the relative amount (a given amount of protein, though sufficient to be detected in pure form, is masked by the presence of an excess of competing material, e.g. due to ion suppression in mass spectrometry) and to the absolute amount (only a limited amount of the material can be loaded onto a gel or a HPLC column). Because of these limitations the concentration range covered by unbiased, hypothesis-free mass spectrometric techniques is 10^3 at best [5], while protein concentrations in body fluids can differ by $>10^{11}$ [3]. Therefore, the analysis of minor components in complex protein mixtures always requires highly efficient and reproducible depletion of the abundant components [6].

Fractionation can be achieved by separating subproteomes based on posttranslational modifications (e.g. glycosylation [7,8], phosphorylation [9]). Alternatively, the most abundant proteins can be depleted by immunodepletion [10]. Immunoaffinity columns for the depletion of the eight, 20 or even more most abundant proteins in human plasma are commercially available. A review of the various prefractionation techniques is given by Pernemalm et al. [11].

1.2. Tandem affinity depletion

The enrichment of disease-specific glycoproteins by a combination of lectin- and immunoaffinity chromatography (“tandem depletion”) has been discussed in detail elsewhere [12]. In short, in the first step of the procedure we generate an immunoaffinity column containing llama heavy chain antibodies directed against a fraction of the plasma glycoproteome of healthy individuals (Fig. 1). By lectin affinity chromatography we isolate from a mixture of plasma samples obtained from healthy individuals a fraction of the glycoproteome. In the present paper we apply wheat germ agglutinin (WGA), but any other lectin can also be used, provided it allows a reproducible fractionation. In this step all non-binding proteins (all proteins not carrying the glycostructure recognized

Abbreviations: CEA, carcinoembryonic antigen; GlcNAc, *N*-acetyl-glucosamine; IAC, immunoaffinity chromatography; SEC, size exclusion chromatography; WGA, wheat germ agglutinine; IAC, immunoaffinity chromatography.

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doi:10.1016/j.ymeth.2011.12.004

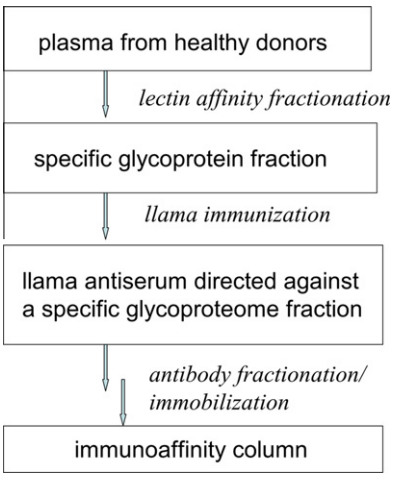

Fig. 1. Workflow 1: making of the llama immunoaffinity column for the lectin affinity chromatography/immunodepletion strategy.

by the particular lectin) are depleted. The lectin-binding plasma protein fraction thus obtained is used to immunize llamas. From the serum of the immunized animals the heavy chain antibody fraction is isolated. Because of their higher stability llama heavy chain antibodies are better suited for the production of immunoaffinity columns than standard antibodies containing light and heavy chains. The heavy chain antibody preparations are immobilized via their sugar moieties to agarose beads.

In the second step, the immunoaffinity columns generated in the first step are used to deplete the respective lectin-binding glycoprotein fractions (in our example the WGA-binding fraction) of patient plasma samples from all proteins also found in the plasma of healthy persons in order to enrich disease-related proteins (Fig. 2). The samples are fractionated on a lectin affinity column, where all proteins not binding to the respective lectin are removed. The lectin-binding fraction is then applied onto the immunodepletion column and the flow-through of this column is used for quantitative mass spectrometric analysis. 500-fold to 1000-fold depletion of most normal plasma proteins can be achieved in this way, enabling the detection of disease-related proteins in the low femtomole range by mass spectrometric methods. An equal number of samples from healthy donors (or other suitable controls) are processed in parallel.

