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Determining the extent of heparan sulfate depolymerisation following heparin lyase treatment

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ABSTRACT

The depolymerisation of porcine mucosal heparan sulfate under the action of heparin lyases and analysis by size-exclusion chromatography (SEC) is described. Heparan sulfate treated to enzymic bond scission producing a $\Delta_{4,5}$ double-bond and quantified by SEC with ultraviolet-visible (UV) spectroscopic detection (230 nm) indicated that the majority of the biopolymer (>85%) was reduced to disaccharides (degree of polymerisation (DP) = 2). However, analysis of the SEC eluant using refractive index (RI), which reflects the mass contribution of the oligosaccharides rather than the molar response of a UV chromophore, indicated that a considerable proportion of the digested HS, up to 43%, was present with DP >2. This was supported by a mass balance analysis. These results contradict the accepted literature where "complete digestion" is routinely reported. Herein we report on the composition and methodology utilised to ascertain the extent of depolymerization and disaccharide composition of this important biopolymer.

1. Introduction

Heparan sulfate (HS) is part of the family of highly complex, linear glycosaminoglycan (GAG) polysaccharides implicated in a huge number of biological processes. The function of HS molecules stems from their ability to bind specifically to a variety of proteins (including growth factors, enzymes, morphogens etc.) and modulate their activity (Whitelock and Iozzo, 2005). The role HS plays in this context has led to significant investigation of this molecule as a human therapeutic, either alone or in conjunction with growth factors (Murali et al., 2013; Wang et al., 2014). HS consists of a repeating disaccharide unit of α-glucosamine (GlcN), linked 1 → 4 to uronic acid, either as glucuronic acid (GlcA) or its C-5 epimer, iduronic acid (IdoA). Heterogeneity of the HS structure arises from a combination of the pattern of uronic acid in the disaccharide moiety, as well as whether the GlcN is *N*-acetylated, *N*-sulfated or unsubstituted. The uronic acid may also be esterified with a sulfate group at the O-2 position and the GlcN sulfated at the O-3 or the O-6 positions (Rabenstein, 2002). The specificity of binding appears to be dependent on chain length, sulfation pattern and subsequent conformation of the HS polysaccharide (Kreuger, Salmivirta, Sturiale, Giménez-Gallego & Lindahl, 2001; Kreuger, Spillmann, Li & Lindahl, 2006; Xu and Esko, 2014).

To obtain data on the overall composition of HS, it is usual to obtain a disaccharide composition by digestion of the polysaccharide with a combination of three heparin lyase (HL) enzymes that cleave the glucosamine-uronic acid linkages to give oligosaccharides with a C4–C5 unsaturated bond (Korir and Larive, 2009). HL I cleaves highly sulfated regions of heparin and HS containing 2-*O*-sulfated iduronic acid residues, while HL III cleaves linkages in regions of lower sulfation. HL II has a broad specificity for regions containing both iduronic and glucuronic acid with varying degrees of *O*-sulfation (LeBrun and Linhardt, 2001). Although enzymatic depolymerisation is widely used for ascertaining the gross composition of HS, there is no standard method in the literature for generating disaccharides by the concerted action of the three HL enzymes. The majority of researchers conduct the reactions at 37 °C and pH 7.0, and use varying concentrations of sodium acetate (10–250 mM), containing a small amount of calcium acetate (0.1–10 mM). This system is not buffered and so the reaction is not necessarily maintained at pH 7.0 throughout (Brickman, Ford, Gallagher, Nurcombe, Bartlett & Turnbull, 1998; Deakin and Lyon, 2008; Galeotti & Volpi, 2013; Skidmore, Guimond, Dumax-Vorzet, Yates & Turnbull, 2010). In some cases reactions have been done

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in sodium phosphate buffer (50 mM, pH 7.0–7.5) with or without 100 mM NaCl and at 30–37 °C, based on the methods of Griffin and Linhardt (Griffin et al., 1995; Linhardt, 2001). There is large variation in the amounts of the enzymes used, how they are added to the reactions (simultaneously, or sequentially) and the incubation times (Brickman et al., 1998; Galeotti and Volpi, 2013; Karamanos, Vanky, Tzanakakis & Hjerpe, 1996; Skidmore, Guimond, Dumax-Vorzet, Atrih, Yates & Turnbull, 2006).

The conventional view has been that the combination of these three enzymes will almost completely digest the HS and heparin molecules to their component disaccharides, with the exception of a small amount of lyase-resistant biopolymer that remains as a tetrasaccharide. Consequently, the disaccharide composition obtained represents the entire molecule. Many workers have reported a >90% yield of disaccharides, based on peak areas with UV detection at 232 nm or using radiolabeled HS substrate and determining the proportion of radioactivity recovered as disaccharide (Brickman et al., 1998; Karamanos, Vanky, Tzanakakis, Tsegenidis & Hjerpe, 1997; Lyon, Deakin & Gallagher, 1994; Murata, Murata & Yoshida, 1995). However, both methods of detection measure a molar rather than a mass contribution. In the case of UV detection the portion of > dp2 material present in the digests is underestimated and, consequently, the disaccharide proportion is overestimated. Indeed, Ziegler and Zaia (2006) showed that, following exhaustive digestion of heparin with the three HL enzymes, more than 50% of the molecule was not depolymerised to disaccharides as determined by mass recovery following preparative size-exclusion chromatography (SEC). Yet integration of the UV signal estimated that >70% of the digest was disaccharides (Ziegler and Zaia, 2006).

