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Capillary electrophoresis: a tool for studying interactions of glycans/proteoglycans with growth factors

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Abstract

Heparan sulfate (HS) and heparin bind to various growth factors and modulate their activities. Interactions of heparin and HS with members of the fibroblast growth factor (FGF) family are prerequisites for binding of FGFs to their high affinity cell receptors. The sulfation patterns of distinct oligosaccharide domains within heparin and HS chains determine their high affinity binding with basic FGF (bFGF). In order to study the structural basis of interactions of HS with bFGF, we developed a capillary electrophoresis (CE) method in order to monitor the ability of HS-derived oligosaccharides to bind this growth factor. HS was degraded to Δ-di- and Δ-oligosaccharides with digestion with heparitinase and the obtained Δ-saccharides were analyzed by capillary zone electrophoresis (CZE), using 50 mM phosphate, pH 3.5, as operating buffer, reversed polarity (30 kV) and detection at 232 nm. Under these conditions all differently sulfated HS Δ-disaccharides and the various Δ-oligosaccharide groups were resolved. Following incubation of the digest with bFGF and re-electrophoresis of the mixture, the bFGF interacting oligosaccharide groups were easily detected and identified. In view of the obtained results, CE is a multipotent analytical tool for determining disaccharide composition in HS, separating the various oligosaccharide groups produced by the action of heparitinase and identifying those interacting with bFGF.

Keywords: Heparin; Heparan sulfate; Disaccharides; Oligosaccharides; Basic fibroblast growth factor; Capillary electrophoresis

1. Introduction

Heparin and heparan sulfate (HS) are linear heteropolysaccharides composed of repeating disaccharide units which contain glucosamine and glucuronate (GlcA) or its C-5 epimer, the iduronate (IdoA). The disaccharide units may be sulfated at any available hydroxyl group and twelve differently sulfated units have been identified so far (Fig. 1); [1,2]. Heparin contains higher

Abbreviations: bFGF, basic fibroblast growth factor; CE, capillary electrophoresis; CZE, capillary zone electrophoresis; GAG, glycosaminoglycan; GlcA, D-glucuronic acid; HS, heparan sulfate; IdoA, L-iduronic acid; PGs, proteoglycans.

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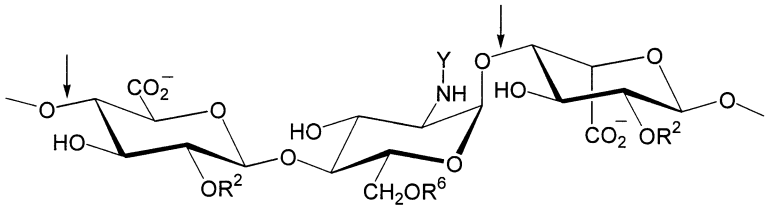

No	Disaccharide type	R^2	R^6	Y
1	aΔdi-nonS	H	H	Ac
2	Δdi-nonS	H	H	H
3	aΔdi-mono2S	SO_3^-	H	Ac
4	aΔdi-mono6S	H	SO_3^-	Ac
5	Δdi-monoNS	H	H	SO_3^-
6	Δdi-mono2S	SO_3^-	H	H
7	Δdi-mono6S	H	SO_3^-	H
8	aΔdi-di(2,6)S	SO_3^-	SO_3^-	Ac
9	Δdi-di(2,N)S	SO_3^-	H	SO_3^-
10	Δdi-di(6,N)S	H	SO_3^-	SO_3^-
11	Δdi-di(2,6)S	SO_3^-	SO_3^-	H
12	Δdi-tri(2,6,N)S	SO_3^-	SO_3^-	SO_3^-

Fig. 1. Representative structure of the heparin/HS chains showing the repeating disaccharide unit and the presence of both GlcA and IdoA. Arrows indicate the sites of degradation by heparin/HS lyases. Heparin lyase III (heparitinase) catalyzes the eliminative cleavage mainly of HS at the glycosidic linkage between GlcNAc (±6S) or GlcNS(±6S) and GlcA and produces Δ-di- and Δ-oligosaccharides.

number of sulfates and IdoA residues than HS and is characterized by the presence of sulfated glucosamine at C-3. Both glycosaminoglycans (GAGs) are biosynthesized as proteoglycans (PGs), i.e. covalently bound to a protein core. HSPGs are integral components of plasma membrane (e.g. syndecan family) and the extracellular matrix (e.g. perlecan). HSPGs via their HS chains participate in several cellular events and biologic processes, such as cell–cell and cell–protein interactions. Furthermore, HS acts as an endogenous receptor for growth factors [3].

