Analytical Biochemistry 427 (2012) 91–98

Contents lists available at SciVerse ScienceDirect

Analytical Biochemistry

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

Analysis of glycosaminoglycan-derived disaccharides by capillary electrophoresis using laser-induced fluorescence detection

Yuqing Chang [a], Bo Yang [a], Xue Zhao [a,b], Robert J. Linhardt [a,c,d,e,*]

[a] Department of Chemistry and Chemical Biology, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180, USA
[b] College of Food Science and Technology, Ocean University of China, Qingdao, Shandong 266003, People's Republic of China
[c] Department of Biology, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180, USA
[d] Department of Chemical and Biological Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180, USA
[e] Department of Biomedical Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180, USA

ARTICLE INFO

Article history:
Received 14 April 2012
Received in revised form 5 May 2012
Accepted 7 May 2012
Available online 15 May 2012

Keywords:
2-Aminoacridone
Capillary electrophoresis
Chondroitin/dermatan sulfate
Heparan sulfate/heparin
Hyaluronan
Glycosaminoglycan

ABSTRACT

A quantitative and highly sensitive method for the analysis of glycosaminoglycan (GAG)-derived disaccharides that relies on capillary electrophoresis (CE) with laser-induced fluorescence detection is presented. This method enables complete separation of 17 GAG-derived disaccharides in a single run. Unsaturated disaccharides were derivatized with 2-aminoacridone to improve sensitivity. The limit of detection was at the attomole level and approximately 100-fold more sensitive than traditional CE–ultraviolet detection. A CE separation timetable was developed to achieve complete resolution and shorten analysis time. The relative standard deviations of migration time and peak areas at both low and high concentrations of unsaturated disaccharides are all less than 2.7 and 3.2%, respectively, demonstrating that this is a reproducible method. This analysis was successfully applied to cultured Chinese hamster ovary cell samples for determination of GAG disaccharides. The current method simplifies GAG extraction steps and reduces inaccuracy in calculating ratios of heparin/heparan sulfate to chondroitin sulfate/dermatan sulfate resulting from the separate analyses of a single sample.

Proteoglycans (PGs)[1] are a group of glycoconjugates ubiquitously presented in the extracellular matrix and on the surface of all eukaryotic cells, as well in basement membranes of various tissues, where they participate in many cellular, physiological, and pathological processes such as chemokine and cytokine activation, microbial recognition, tissue morphogenesis during embryonic development, immune response, and tumor progression and invasion [1–6]. PGs are composed of various core proteins posttranslationally modified with 1 to more than 100 long, unbranched, and anionic polysaccharides called glycosaminoglycans (GAGs) [7]. GAGs are composed of repeating disaccharide units of hexuronic acid, D-glucuronic acid (GlcA) and/or its C-5 epimer L-iduronic acid (IdoA), and hexosamine, D-glucosamine (GlcN) or D-galactosamine (GalN) [8–10]. With the exception of hyaluronic acid (HA), GAGs are sulfated polysaccharides with complex structures that are different based on degree of charge, pattern of sulfo group substitution, and hexuronic acid epimerization [4,5]. There are three major classes of GAGs in animals differing by their polysaccharide backbone structure: HA, heparan sulfate (HS)/heparin (HP), and chondroitin sulfate (CS)/dermatan sulfate (DS). HA, the simplest GAG that neither contains sulfo groups nor is attached to a core protein, is composed of a → 3) β-GlcNAc (1 → 4) β-GlcA (1 → repeating unit (where Ac is acetyl). HS/HP are *O*-sulfo group substituted GAGs with → 4) α-GlcNAc or α-GlcNS (1 → 4) β-GlcA or α-IdoA (1 → repeating units (where S is sulfo). CS/DS are *O*-sulfo group substituted GAGs with → 3) β-GalNAc (1 → 4) β-GlcA or α-IdoA (1 → repeating units [5]. Biosynthesis of GAGs takes place in Golgi and begins with the stepwise addition of four monosaccharides acting as a tetrasaccharide linker on a core protein serine residue in the endoplasmic reticulum [7]. Sugar chain elongation of GAGs then occurs as the sequential addition of uridine diphosphate (UDP)–GlcNAc and UDP–GlcA, determining that the GAG chain belongs to the HS/HP family, or as the addition of

* Corresponding author at: Department of Chemistry and Chemical Biology, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180, USA. Fax: +1 518 276 3405.
E-mail address: linhar@rpi.edu (R.J. Linhardt).

