Analytical Biochemistry 443 (2013) 172–180

Contents lists available at ScienceDirect

Analytical Biochemistry

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

An optimized method for extraction and quantification of nucleotides and nucleotide sugars from mammalian cells

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ARTICLE INFO

Article history:
Received 3 May 2013
Received in revised form 16 August 2013
Accepted 4 September 2013
Available online 11 September 2013

Keywords:
HPLC
Nucleotides
Nucleotide sugars
Protein glycosylation
CHO cells
Metabolite extraction

ABSTRACT

Glycosylation is a critical attribute of therapeutic proteins given its impact on the clinical safety and efficacy of these molecules. The biochemical process of glycosylation is inextricably dependent on metabolism and ensuing availability of nucleotides and nucleotide sugars (NSs) during cell culture. Herein, we present a comprehensive methodology to extract and quantify these metabolites from cultured cells. To establish the full protocol, two methods for the extraction of these compounds were evaluated for efficiency, and the requirement for quenching and washing the sample was assessed. A chromatographic method based on anion exchange has been optimized to separate and quantify eight nucleotides and nine NSs in less than 30 min. Degradation of nucleotides and NSs under extraction conditions was evaluated to aid in selection of the most efficient extraction protocol. We conclude that the optimized chromatographic method is quick, robust, and sensitive for quantifying nucleotides and NSs. Furthermore, our results show that samples taken from cell culture should be treated with 50% v/v acetonitrile and do not require quenching or washing for reliable extraction of nucleotides and NSs. This comprehensive protocol should prove useful in determining the impact of nucleotide and NS metabolism on protein glycosylation.

Approximately 60% of all currently manufactured therapeutic proteins are glycosylated [1]. Given the complexity and importance of this posttranslational modification for biological activity, these glycoproteins are produced by culturing mammalian cells to ensure efficacy and safety. Several reports indicate that the presence and composition of the carbohydrates bound to glycoproteins greatly influence their therapeutic function by determining their serum half-life [2,3], eliciting undesired immune response in patients [4], or modulating the mechanisms by which they act in vivo [5,6]. In addition, cell culture conditions have been shown to determine the extent of glycosylation as well as the monosaccharide composition of the carbohydrates bound to glycoproteins [7–9].

The two fundamental types of glycosylation are defined by the amino acid residue onto which carbohydrates are bound. In O-linked glycosylation, carbohydrates are bound to serine, threonine, and tyrosine residues of proteins, whereas N-linked glycosylation refers to carbohydrates that are bound to the asparagine in the amino acid sequon Asn-X-Ser or Asn-X-Thr, where X is any amino acid except proline or asparagine. Despite differences in the initiation of the process, both O- and N-linked glycosylation require the addition of monosaccharides to the protein-bound carbohydrate [10]. The addition of each monosaccharide is catalyzed by a glycosyl-transferase enzyme, which requires nucleotide sugars as cosubstrates for the reaction. The nucleotide sugars most commonly involved in mammalian glycosylation are uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc), uridine diphosphate *N*-acetylgalactosamine (UDP-GalNAc), uridine diphosphate glucose (UDP-Glc), uridine diphosphate galactose (UDP-Gal), guanosine diphosphate mannose (GDP-Man), guanosine diphosphate fucose (GDP-Fuc), cytidine monophosphate *N*-acetylneuraminic acid (CMP-Neu5Ac), uridine diphosphate glucoronic acid (UDP-GlcA), and cytidine monophosphate N-glycolylneuraminic acid (CMP-Neu5Gc). Additional metabolites, such as ribonucleotide triphosphates, are key metabolites for the biosynthesis of nucleotide sugars. These molecules are also prevalent determinants of nucleic acid synthesis, cellular growth, and energy metabolism. The most common nucleotide triphosphates in living organisms are adenosine triphosphate (ATP), cytidine triphosphate (CTP), guanosine triphosphate (GTP), and uridine triphosphate (UTP). Other nucleotides that are closely involved in energy metabolism, nucleotide sugar biosynthesis, and protein glycosylation are the mono- and diphosphate versions of the nucleotides mentioned above: adenosine diphosphate (ADP), adenosine monophosphate (AMP), cytidine diphosphate, cytidine monophosphate (CMP), guanosine

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0003-2697/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ab.2013.09.005

diphosphate (GDP), guanosine monophosphate (GMP), uridine monophosphate (UMP), and uridine diphosphate (UDP).

Previous studies have found that the availability of nucleotide sugar donors determines the presence or absence of complex carbohydrates on the peptide backbone of the glycoprotein (macroheterogeneity) [11,12] and also the monosaccharide composition of the carbohydrate bound to the protein (microheterogeneity) [13–16]. Because of these effects, several attempts have been made to extract and quantify these compounds from cells in culture. Analytical techniques for nucleotide and nucleotide sugar analysis include high-performance anion-exchange chromatography (HPAEC) [17,18], reverse-phase ion-pairing high-performance liquid chromatography [19–22], and capillary electrophoresis [23,24]. Despite the encouraging results from these reports, many of the analytical techniques have limitations regarding the length of analysis time [17,18] (>50 min) or detection of certain species [19–21] and, in some cases, are based on sophisticated detection or separation methods [22,25] or require laborious preparation of the mobile phase(s) [21,24].

In addition to the analytical method, recent reports suggest that cell culture sample treatment prior to extraction (washing and quenching), as well as the metabolite extraction protocol, may greatly influence the measurement of intracellular metabolite pools [25–28]. The objective of these procedures is to ensure that (i) the extraction method allows for reproducible retrieval of the desired metabolites, (ii) the metabolite pools to be extracted are representative of the metabolic state of the cells at the moment of extraction, and (iii) components from the cell culture medium do not contaminate the intracellular samples.

