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Chondroitin C lyase [4.2.2.] is unable to cleave fructosylated sequences inside the partially fructosylated *Escherichia coli* K4 polymer

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Abstract Chondroitin C lyase was demonstrated to be unable to act on fructosylated sequences inside a partially fructosylated polysaccharide having the chondroitin backbone structure, the *Escherichia coli* K4 polymer, using different analytical approaches. Chondroitin C lyase produced various unsaturated oligosaccharides by acting on an approximately 27%-fructosylated K4 polymer. The online HPLC-ESI-MS approach showed the disaccharide nature of the main species produced by chondroitinase C as ΔHexA-GalNAc. Furthermore, the non-digested sequences inside the K4 polymer were demonstrated to be oligosaccharides bearing a fructose for each glucuronic acid unit. In fact, unsaturated fully fructosylated oligomers, from tetrasaccharide to decasaccharide (ΔHexA(Fru)-GalNAc-$[GlcA(Fru)\text{-}GalNAc]_n$ with *n* between 1 and 4), at decreasing percentages, were produced by the enzyme. These results clearly indicate that chondroitinase C cleaved the innermost glucuronic acid-*N*-acetylgalactosamine linkage without affecting the 1,4 glycosidic linkage between fructosylated glucuronic acid and *N*-acetylgalactosamine residues, confirming that the 3-*O*-fructosylation of the GlcA residue renders the polysaccharide resistant to the enzyme action. This novel specific activity of chondroitinase C was also useful for the production of discrete microgram amounts of fully fructosylated oligomers, from 4- to 10-mers, from *E. coli* K4 for possible further studies and applications.

Keywords K4 polysaccharide · Chondroitinase C · HPLC · ESI-MS · Glycosaminoglycans · Chondroitin

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Abbreviations

ΔHexA	ΔUA, $\Delta^{4,5}$-unsaturated hexuronic acid
ΔDi-6S	ΔUA1→3GalNAc($6SO_4$)
ΔDi-4S	ΔUA1→3GalNAc($4SO_4$)
ΔDi-HA	Δ-hexuronic acid-*N*-acetyl-glucosamine
GlcA	D-glucuronic acid
GalNAc	*N*-acetyl-D-galactosamine
Fru	D-fructose
CS-C	Chondroitin sulfate C, chondroitin 6-sulfated
BSA	Bovine serum albumin
ESI-MS	Electrospray mass spectrometry
HPLC	High-performance liquid chromatography
LC	Liquid chromatography
RPIP	Reversed-phase ion-pairing
SAX	Strong anion exchange
TIC	Total ion chromatogram
TOF	Time-of-flight

Introduction

Chondroitin C lyase [4.2.2.-], chondroitinase C, was isolated for the first time from *Flavobacterium heparinum* [1]. This enzyme is able to act on chondroitin sulfate C (CS-C) producing tetrasaccharide plus an unsaturated 6-sulfated disaccharide (ΔDi-6S) and hyaluronic acid forming the unsaturated disaccharide ΔDi-HA. Chondroitin sulfate A is also degraded, producing oligosaccharides and ΔDi-6S but not unsaturated 4-sulfated disaccharides (ΔDi-4S) [1].

Escherichia coli K4 bacteria (05:K4:H4) synthesize a polysaccharide composed of disaccharides [GlcA (β1->3) GalNAc $(\beta 1\text{->}4)]_n$ to which β-fructofuranose (Fru) units are linked to C-3 of D-glucuronic acid (GlcA) residues [2]. As a consequence, the structure of this polyanion consists

of a chondroitin backbone having D-fructose units linked to GlcA. Oligosaccharides having a defined length may be produced from this polysaccharide by means of enzymatic treatment and preparative chromatographic approaches [3] and may be useful for the evaluation of possible biological activities. Moreover, fructose residues may be removed by hydrolytic treatment in acidic conditions [1, 4, 5] to produce a chondroitin backbone useful to gain detailed insight into the mode of action of several enzymes [6, 7]. Furthermore, bacterial polysaccharide lyases presumably play a role in the initial catabolism of GAGs [8], and they have found many applications, including the preparation of new therapeutic agents from natural GAGs [9], the analysis of GAGs found in tissues and biological fluids [10, 11], and the removal of GAGs from the circulation [12]. Finally, due to their highly specific action, these enzymes are potentially useful tools for structural studies of complex polysaccharides [8, 13].

To date, the action of chondroitinase C on polysaccharide K4 and its totally defructosylated polyanion derivative has never been studied also in connection with the possibility and capacity of this lyase to act on fructosylated carbohydrate sequences. Furthermore, this enzymatic approach may be useful for the production of oligomers having specific and particular sequences and also for the quantification of the fructose content inside the K4 carbohydrate backbone.