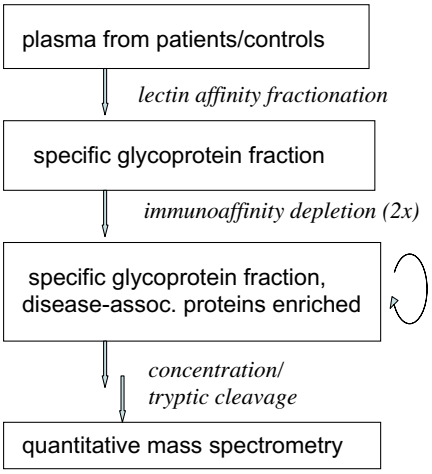

Fig. 2. Workflow 2: the lectin affinity chromatography/immunodepletion strategy.

2. Materials and methods

All reagents and solvents were analytical grade or better.

2.1. Generation of the llama antibody immunodepletion column

2.1.1. Lectin affinity fractionation of human plasma from healthy donors

One milliliter of pooled human heparin plasma (DGH, Koblenz, Germany) of healthy donors (61 mg total protein) was centrifuged at 10,000 rpm and filtered through a 0.22 μm spin centrifugal unit (Omnilab, Bremen, Germany), diluted 1:10 with WGA binding buffer (0.2 M Tris–HCl pH 7.5, 0.1 M NaCl, 1 mM EDTA) and loaded onto the lectin column (7 ml WGA agarose, 4.8 mg lectin/ml (Sigma Aldrich) in a C 10/10-column on an ÄKTA Explorer FPLC system (GE Healthcare)). The column was washed with 100 ml WGA binding buffer (flow rate 0.3 ml/min) and the lectin bound proteins eluted with 10 ml elution buffer (0.2 M Tris–HCl pH 7.5, 0.1 M NaCl, 1 mM EDTA with 0.5 M GlcNAc (Sigma Aldrich)). The eluted proteins were collected into LoBind tubes (Eppendorf, Hamburg, Germany) and concentrated on centrifugal filter units (molecular weight cut-off 10 kDa, Microcon YM-10, Millipore) to a protein concentration of 2 mg/ml. After each run, the column was rinsed with WGA binding buffer (>100 ml) until complete removal of the GlcNAc.

2.1.2. Llama immunization

A llama (*Lama glama*) was immunized by the injection of a mixture of 0.5 ml (2 mg/ml) antigen (WGA-bound plasma proteins isolated from a plasma pool of healthy individuals) and 0.625 ml adjuvant (Stimmune, Cedi Diagnostics B.V., The Netherlands). Three weeks before each blood withdrawal the animal was boosted as described above. The blood samples were centrifuged for 10 min with 2 500 rpm in order to separate the cellular components from the serum. The llama antiserum was stored at −20 °C.

The antibody titer of the llama serum was monitored by an ELISA assay. In the wells of a microtiter plate (MaxiSorp Nunc Immuno Moldule, Nunc, Denmark, Roskilde) 100 μl of 0.004 μg/μl WGA-bound proteinfraction was pipetted for seven repeat determinations and eight blank values. The microtiter plate was incubated overnight at 4 °C, washed three times with 200 μl TBST, blocked with 200 μl of 3%-BSA (dissolved in PBS) (Carl Roth, Karlsruhe, Germany) for 2 h at ambient temperature and again washed three times with TBST. Each well was incubated with 100 μl of 0.01 μg/μl llama serum for 1 h at ambient temperature and washed three times with 200 μl TBST. In each well 100 μl of a 1:30,000 goat anti llama IgG (h + l) HRP conjugated antibody solution (BETHYL Laboratories, Montgomery, TX) was added and incubated for 1 h at ambient temperature, washed three times with 200 μl TBST and 200 μl ABTS solution (PCR ELISA DIG Detection, Roche, Mannheim, Germany) added. The microtiter plate was incubated for 30 min in the dark and the absorptivity at 405 nm measured in a ELISA reader.

2.1.3. Llama antibody fractionation

Llama serum was fractionated into heavy chain and conventional IgG fractions based on their different affinities to protein G and protein A. Protein G and protein A Sepharose 4 Fast Flow™ were obtained from GE Healthcare and the chromatography done as described in Ref. [13]. 2 ml of llama serum were thawed on ice, centrifuged (10 min at 10,000 RPM), filtered 0.22 μm centrifugal unit (Spin-X, Omnilab, Bremen), diluted with an equal volume of PBS, loaded onto 5 ml protein G sepharose and incubated for 2 h at ambient temperature. The column was then washed with PBS until the OD_{280} was <0.01. The bound antibodies were eluted in