This article describes an optimized method for nucleotide and nucleotide sugar extraction and quantification from mammalian cells. Extractions with acetonitrile (ACN) and perchloric acid (PCA) were compared to define the most suitable protocol for nucleotide and nucleotide sugar (NS) recovery. The need for quenching and washing of the cell culture sample was also evaluated to assess the impact of these processes on the observed intracellular nucleotide and NS pools. Finally, an HPAEC method for nucleotide and NS quantification, based on that of Tomiya et al. [18], was optimized to increase throughput and resolution power. The result was a method able to resolve nine nucleotide sugars and eight nucleotides in under 30 min (including reequilibration of the column). We report the validity of the optimized analytical method by discussing its linearity, reproducibility, and sensitivity. The overall platform allows for rapid, convenient, and accurate measurement of intracellular nucleotide and NS pools in the context of evaluating the effect of cellular metabolism on recombinant protein glycosylation.

Materials and methods

HPAEC analysis

The HPAEC analysis was performed on an Alliance HPLC system (Waters, UK), which is composed of a Series 2695 separations module coupled to a Model 2998 photodiode array detector and controlled by the Empower 2 software (Waters, UK). Separation was carried out with a CarboPac PA-1 column with a PA-1 guard column (Dionex, USA). The mobile phases used as eluents for the chromatographic method were 3 mM NaOH (E1) and 1.5 M sodium acetate in 3 mM NaOH (E2). These eluents were prepared on the day of analysis and filtered using 0.2-μm filter units (Nalgene, UK). The column temperature was maintained at 30 °C throughout separation. Elution of the sample was carried out using the following gradient: $t_{0\ min}$, 20% E2; $t_{6\ min}$, 66% E2; $t_{18\ min}$, 66% E2; $t_{23\ min}$, 20% E2; $t_{30\ min}$, 20% E2. Detection was performed at 262.1 and 271.6 nm simultaneously to ensure maximum absorbance of all species present in the mixtures (all cytidine-containing species were detected at 271.6 nm and all others were detected at 262.1 nm). Analysis of the chromatographic data was performed with the Empower 2 software (Waters), and all peak assignments and integrations were performed automatically with a user-defined data processing method on the software. The 19 compounds that were used as standards for method optimization and calibration were ATP, CTP, GTP, UTP, CMP-Neu5Ac, UDP-GalNAc, UDP-GlcNAc, UDP-Gal, UDP-Glc, GDP-Fuc, GDP-Man, GDP-Glc, UDP-GlcA, AMP, ADP, CMP, GMP, UMP, and UDP, and all were purchased from Sigma–Aldrich, UK. Calibration of the HPAEC method was achieved by serially diluting a stock mixture (each analyte at 0.08 mM) in deionized water (18.2 MΩ).

Cell culture

Suspension Chinese hamster ovary (CHO) cells (Invitrogen, UK) were cultured as indicated by the manufacturer. The culture medium employed was CD-CHO (Invitrogen) supplemented with 8 mM glutamine (Invitrogen) and 10 ml/L 100× HT supplement (Invitrogen). The cells were cultured in 250-ml shake flasks (Corning, UK) with a working volume of 50 ml at 8% v/v CO_2 and 37 °C in a temperature- and CO_2-controlled incubator (NuAire, UK). The cells were maintained in suspension by stirring at 125 rpm on an orbital shaker (Stuart, UK). Cell numbers and viability were determined by the trypan blue dye exclusion method.

Extraction of intracellular nucleotides and nucleotide sugars

The procedure for perchloric acid extraction was based on previous reports [21,27] and was conducted as follows: 1.5×10^7 cells were removed from the culture and centrifuged (1000*g*, 1 min). After the supernatant was discarded, the pellet was resuspended in 200 μl of ice-cold 0.5 M PCA (Sigma–Aldrich). At this point, 2 μl of 20 mM GDP-Glc (a NS that does not naturally occur in CHO cells) was added as an internal standard. The pellet/PCA mixture was incubated on ice for 5 min and then centrifuged (0 °C, 18,000*g*, 5 min) in a refrigerated centrifuge (Hermle, Germany). The supernatant was transferred to a new microcentrifuge tube containing 40 μl of ice-cold 2.5 M KOH (Sigma–Aldrich) in 1.1 M K_2HPO_4 (Sigma–Aldrich). This mixture was incubated on ice for 5 min and then centrifuged (0 °C, 18,000*g*, 5 min). The supernatant from this sample was removed and stored at −80 °C until HPAEC analysis. Immediately prior to HPAEC analysis, the sample was quickly thawed at room temperature and filtered using 0.2-μm syringe filter units (Fisher Scientific, UK).

Acetonitrile extraction, based on [27,29], consisted of adding 3 ml of ice-cold 50% v/v aqueous acetonitrile (Sigma–Aldrich) and 2 ml of 20 mM GDP-Glc to 1.5×10^7 cells. The resulting suspension was incubated on ice for 10 min, after which it was centrifuged (0 °C, 18,000*g*, 5 min). The supernatant was collected and dried using a SpeedVac (Savant, USA). The dried extract was resuspended in 240 μl of deionized water and stored at −80 °C until HPAEC analysis. The sample was thawed at room temperature and filtered using 0.2-μm syringe filter units (Fisher Scientific) immediately prior to HPAEC analysis.

Cell pellet quenching and washing

Where applicable, the cell culture sample was either quenched or both quenched and washed prior to metabolite extraction. Based on previous findings, where various quenching methods were compared for nucleotide and NS extraction [27,28], ice-cold 0.9% w/v aqueous NaCl (Sigma–Aldrich) was selected as the quenching and washing solution. For quenching alone, 1 volume of cell