Material and methods

Materials

E. coli strain K4 U1-41, serotype O5:K4(L):H4, was from American Type Culture Collection. Chondroitinase C from *F. heparinum* [E.C. 4.2.2.-, >200 units/mg solid, one unit will form 0.1 μmole of unsaturated uronic acid per h at pH 8.0 at 25°C using CS-C as substrate] and chondroitin ABC lyase [E.C. 4.2.2.4, 50–250 units/mg protein, one unit will liberate 1.0 μmole of ΔDi-6S from CS-C per min at pH 8.0 at 37°] were purchased from Sigma-Aldrich. Ion-exchange resin QAE Sephadex® A-25 was from Pharmacia Biotech. Dialysis tubes having a cut-off of 5,000 Da and 100 Da were from Spectrum Labs. Acetonitrile, MS-grade, and all other reagents, of the purest grade available, were from Sigma-Aldrich. Totally defructosylated K4 polysaccharide was produced as previously reported [4, 5] by acidic treatment.

Purification of K4 polysaccharide

E. coli Ul-41 cells were cultured overnight at 37°C in the Luria-Bertani medium [6] (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl] containing 0.5% glucose. Half of the culture was added to 200 ml of this medium and incubated at 37°C for various lengths of time. After centrifugation at 4,000 *g* for 30 min, polysaccharide was precipitated from the supernatant by addition of 5 volumes of acetone and stored at −20°C overnight. The precipitate was recovered by centrifugation at 5,000 *g* for 15 min and dried at 60°C for 12 h. The dried precipitate was dissolved in 5 ml of 50 mM NaCl. After centrifugation at 10,000 *g* for 10 min, the supernatant was applied to a column (1 cm×20 cm) packed with QAE Sephadex® A-25 anion-exchange resin equilibrated with the same NaCl solution. K4 polysaccharide was eluted with a linear gradient of NaCl from 50 mM to 2 M from 0 to 150 min using low-pressure liquid chromatography (LP chromatography system from BioRad) at a flow of 1 ml/min. Fractions of 2 ml were collected. K4 polysaccharide was detected in the collected fractions by UV monitoring at 214 nm, agarose-gel electrophoresis [5] and capillary electrophoresis [4, 5]. The collected fractions corresponding to K4 polysaccharide were dialyzed by double distilled water and freeze–dried. Approximately 80 mg/l were recovered.

The total content of fructose in the K4 polysaccharide and the purity of the defructosylated K4 polysaccharide were determined by SAX-HPLC as reported below.

Treatment of K4 and totally defructosylated K4 with chondroitinase C

To a solution containing 2 mg of polysaccharides, K4 or totally defructosylated K4, in 2 ml of 50 mM Tris-acetate buffer pH 7.0 containing 20 mM $CaCl_2$ and 0.05% BSA, approx. 30 U chondroitinase C were added, and enzymatic digestion was performed at 37°C for 24 h. The reactions were stopped by boiling for 20 min. The samples were centrifuged at 10,000 rpm for 30 min at 5°C, and the supernatants were run in strong anion-exchange high-performance liquid chromatography (SAX-HPLC) and HPLC/ESI-MS.

SAX-HPLC

Twenty microliters of the enzyme-treated samples (40 μg) were injected for analytical purposes. The generated unsaturated oligosaccharides were separated by (SAX)-HPLC using a 150×4.6-mm stainless-steel column spherisorb 5-SAX using an isocratic separation with 50 mM NaCl pH 4.00 for 5 min and a gradient separation from 50 mM NaCl pH 4.00 at 5 min to 1.2 M NaCl pH 4.00 at 60 min at a flow rate of 1.2 ml/min. The effluent was monitored at 232 nm.

For preparative approaches, 50 μg of the chondroitinase C treated fructosylated K4 were injected in the 150×4.6-mm (SAX)-HPLC column and the oligosaccharide species were eluted with the above-reported linear gradient at a flow rate of 1.2 ml/min. The effluent was monitored at 232 nm and fractions of 0.6 ml were collected. The collected fractions

corresponding to single oligosaccharide species were exhaustively dialyzed by double distilled water for 48 h in 100 Da cut-off dialysis tubes and freeze–dried. The single purified oligosaccharides were further analyzed by means of the analytical (SAX)-HPLC in the presence and absence of treatment with chondroitinase ABC.

Reversed-phase ion-pairing (RPIP) HPLC/ESI-MS

HPLC separation was performed as previously reported [14, 15] on a 3-μm Gemini C18 110Å column (4.6×150 mm) from Phenomenex (Torrance, CA, USA). Approximately 200 μg of the sample was injected.

