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Simultaneous determination of intracellular UDP-sugars in hyaluronic acid-producing *Streptococcus zooepidemicus*

Lukáš Franke, Dagmar Čožíková*, Dzianis Smirnou, Martina Hermannová, Tereza Hanová, Andrea Růžičková, Vladimír Velebný

Contipro Pharma, Dolní Dobrouč 401, 561 02 Dolní Dobrouč, Czech Republic

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ABSTRACT

Two chromatographic methods for the quantitative analysis of uridine diphosphate (UDP) sugars involved in hyaluronan pathway of *Streptococcus zooepidemicus* (SEZ) were developed and compared. The sample preparation protocol using centrifugation and extraction in hot ethanol was employed prior to the analyses. Separation was achieved using an anion exchange Spherisorb SAX column or a Shodex QA-825 column connected with a photodiode array (PDA) detector. To increase the throughput of the chromatography method employing the Spherisorb SAX column, the solid phase extraction (SPE) procedure was introduced. Method validation results displayed that limits of detection (LODs) of UDP-glucose (UDP-Glc), UDP-N-acetylglucosamine (UDP-GlcNAc) and UDP-glucuronic acid (UDP-GlcA) calculated according to QC Expert software were in the low micromolar range and the coefficient of correlation (R^2) was above 0.997. However, the analytical technique using the Spherisorb SAX column resulted in 80–90% recoveries and low LODs ($\leq$6.19 μM), the Shodex QA-825 column showed better long-term stability and reproducible chromatographic properties (RSD $\leq$ 5.60%). The Shodex QA-825 column was successfully used to monitor UDP-sugar levels during the growth rate of SEZ cells.

1. Introduction

Hyaluronan (hyaluronic acid, HA) is a linear polysaccharide composed of alternating D-glucuronic acid and *N*-acetylglucosamine linked by β(1,4) and β(1,3) glycosidic bonds. This macromolecule is found in various tissues of vertebrates, where it has different biological functions. Nowadays, HA has become one of the nature's most versatile and fascinating materials. The quality of HA is determined by its molecular weight (MW), with high MW HA having applications in the biomedical industries, while low MW HA is used in cosmetics [1,2].

Industrially, HA has been manufactured by fermentation of group C streptococci since the early 1980s. One such bacterial producer of HA is *Streptococcus equi* subspecies *zooepidemicus* (SEZ). Various strains of streptococci, including wild-type and HA-high-producing mutants, were adopted to attain high yields of HA. Currently, quality factors such as purity and size distribution rather than quantity have been the focus of strain and process development in HA production [3].

HA is produced by processive synthase from the activated UDP-sugars (UDP-GlcA and UDP-GlcNAc). In addition to the HA synthase (*hasA*), streptococcal *has* operons encode one or more enzymes involved in biosynthesis of UDP-sugars [4,5]. Whereas, the biosynthetic pathway for HA in streptococci is well established [6–8], little is known about what controls HA molecular weight. Several studies have proven that MW is affected by culture parameters, e.g. temperature and aeration [9–12]. Although culture conditions affect the physicochemical environment of the HA synthase, a more likely explanation is that MW is influenced by the availability of activated UDP-sugars (UDP-GlcNAc and UDP-GlcA) as well as the concentration of possible effector molecules, such as free UDP [4,13,14].

Investigation of the metabolism of activated UDP-sugars requires rapid analytical method that allows the separation, structural characterization and quantification of substrates, intermediates and end products. Several high-performance liquid chromatography (HPLC) methods for the analysis of UDP-sugars have been developed, including ion exchange chromatography (IEC), reversed-phase liquid chromatography (RPLC) and more recently the ion-pair chromatography (IPC) [15–18]. HPLC has been shown to be one of the most simple and fast technique for measuring these metabolites. UDP-sugars detection in these methods is based on absorption of light at a wavelength around

* Corresponding author.
E-mail address: Dagmar.Cozikova@contipro.com (D. Čožíková).

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260 nm [19–21]. IEC methods are also coupled with conductometric [22,23] or pulsed amperometric detection [24–26]. However, major concerns reported in the literature regarding such columns are long separation times, and poor long-term stability. Similarly, in studies using well established IPC method widely applied for the analysis of UDP-sugars in fermentation media, UDP-hexose and UDP-hexosamines cannot be quantified individually in the presence of common nucleotides [27,28].

There are also reports on the use of mass spectrometry (MS) and nuclear magnetic resonance (NMR) to identify and quantify UDP-sugars from cells [17,29], however these methods require equipment and expertise not readily available in many laboratories. Furthermore, mass spectrometry-assisted identification cannot be combined with all separation methods. Capillary electrophoresis (CE) has likewise been used for the separation and determination of UDP-sugars and related compounds with promising results [30,31]. Despite the encouraging results from a wide variety of above mentioned methods, many of these techniques have limitations regarding the detection of certain molecules and in some cases, are based on sophisticated detection or separation methods or require laborious preparation of mobile phases. In addition, metabolite samples are very complex and the method needs to be accurate and sensitive enough to determine low concentrations.

This paper describes two newly developed IEC methods with UV detection for the determination of intracellular UDP-sugars (UDP-Glc, UDP-GlcA and UDP-GlcNAc) involved in HA biosynthesis. The aim was to resolve the target UDP-sugars and quantify them in the SEZ cells extract samples. Besides, the validity of both chromatographic techniques is reported by discussing its linearity, reproducibility and recovery.