Biologic activities of heparin and HS generally depend on interactions of the GAG chain with proteins. These interactions are mediated by distinct oligosaccharide sequences. Binding of heparin/HS oligosaccharide domains to proteins is generally (although not exclusively) ionic, and thus involves positively charged, usually clustered, amino acid residues of the protein components. Conversely, the anionic protein binding domains of GAG chains may differ with regard to structure, degree of binding specificity and organization at the macromolecular level.

Basic fibroblast growth factor (bFGF or FGF-2) is a member of a large family of structurally related proteins that affect the growth, differentiation, migration and survival of a wide variety of cell types. It was initially identified as a 15 kDa protein (pI 9.6) [4] that was later found to represent a proteolytic product of a primary 18 kDa form [5]. Although the binding of heparin and

HS has little effect on bFGF structure, it may facilitate the self-association of bFGF molecules into dimer and higher-order oligomers [6]. It has been proposed that bFGF has two separate receptor binding sites, which might allow a single bFGF to bind to two receptors or to interact with a single receptor in two separate positions [7]. HSPGs can increase the affinity of bFGF for its receptors and potentially act as a bridge to facilitate dimerization of receptors [8,11].

Interaction of bFGF with heparin/HS protects the growth factor against heat and acid denaturation, and protease cleavage [9]. Heparin and HS oligosaccharides that bind bFGF have been found to be rich in IdoA(2-OSO_3^-) containing disaccharides, with affinity increasing with chain length. The minimal bFGF binding sequence in HS has been identified as a pentasaccharide, which contains the disaccharide units IdoA (2-OSO_3^-)–GlcNSO_3^- or IdoA(2-OSO_3^-)–GlcNSO_3^-–(6-OSO_3^-) [10]. Accordingly, binding studies involving chemically modified heparins or HS preparations have shown that 2-OSO_3^- and *N*-sulfonyl groups are important for their interaction with bFGF. It should be pointed out that carboxyl groups of heparin hexuronic acid contribute to bFGF interaction and that small non-sulfated di- and trisaccharides compete with heparin for binding to bFGF. It is, therefore, obvious that distinct structural domains are necessary for the interaction of heparin/HS with bFGF.

In order to study the interactions of HS with bFGF, we developed a method combining the high-resolution efficiency of capillary electrophoresis (CE) with the specificity of hepatitinase against HS for monitoring the ability of the variously sulfated HS oligosaccharides to bind bFGF.

2. Experimental

2.1. Biologic material and chemicals

HS (Na^+ salt) from bovine intestinal mucosa, the various sulfated heparin- and HS-derived Δ-disaccharides and tris[hydroxymethyl]aminomethane were from Sigma (St. Louis, MO, USA). Heparitinase (heparin lyase III, EC 4.2.2.8) from *Flavobacterium heparenium* was purchased from Seikagaku Kogyo (Tokyo, Japan). bFGF was obtained from Serotec (Oxford, UK). All other chemicals used were of analytical reagent grade.

2.2. Enzymic degradation of GAGs and interaction with bFGF

Enzymic treatment of HS was performed as previously described [12,13]. In brief, HS was dissolved in 20 mM acetate buffer, pH 7.0, containing 1 μmol calcium acetate and 0.3 units of heparitinase per mg of polysaccharide dry weight, and incubated at 37 °C for 90 min. Digestion was terminated by boiling the mixture for 1 min. The degraded HS was then incubated with bFGF (1:1, w/w), which had been dissolved in 5 mM Tris–HCl buffer, pH 7.6, for 2 h at 37 °C. Aliquots were then taken for CE analysis.