ESI mass spectra were obtained using an Agilent 1100 VL series (Agilent Technologies, Inc.) in negative ionization mode with the capillary voltage at 3,500 V and a heat source of 325°C. Software versions were 4.0 LC/MSD trap control 4.2 and Data Analysis 2.2 (Agilent Technologies, Inc.).

Results and discussion

E. coli Ul-41 cells were cultured at different times to produce a partially fructosylated K4 polysaccharide. After extraction and purification, a K4 polymer composed of approx. 27% fructosylated disaccharides, *i.e.* GlcA(Fru)-GalNAc, and 73% defructosylated disaccharides, *i.e.* chondroitin disaccharide GlcA-GalNAc, as evaluated by SAX-HPLC (Fig. 1) of the unsaturated disaccharides generated by treatment with chondroitin ABC lyase [4], was produced.

Partially fructosylated K4 was treated with chondroitinase C and the related products separated by analytical SAX-HPLC. Figure 2a illustrates the separation of the oligosaccharides produced by partially fructosylated K4 polymer in comparison with the unique non-fructosylated disaccharide produced by totally defructosylated K4 polysaccharide (Fig. 2b) after treatment with chondroitin C lyase. As is evident, chondroitinase C produced various oligosaccharide species from partially fructosylated K4 while a unique species, identified as the non-fructosylated disaccharide GlcA-GalNAc (named A in Fig. 2, see also below for the characterization of this disaccharide), was produced from totally defructosylated K4 polymer. The species A in the chromatogram of Fig. 2a accounted for approximately 74% of the total oligosaccharide species in accordance with the non-fructosylated portion of the polymer calculated after treatment with chondroitinase ABC (see also Fig. 1). The other species accounted for approx. 26% with the relative proportion of approx. 18% for the species named B in Fig. 2a, 5% for C, 2% for D and 0.5% for E. The structures of these oligosaccharides were further investigated.

The online HPLC/ESI-MS approach for the direct separation and characterization of complex oligosaccharides [14, 15] was used for the full identification of the structures of K4 oligosaccharides generated by means of chondroitinase C. K4 oligosaccharides from two monomers (2-mers, the disaccharide species named A) up to 10-mers, the oligosaccharides named from B to E, are separated in approximately 15 min (Fig. 3). The negative ESI-MS spectrum of the peak at the retention time of 6.8 min shows the main ion at *m/z* 378.1 corresponding to the unsaturated non-fructosylated K4 disaccharide (ΔHexA-GalNAc) having a molecular mass of 379.1 (Fig. 4a). As a consequence, the MS confirms that the peak A in Fig. 2a, and obviously in Fig. 2b, corresponds to the non-fructosylated disaccharide produced by the action of chondroitinase C on the

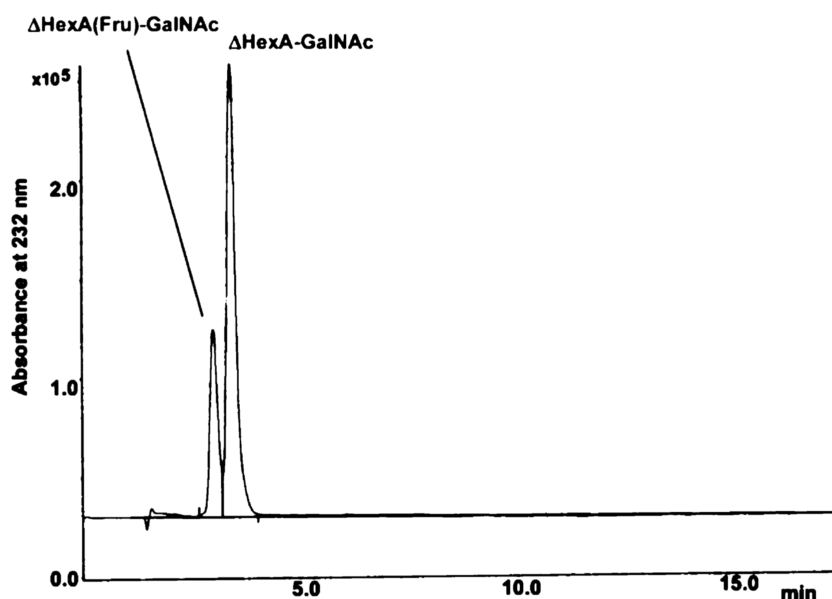

Fig. 1 Strong anion-exchange HPLC at 232 nm of K4 unsaturated disaccharides [the fructosylated ΔHexA(Fru)-GalNAc and the defructosylated ΔHexA-GalNAc] produced by treatment with chondroitin ABC lyase