We have an ongoing interest in the composition of HS and subtle differences in the composition of disperse HS populations can be associated with notable differences in biological activity (Murali et al., 2013; Wang et al., 2014). Therefore, in the present work we investigated the extent of digestion of commercially available HS (porcine mucosa and bovine kidney) and heparin (porcine mucosa) by the combination of commercially available HL I, II and III using SEC, comparing UV (230 nm) and refractive index (RI) detection, and quantitative disaccharide analysis by strong anion exchange-high performance liquid chromatography (SAX-HPLC) with UV (232 nm) detection.

2. Materials and methods

2.1. Materials

Porcine mucosal HS was from Celsus Laboratories Inc. (HO-03102, Lot #HO-10595 and HO-03103, Lot #HO-10697), bovine kidney HS (H7640, Lot #SLBF5723V) and porcine intestinal mucosal heparin (H3149, Lot #63H1017) were from Sigma-Aldrich. For nuclear magnetic resonance spectroscopy (NMR) reference materials chondroitin sulfate A (Sigma, C-8529, bovine trachea, Lot #58F0616), chondroitin sulfate C (contains ~10% chondroitin sulfate A, Sigma, C-4384, shark cartilage, Lot #87F0611), deramatan sulfate (Sigma, C3788, Lot #046K1411) and heparin (New Zealand Pharmaceuticals, porcine mucosa, Lot #5108356) were procured. Twelve disaccharide standards, derived from the digestion of porcine heparin, were purchased from Iduron Limited, Manchester, UK. HL I (heparinase I or heparitinase III (EC 4.2.2.7)), HL II (heparinase II or heparitinase II (no EC number assigned)) and HL III (heparinase III or heparitinase I (EC 4.2.2.8)) were obtained from Seikagaku Corporation, Japan or Ibex Technologies Incorporated, Montreal, Canada (the action of enzymes from these two sources was found to be identical). All enzymes were diluted with BSA solution (Fraction V, Gibco BRL; 0.1% w/v in 50 mM sodium phosphate buffer, pH 7.1 for HL II, pH 7.6 for HL III and pH 7.1 containing 100 mM NaCl for HL I) and frozen (−80 °C) in 5 μL aliquots (containing 5 mIU according to the suppliers specification) until needed. Prior to use, the enzyme activities were assayed (LeBrun and Linhardt, 2001), using Celsus HS (HO-03103, Lot #HO-10697) and the digestion conditions described below. Five microlitres of HL I, II and III contained 2.8, 5.0 and 13.8 mIU of activity respectively. Water was distilled then double RO filtered (>18.5 MΩ).

2.2. Digestion of HS and heparin with heparin lyases

The enzymatic hydrolysis of HS in initial experiments was based on methods published by Skidmore and co-workers (Skidmore et al., 2006, 2010).

HS (10 × 1 mg portions of Celsus Lot # HO-10697) were each dissolved in 500 μL (100 mM NaOAc with 10 mM CaOAc, pH 7) and HL I, II and III (2.5 mIU of each enzyme according to the suppliers specification) was added to each aliquot. The solutions were incubated at 37 °C for 24 h (gentle inversion; 9 rpm) before a second aliquot of each enzyme was added and the solutions incubated for a further 24 h. Digests were terminated (100 °C, 5 min), samples centrifuged (13,000 rpm, 10 min) to remove insoluble material and analysed as described below.

In subsequent experiments HS and heparin materials (1 mg, in duplicate) were dissolved in 470 μL of either NaOAc (100 mM, pH 7.0) with or without CaOAc (2 mM), or sodium phosphate buffer (50 mM, pH 7.0). HL I (5 mIU according to the suppliers specification) was added and the solutions were incubated (37 °C, 2 h) with gentle inversion (9 rpm). Then, HL III (5 mIU) was added and the solutions incubated (37 °C, 1 h), followed by HL II (5 mIU) with incubation (37 °C, 18 h). A second aliquot of each enzyme was added simultaneously and the solutions incubated for a further 24 h. To test the effect of adding additional enzyme, 250 μL of the digestion mixture was removed and another aliquot of each of the three HL enzymes (5 mIU each according to the manufacturer's specification) was added simultaneously and the samples incubated for a further 48 h. Digests were terminated (100 °C, 5 min), samples centrifuged (13,000 rpm, 10 min) to remove insoluble material and analysed as described below.

2.3. Isolation of oligosaccharides >dp 2 from HS digests

Enzyme digests obtained in initial experiments from a total of 10.4 mg HS were pooled and fractionated using Vivaspin 5000 and 2000 Da nominal molecular weight cut-off (NMWCO) spin columns (centrifuged at 3500*g*, 30 min; Satorius AG, Goettingen, Germany), sequentially. The retentates from the Vivaspin 5000 Da NMWCO membranes were mixed with water (2 × 2 mL) and recentrifuged. The combined filtrates were transferred to a Vivaspin 2000 Da NMWCO spin column, centrifuged and the retentates washed with water as before. Retentates from both membranes were recovered by resuspending in deionised water (4 × 500 μL) and freeze-dried. Fractions >5000 Da NMWCO (3.2 mg) and 2000–5000 Da NMWCO (2 mg) were recovered and analysed by SEC—HPLC and proton NMR spectroscopy.

2.4. HPLC-SEC of digests

The SEC chromatograms were obtained using two Superdex™ Peptide 10/300 GL columns in series (300 × 10 mm, GE Healthcare, Buckinghamshire, UK) on a Waters 2690 Alliance system. The eluent was monitored by UV (230 nm) using a Waters 490E variable wavelength detector and RI using a Waters 2410 refractive index detector. Samples (2 mg/mL) were injected (50 μL) and eluted with 50 mM ammonium acetate (0.5 mL/min) at 20 °C. For quantifica-