2.3. Capillary electrophoresis

The analysis was performed with a $HP^{3D}CE$ (Agilent Technologies, Waldbronn, Germany) instrument with a built-in diode array detector set at 232 nm for detection of migrated species. Recording of spectra at 191–600 nm for these peaks was also used. Analysis was carried out on an uncoated fused silica extended light path capillary tube (50 μm i.d., 64.5 cm total and 56 cm effective length) at 25 °C, using 50 mM phosphate buffer, pH 3.5, at 30 kV. The operating buffer was filtrated through a 0.2 μm membrane filter. Samples were introduced using the pressure mode (50 mbar × 5 s) at the cathodic end (reversed polarity), so that the di- and oligosaccharides would migrate from the negative to the positive electrode by their electrophoretic mobility and against the electroosmotic flow of the buffer [12]. Before each run the capillary tube was washed with 0.1 M NaOH for 1 min, 2 × distilled water for 1 min and with the operating buffer for 5 min. The variously sulfated Δ-disaccharides were identified using external standards of known structure as well as by spiking the samples with known amounts of standard Δ-disaccharides.

3. Results and discussion

Capillary zone electrophoresis (CZE) analysis of standard Δ-disaccharides under the conditions described showed the complete separation of all 12 differently sulfated Δ-disaccharides within 17 min (Fig. 2), as previously described by our research group [1]. It has been well documented that treatment of HS with heparin/HS lyases I, II and III in combination results in almost complete degradation of HS ($>92\%$) to Δ-disaccharides [1,2]. Therefore, determination of the disaccharide composition of HS was performed by treatment of HS with an equi-unit mixture of heparin/HS lyases I, II and III. CZE analysis of the obtained digestion mixture showed that HS is mainly composed of a Δdi-nonS (ca. 35%) and various amounts of mono-, di- and trisulfated disaccharides, which were in good agreement with previously reported values [1,2,13]. This confirmed that this CZE method can be easily and accurately used for the determination of HS disaccharide composition.

In order to obtain variously sulfated and sized Δ-oligosaccharides, HS was treated with heparitinase [14] (for specificity of this enzyme see Fig. 1). As shown in Fig. 3A, CZE analysis shows that the enzymic action on HS produced five groups of oligosaccharides and disaccharides with different charge to mass ratios. The first population (IIIa) migrates with Δdi-triS, i.e. with a charge to mass ratio equal to 3. The next detected cluster of peaks (IIIb) represents a group of oligosaccharides with a charge density close to 2, i.e. they contain two sulfate groups per repeating disaccharide unit. Two other (IIIc and IIId) oligosaccharide populations migrate in the region of the monosulfated Δ-disaccharides (Fig. 3A), i.e. with a charge to mass ratio close to 1. Taking into account that the Δdi-monoS migrates at 8 min and the *N*-acetylated ones at 14 min, it is concluded that the group IIIc contains both *N*-acetylated and non-acetylated units and group IIId contains only the acetylated ones. The last oligosaccharide population (IIIe) migrates with a charge to mass ratio close to 0, i.e. this population does not carry any sulfate group in

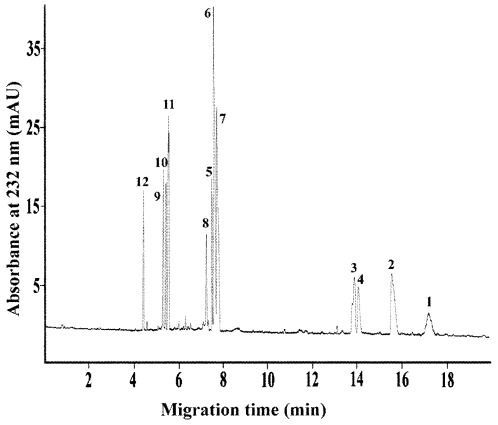

Fig. 2. Typical electropherogram showing the resolution of all 12 known heparin- and HS-derived Δ-disaccharides. Analysis was performed at 25 °C, using a 50 mM phosphate buffer, pH 3.5, reversed polarity at 30 kV and detection at 232 nm.