2. Experimental

2.1. Chemicals

UDP-sugars (uridine 5′-diphosphoglucose disodium salt, uridine 5′-diphosphoglucuronic acid trisodium salt, uridine 5′-diphospho-*N*-acetylglucosamine sodium salt), sodium hydroxide, sodium phosphate monobasic dehydrate, cetyltrimethylammonium bromide (CTAB) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Sigma (Prague, Czech Republic). Ammonium acetate, methanol, sodium acetate, acetic acid, sodium chloride, trisodium citrate dihydrate and ethanol were obtained from Lach:ner (Neratovice, Czech Republic). Water was deionized and filtered through a 0.20 μm nylon membrane filter (Millipore, Prague, Czech Republic) by using a Labicom water generation system (Olomouc, Czech Republic).

2.2. Strain culture and medium

HA producing strain was obtained from Czech Collection of Microorganisms (CCM, Masaryk University, Brno, Czech Republic). Freeze-dried cells were revived by plating on THY agar (37 g/l Todd Hewitt Broth, 3 g/l yeast extract, 20 g/l agar). Colonies were inoculated into Erlenmeyer flasks with 100 ml of medium (37 g/l Todd Hewitt Broth, 5 g/l yeast extract, 40 g/l sucrose) and incubated in a rotary shaker for 7 h at 37 °C and 150 rpm. The 32 ml of seed culture was inoculated into a bench-top bioreactor (Multifors, Infors HT, Switzerland) containing 800 ml of medium with 200 μl/l Antifoam 204 (Sigma, Prague, Czech Republic). The bioreactor was sparged with filter-sterilized air at 1 vvm and agitated at 300 rpm. The pH was measured by pH electrode (Hamilton, Switzerland) and automatically regulated at 7.0 by addition of 20% NaOH. The reactor was operated at 37 °C for 16 h.

2.3. Preparation of cellular extracts

At 4th, 8th and 14th hours of cultivation 20 ml cell suspension were diluted with 20 ml of deionized water, centrifuged (100,000 × *g*, 2 min, 37 °C, Sorvall LYNX 6000, Thermo Scientific, USA) and extracted by hot ethanol according to an established protocol described previously by Marcellin et al. [25]. Finally, 10 min of sonication on ice bath (U200S control, IKA Labortechnik, Staufen, Germany) was employed to optimize the extraction procedure. Ethanol was evaporated till dryness (waterbath T = 30 °C, Rotavapor Büchi, Flawil, Switzerland) and the samples were reconstituted in 1 ml of water. Samples were stored at −40 °C until analyzed. UDP-sugars concentrations were normalized using the corresponding cell dry weight (CDW). Values were expressed as μmol/g of CDW.

One half of SEZ cell extract samples was injected directly into chromatography system whereas the other half was subjected to solid phase extraction (SPE) procedure using 500 mg Strata-X-A strong anion exchange & reversed phase columns (6 ml reservoir, Phenomenex, Prague, Czech Republic). The SPE column was activated by flushing with 3 ml of MeOH and 3 ml of deionized water. 3 ml of the sample was loaded onto the column. To remove impurities from the sample the SPE column was flushed with 3 ml of 25 mM CH_3COONH_4 followed by flushing with 3 ml of MeOH. Finally, the metabolites were eluted from the column by 3 ml of 0.2 M sodium citrate. The eluent was collected into 5 ml glass tubes and evaporated till dryness (waterbath T = 39 °C). The purified sample of the metabolite extract was dissolved in 1 ml of water, filtered through a 0.22 μm nylon syringe filters (Chromservis, Prague, Czech Republic) and injected into chromatography system.

2.4. Chromatographic conditions

All experiments were carried out on an Alliance HPLC system (e2695 Separation Module) equipped with 2998 photodiode array (PDA) detector (Waters, Prague, Czech Republic). UV signal was simultaneously measured at 260 nm. Injection volume was 10 μl. The temperature in autosampler was set at 5 °C. Data were collected using the Empower3 chromatography software. The analyses were performed on an anion exchange Spherisorb 5 μm SAX column (Waters, Prague, Czech Republic) or on a Shodex QA-825 column (Chromservis, Prague, Czech Republic) at ambient temperature. When the Spherisorb SAX column was used, the chromatographic eluent consisted of a binary phase made from 10 mM NaH_2PO_4 (A) and 10 mM NaH_2PO_4 with 0.5 M NaCl (B) was pumped at a constant flow rate of 1 ml/min. The following gradient mode was used for the separation: B increased from 0 to 100% over 40 min, decreased to 0% over 2 min, held at 0% for 8 min. Shodex QA-825 column was used with a flow rate of 1 ml/min. UDP-sugars were eluted with the gradient of eluent A (10 mM NaCl) and eluent B (250 mM NaCl) as follows: B increased from 2 to 100% over 50 min, decreased to 2% over 2 min, held at 2% for 8 min.

2.5. Validation procedure

Primary stock solution was prepared in water at a concentration of 300 μM for each standard (UDP-Glc, UDP-GlcA and UDP-GlcNAc). Calibration curves were obtained by analysing stock standard mixture at eleven concentrations, ranging from 4 μM to 200 μM. Calibration curves were performed in triplicate to enable statistical assessment of the variation in measured concentration. The area under each peak was plotted against the known concentration of each compound added to the standard mixture. The limits of detection (LODs) and limits of quantification (LOQs) were calculated according to QC Expert software (TriloByte Statistical Software, Pardubice, Czech Republic).