Analytical Biochemistry 497 (2016) 76–82

Contents lists available at ScienceDirect

Analytical Biochemistry

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

Enrichment and identification of glycoproteins in human saliva using lectin magnetic bead arrays

Michael Caragata [a], Alok K. Shah [a], Benjamin L. Schulz [b], Michelle M. Hill [a, **], Chamindie Punyadeera [c, *]

[a] The University of Queensland Diamantina Institute, The University of Queensland, Translational Research Institute, Woolloongabba, Queensland, 4102, Australia
[b] School of Chemistry and Molecular Biosciences, The University of Queensland, St. Lucia, Queensland, 4072, Australia
[c] School of Biomedical Sciences, Institute of Biomedical Innovations, Queensland University of Technology, Kelvin Grove, and Translational Research Institute, Woolloongabba, Queensland, 4102, Australia

ARTICLE INFO

Article history:
Received 14 August 2015
Received in revised form
23 November 2015
Accepted 24 November 2015
Available online 30 December 2015

Keywords:
Saliva proteomics
Biomarker discovery
Glycosylation
Lectins
Magnetic beads

ABSTRACT

Aberrant glycosylation of proteins is a hallmark of tumorigenesis and could provide diagnostic value in cancer detection. Human saliva is an ideal source of glycoproteins due to the relatively high proportion of glycosylated proteins in the salivary proteome. Moreover, saliva collection is noninvasive and technically straightforward, and the sample collection and storage is relatively easy. Although differential glycosylation of proteins can be indicative of disease states, identification of differential glycosylation from clinical samples is not trivial. To facilitate salivary glycoprotein biomarker discovery, we optimized a method for differential glycoprotein enrichment from human saliva based on lectin magnetic bead arrays (saLeMBA). Selected lectins from distinct reactivity groups were used in the saLeMBA platform to enrich salivary glycoproteins from healthy volunteer saliva. The technical reproducibility of saLeMBA was analyzed with liquid chromatography–tandem mass spectrometry (LC–MS/MS) to identify the glycosylated proteins enriched by each lectin. Our saLeMBA platform enabled robust glycoprotein enrichment in a glycoprotein- and lectin-specific manner consistent with known protein-specific glycan profiles. We demonstrated that saLeMBA is a reliable method to enrich and detect glycoproteins present in human saliva.

Glycosylation, the covalent addition of sugars to proteins, is a common post-translational modification, with more than 50% of all eukaryotic proteins thought to be glycosylated [1]. Glycoproteins and glycosylation play fundamental roles in many biological processes and have important cellular functions such as increasing protein stability, protecting proteins from degradation, increasing protein solubility, and regulating protein activity [2]. Glycosylation is also known to be organism, tissue, and cell type specific, regulated by the quantity and localization of glycosyltransferase enzyme and the available amount of substrate [3]. However, the equilibrium of this relationship can be disrupted in a disease state. Glycosylation is known to change within a cell from “healthy” to a “disease” state [4]. For instance, aberrant glycosylation is associated with cancer progression in breast [5], prostate [6], ovarian [7], lung [8], and hepatocellular carcinoma [9].

Human saliva is emerging as a promising biological fluid for diagnostic testing. There is 20–30% overlap in protein content between saliva and blood/plasma, suggesting that saliva could be an attractive fluid for biomarker discovery for systemic diseases and may serve as a diagnostic alternative to blood tests [10]. Compared with other body fluids such as blood, cerebral spinal fluid, and urine, the collection of whole saliva is easy and relatively stress free for the person donating it, noninvasive, and simple. As such, it allows collection of multiple samples at one time without imposing

Abbreviations used: LeMBA, lectin magnetic bead array; saLeMBA, saliva optimized lectin magnetic bead array; AAL, *Aleuria aurantia* lectin; BPL, *Bauhinia purpurea* lectin; JAC, Jacalin; SBA, soybean agglutinin; NPL, *Narcissus pseudonarcissus* lectin; SNA, *Sambucus nigra* agglutinin; ConA, concanavalin A; ECA, *Erythrina cristagalli* agglutinin; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; ABC, ammonium bicarbonate; LC–MS/MS, liquid chromatography–tandem mass spectrometry; PCA, principal component analysis.