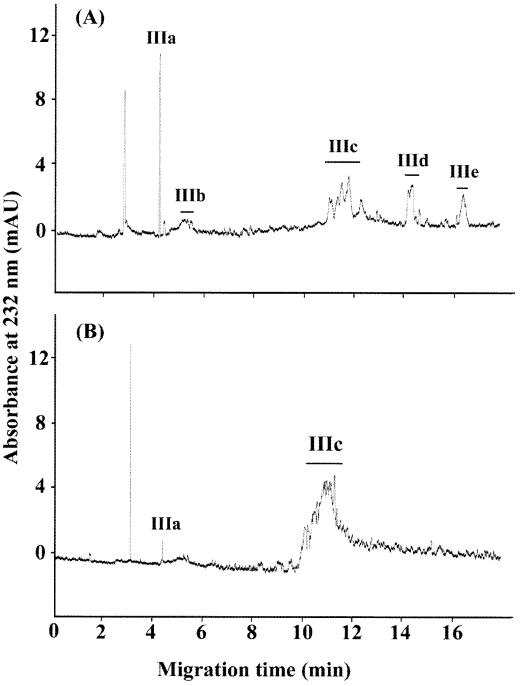

Fig. 3. Electropherograms obtained by CZE analyses of partially degraded HS with heparitinase before (A) and after incubation with bFGF (B). Five groups of oligosaccharides have been separated. As shown population IIIa co-migrates with Δdi-triS, group IIIb migrates with a charge mass to ratio close to 2, two of them (IIIc and IIId) migrate with a charge mass to ratio close to 1 and population IIIe with a charge density equal to 0. Groups IIIb, IIId and IIIe interact with bFGF and are not recorded in the electropherogram (B).

its disaccharide units, and furthermore the migration time reveals that the units of this population are *N*-acetylated. The presence of more than one oligosaccharide populations in each of the groups (IIIb, IIIc and IIId) may well be due to the presence of oligosaccharides which contain esterified sulfate groups in different positions of the repeating unit.

In order to identify the groups of oligosaccharides that interact with bFGF, the heparitinase digest was incubated with bFGF and the obtained mixture was re-electrophoresed under the same conditions. At the analytical conditions used, low pH and reversed polarity, bFGF is positively charged, does not migrate at all and, therefore, is not detected and recorded. It is worth noticing that the groups of oligosaccharides IIIb, IIId, and IIIe were not recorded in the electropherogram following incubation with bFGF, whereas population IIIa was significantly decreased (Fig. 3B). Taking into account that the negative groups of oligosaccharides are occupied by the positive ones of bFGF and that the bFGF-HS complex is acid-resistant [6], i.e. there is no dissociation at the low pH used for CZE analysis, it can be deduced that the bFGF-HS oligosaccharide complexes cannot migrate to the anode. Therefore, the non-recorded peaks after incubation with bFGF represent the bFGF interacting oligosaccharides of HS. The small peak identified in IIIa after incubation with bFGF represents the trisulfated Δ-disaccharide that co-migrates with the HS-derived Δ-oligosaccharides of the same charge density. The group IIIc of oligosaccharides showed similar profile with that before incubation with bFGF, indicating that they do not interact with this growth factor. This may well be attributed to the presence of oligosaccharides in these groups with lower molecular size than that required for high affinity binding to bFGF. In view of the fact that 2-OSO_3^- and *N*-sulfonyl groups are essential for bFGF binding to HS as well as the specific lyase action of heparitinase, it is plausible to suggest that the interacting groups of oligosaccharides (IIIa, IIIb, IIId and IIIe) are characterized by the presence of these sulfated structures.

4. Concluding remarks

In the present report, we describe a simple CZE method that can be used to resolve the variously sulfated HS groups of oligosaccharides. Combining all three heparin/HS lyases the disaccharide composition of HS can be easily determined. Partial degradation of HS with heparitinase resulted in five Δ-oligosaccharide groups of different electrophoretic mobility. Incubation of degraded HS with bFGF followed by CZE analysis revealed that this growth factor interacts with specific groups of HS-derived oligosaccharides. It seems, therefore, that the present CZE method is a useful analytical tool for the identification of bFGF interacting HS oligosaccharides.

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