[1] *Abbreviations used:* PG, proteoglycan; GAG, glycosaminoglycan; GlcA, D-glucuronic acid; IdoA, L-iduronic acid; GlcN, D-glucosamine; GalN, D-galactosamine; HA, hyaluronic acid; HS, heparan sulfate; HP, heparin; CS, chondroitin sulfate; DS, dermatan sulfate; Ac, acetyl; S, sulfo; UDP, uridine diphosphate; HPLC, high-performance liquid chromatography; UPLC, ultra-performance liquid chromatography; GPC, gel permeation chromatography; PAGE, polyacrylamide gel electrophoresis; CE, capillary electrophoresis; UV, ultraviolet; MS, mass spectrometry; NMR, nuclear magnetic resonance; LIF, laser-induced fluorescence; ΔUA, 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid; 2-AP, 2-aminopyridine; APTS, 8-aminopyrene-1,3,6-trisulfonate; ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; AMAC, 2-aminoacridone; Et, ethyl; IS, internal standard; CHO, Chinese hamster ovary; HT, hypoxanthine/thymidine mixture; MWCO, molecular weight cutoff; RSD, relative standard deviation; LOQ, limit of quantification; RP, reversed-phase.

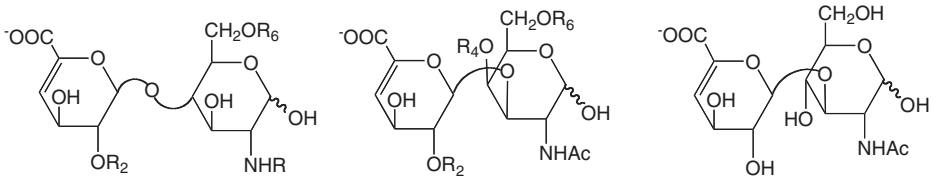
0003-2697/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ab.2012.05.004

UDP–GalNAC and UDP–GlcA, determining that the GAG chain belongs to the CS/DS family. Then the growing chains of GAGs can be modified at various positions: N-deacetylation/N-sulfonation of GlcNAc units catalyzed by *N*-deacetylase/*N*-sulfotransferases in HS/HP chains, C-5 epimerization of GlcA to IdoA catalyzed by C-5 epimerase in HS/HP and also DS chains (and modification of IdoA catalyzed by 2-*O*-sulfotransferase can then take place), O-sulfonation in GlcNAc units catalyzed by 3-*O*-sulfotransferase and 6-*O*-sulfotransferase in HS/HP, and O-sulfonation in GalNAc units catalyzed by 4-*O*-sulfotransferase or 6-*O*-sulfotransferase [7,11,12]. These postpolymerization enzymatic modification steps result in the heterogeneity and diversity in the disaccharide residues of GAG polysaccharide chains as well as in the polydispersity of chain sizes, all of which are responsible for the crucial biological functions of GAGs; for example, HS/HP are implicated in critical biological processes, such as regulation of enzymatic catalysis in the coagulation cascade and cell–cell interactions [13,14], and CS/DS may be involved in participating and mediating cell–cell interactions and cellular communication [15].

GAGs are extremely difficult to analyze because of their negative charge, polydispersity, and structural heterogeneity [16]. A common strategy of detailed structural analysis of GAGs involves the enzymatic depolymerization of GAGs to obtain their disaccharide constituents. The GAG disaccharides produced by exhaustive lyase-catalyzed digestion contain a Δ-unsaturated hexuronic acid at their nonreducing end with a unique absorbance at 232 nm [17,18] and a molar extinction coefficient of approximately 6000 $M^{-1}\,cm^{-1}$ [19]. Exhaustive heparin lyase treatment of HS/HP affords eight major HS/HP-derived disaccharides. There are also several rare HS/HP-derived disaccharides that can be formed from among the 23 possible known disaccharide sequences [11]. Chondroitin lyase treatment of CS/DS or HA produces eight CS/DS disaccharides and one HA disaccharide. The structures of 17 commercially available, lyase-prepared HS/HP, CS/DS, and HA disaccharides are shown in Table 1. Following the sequential enzymatic treatment, quantitative disaccharide analysis was applied to explore the structural information, which is directly related to biological functions of GAGs. Modern separation techniques, such as high-performance liquid chromatography (HPLC) [20–22], ultra-performance liquid chromatography (UPLC) [23,24], gel permeation chromatography (GPC) [25,26], polyacrylamide gel electrophoresis (PAGE) [27], and capillary electrophoresis (CE), have been applied to GAG analysis to help solve many complex structures [28–31].