* Corresponding author. The School of Biomedical Sciences, The Institute of Biomedical Innovations, Queensland University of Technology, 60 Musk Avenue, Kelvin Grove and the Translational Research Institute, Woolloongabba, Queensland, 4102, Australia.

** Corresponding author. The University of Queensland Diamantina Institute, The University of Queensland, Translational Research Institute, 37 Kent Street, Woolloongabba, Queensland, 4102, Australia.

E-mail addresses: m.hill2@uq.edu.au (M.M. Hill), chamindie.punyadeera@qut.edu.au (C. Punyadeera).

http://dx.doi.org/10.1016/j.ab.2015.11.024
0003-2697/

too much discomfort, is relatively safe for hospital staff to handle, and is cost-effective [11,12]. Saliva has been proposed as a diagnostic medium to detect many systemic diseases [13] such as breast cancers, lung cancers, pancreatic cancers [14], Sjögren's syndrome [15], heart failure [16], and head and neck squamous cell carcinomas [17,18].

Although glycosylation is critical for modulating protein activity, efficient and sensitive analysis of protein glycosylation frequently requires enrichment of glycoproteins or glycopeptides. Current methods for glycoprotein enrichment include hydrazide chemistry [19–21], hexapeptide libraries [22], magnetic nanoprobes [23], phenylboronic acid [24], and lectin-based techniques, including single lectin affinity chromatography, multiple lectin affinity chromatography [25], lectin microarrays [26], and lectin magnetic bead arrays [27–29]. Lectins recognize specific glycan structures with high specificity and affinity, allowing targeted enrichment of proteins modified with specific subclasses of glycans. Methods such as single/multiple and serial lectin affinity chromatography can greatly reduce the complexity of the sample being analyzed while simultaneously enriching for specific glycosylation structures of interest. However, the use of lectins for glycoprotein enrichment has drawbacks. The process can be time-consuming, lectin affinity chromatography methods are not suitable for large population-based screening studies, and no single lectin has the ability to enrich the entire glycoproteome within a biological sample [25,30,31]. Previously, we have reported [27–29] the development of lectin magnetic bead arrays (LeMBA) for efficient high-throughput glycoprotein enrichment in serum samples using a panel of lectins.

In the current study, we have optimized and developed the LeMBA methodology for use with complex biological matrices with relatively higher viscoelastic properties such as human saliva (saLeMBA). We describe an optimized protocol for saliva collection and processing, and we demonstrate that the saLeMBA technology platform is robust and reproducible and can identify protein-specific differences in glycan structures in human saliva. We anticipate that saLeMBA will be useful in identifying modifications in human salivary glycoproteins, opening up new avenues of research in the field of salivary glycoproteomics.

Materials and methods

Study design

This study was approved by The University of Queensland medical ethical institutional board and the Queensland University of Technology ethics committee (QUT 1400000617). We recruited 4 healthy nonsmoker volunteers under 30 years of age with no underlying medical conditions. Signed informed consent was obtained before saliva sample collection.

Saliva sample collection and processing

Volunteers were requested not to eat food or drink (except water) for 1 h prior to donating saliva, adhering to our previously published saliva collection protocols [12,32]. We asked the volunteers to rinse their mouths to remove any food debris and then collected unstimulated whole mouth saliva as described previously [33]. In brief, unstimulated saliva was collected by asking volunteers to tilt their head down and pool saliva in the front of their mouth for 2 min before the saliva sample was expectorated into a 50-ml Falcon tube kept on ice. The saliva was then clarified at 500 rcf for 10 min at 4 °C to remove cellular debris, and the supernatant was aliquoted into separate protein LoBind tubes (Eppendorf, Hamburg, Germany). All samples were stored at −80 °C until required. Serum was obtained from whole blood by centrifuging at 500 rcf for 15 min at 24 °C. Samples were stored at −80 °C until analysis.