CE is one of the most powerful techniques for GAG analysis because of its high sensitivity, resolving power, and separation efficiency combined with its short analysis time, straightforward operation [32], and compatibility with a variety of detection methods, including ultraviolet (UV) spectroscopy, mass spectrometry (MS) [33], nuclear magnetic resonance (NMR) spectroscopy, and laser-induced fluorescence (LIF) [28,34,35]. Analysis of GAGs, performed by CE with both normal and reversed polarity [36,37], has been reviewed previously [32,38]. In reversed polarity, low pH is used to reduce electroosmotic flow, and the analyte, applied at the cathode, migrates under electrophoresis toward the detector at the anode. Determination of disaccharides from complex biological sample containing only a few micrograms of GAGs often requires higher sensitivity than is typically associated with UV detection, and some GAG disaccharides do not have a 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid (ΔUA) residue for UV detection at 232 nm [39,40]. Moreover, limited repeatability of migration time and peak areas resulting from complex biological matrix and microheterogeneity in separation environment poses significant challenges to GAG analysis by CE. The conjugation of a fluorophore can greatly increase detection sensitivity using LIF [41]. One of the most frequently used derivatization methods is reductive amination of carbohydrates, which frequently relies on aromatic fluorescent amines, including 2-aminopyridine (2-AP) [42], 8-aminopyrene-1,3,6-trisulfonate (APTS) [43], 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) [44], and 2-aminoacridone

Table 1
Structures of 17 ΔUA–disaccharide standards from HP/HS, CS/DS, and HA



Reference number	Disaccharide	Structure	R		
HS/HP disaccharides, up left			R_2	R_6	R
1	$TriS_{HS}$	ΔUA(2S)–GlcNS(6S)	SO_3^-	SO_3^-	SO_3^-
2	$2S6S_{HS}$	ΔUA(2S)–GlcNAc(6S)	SO_3^-	SO_3^-	Ac
3	$2SNS_{HS}$	ΔUA(2S)–GlcNS	SO_3^-	H	SO_3^-
4	$NS6S_{HS}$	ΔUA–GlcNS(6S)	H	SO_3^-	SO_3^-
5	$2S_{HS}$	ΔUA(2S)–GlcNAc	SO_3^-	H	Ac
6	$6S_{HS}$	ΔUA–GlcNAc(6S)	H	SO_3^-	Ac
7	NS_{HS}	ΔUA–GlcNS	H	H	SO_3^-
8	$0S_{HS}$	ΔUA–GlcNAc	H	H	Ac
CS/DS disaccharides, up middle			R_2	R_4	R_6
9	$TriS_{CS}$	ΔUA(2S)–GalNAc(4S)(6S)	SO_3^-	SO_3^-	SO_3^-
10	SD_{CS}	ΔUA(2S)–GalNAc(6S)	SO_3^-	H	SO_3^-
11	SB_{CS}	ΔUA(2S)–GalNAc(4S)	SO_3^-	SO_3^-	H
12	SE_{CS}	ΔUA–GalNAc(4S)(6S)	H	SO_3^-	SO_3^-
13	$2S_{CS}$	ΔUA(2S)–GalNAc	SO_3^-	H	H
14	$6S_{CS}$	ΔUA–GalNAc(6S)	H	H	SO_3^-
15	$4S_{CS}$	ΔUA–GalNAc(4S)	H	SO_3^-	H
16	$0S_{CS}$	ΔUA–GalNAc	H	H	Ac
HA disaccharide, up right					
17	$0S_{HA}$	ΔUA–GlcNAc	H	H	H