Reagents

Dynabeads MyOne Tosylactivated were purchased from Life Technologies (Carlsbad, CA, USA). Lectins—*Aleuria aurantia* lectin (AAL), *Bauhinia purpurea* lectin (BPL), Jacalin (JAC), soybean agglutinin (SBA), *Narcissus pseudonarcissus* lectin (NPL), and *Sambucus nigra* agglutinin (SNA)—were purchased from Vector Laboratories (Burlingame, CA, USA), whereas lectins concanavalin A (ConA) and *Erythrina crista-galli* agglutinin (ECA) were purchased from Sigma (St. Louis, MO, USA). A Bradford assay kit, Triton X-100, 30% bisacrylamide, 5× Laemmli sample buffer, and TEMED (tetramethylethylenediamine) were purchased from Bio-Rad (Hercules, CA, USA). All other chemicals were obtained from Sigma.

Coupling lectins to DynaBeads, enriching for specific glycoproteins and SDS–PAGE

Coupling of lectins to Dynabeads MyOne Tosylactivated was performed according to previously established methods [27–29]. Saliva (50 μg of total protein and typically a protein concentration of 1–2 μg/μl) was added to denaturing buffer (final concentration 20 mM Tris–HCl buffer [pH 7.4], 1% sodium dodecyl sulfate [SDS], 5% Triton X-100, and 20 mM dithiothreitol [DTT]) to give a final volume of 75 μl and incubated at 65 °C for 30 min. Reduced cysteines in the denatured samples were alkylated by the addition of iodoacetamide to a final concentration of 100 mM and incubated in the dark for 30 min. Reduced/alkylated saliva was diluted with 1425 μl of binding buffer (20 mM Tris–HCl buffer [pH 7.4], 300 mM NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 1% Triton X-100, and 1× Protease Inhibitor Cocktail) and incubated with 50 μl of lectin-coupled magnetic beads for 1 h at 4 °C with constant rotation. The beads with captured salivary glycoproteins were washed three times in washing buffer (20 mM Tris–HCl buffer [pH 7.4], 0.05% SDS, 1 mM DTT, 300 mM NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 1% Triton X-100). For initial analysis and optimization of saLeMBA, salivary glycoproteins were eluted from the beads by resuspending them in 20 μl of 2× Laemmli sample buffer and incubating at 95 °C for 10 min. Eluted proteins were then separated by SDS–PAGE (polyacrylamide gel electrophoresis) using hand-cast 1.0-mm-thick 15% polyacrylamide gels with a Bio-Rad Mini-PROTEAN Quad 4 system. The gels were electrophoresed at a constant 100 V until the bromophenol blue reached the bottom of the gel and stained overnight in colloidal Coomassie G-250 [34]. Gels were then destained in 1% glacial acetic acid until background color was completely removed. Gels were scanned and protein bands were quantified using ImageJ (National Institutes of Health, Bethesda, MD, USA).

Identification of glycoproteins enriched with saLeMBA using LC–MS/MS

For the identification of captured salivary glycoproteins, beads were washed seven times with 20 μl of 50 mM ammonium bicarbonate (ABC), resuspended in 20 μl of 50 mM ABC with 1 μg of trypsin (Promega, Madison, WI, USA), and incubated at 37 °C for 16 h with constant rotation. The supernatant was removed and kept, and the beads were washed in 20 μl of 50 mM ABC, before combining supernatants of digested peptides. Peptide samples were transferred to U-bottom microtiter plates (Greiner Bio-One, Kremsmünster, Austria), dried in a speed vacuum at 45 °C for approximately 1 h, and stored at −80 °C until further use.

For liquid chromatography–tandem mass spectrometry (LC–MS/MS), samples were resuspended in 20 μl of 0.1% (v/v)