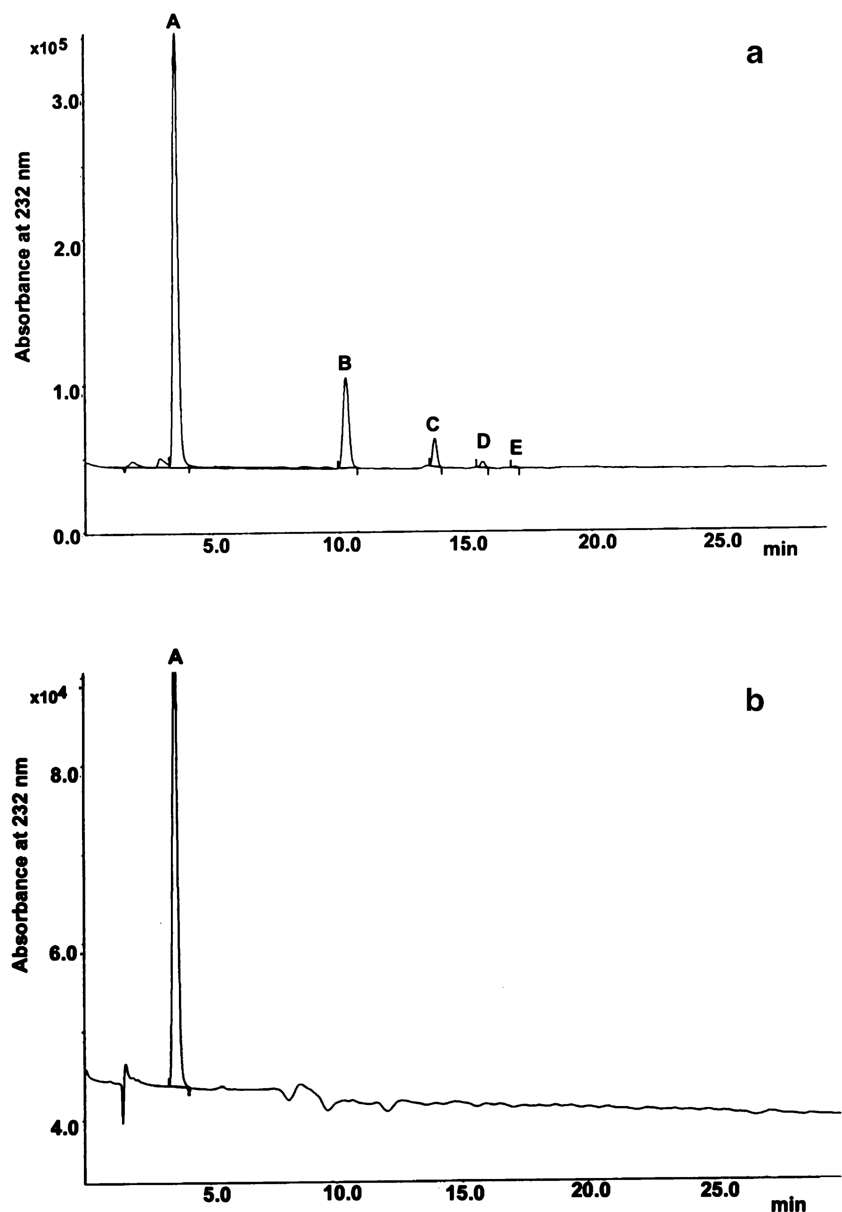

Fig. 2 Strong anion-exchange HPLC at 232 nm of (**a**) K4 unsaturated di/oligosaccharides (40 µg) and (**b**) fully defructosylated K4 unsaturated disaccharide produced by treatment with chondroitin C lyase

partially fructosylated K4 polysaccharide (and from totally defructosylated K4 polymer species). The peak with the retention time of 7.8 min, corresponding to the species named B in Fig. 2a, was identified as the unsaturated difructosylated K4 4-mer oligosaccharide ΔHexA(Fru)-GalNAc-GlcA(Fru)-GalNAc with the main ion at *m/z* 1081.2 (having a charge state of -1, Fig. 4b) possessing a molecular mass of 1082.0. The ESI-MS spectrum of the peak at the retention time of 9.2 min (species named C in Fig. 2a) is reported in Fig. 4c, showing a ion having a charge state of −1 at *m/z* 1623.5 belonging to the unsaturated fully fructosylated hexasaccharide ΔHexA(Fru)-GalNAc-[GlcA(Fru)-GalNAc]$_2$ with a molecular mass of 1,624.5. Other various ions having a charge state of −2 were identified, at *m/z* 811.2 belonging to the unsaturated fully fructosylated hexasaccharide above reported, at *m/z* 700.5 corresponding to the unsaturated trifructosylated species ΔHexA(Fru)-GalNAc-GlcA(Fru)-GalNAc-GlcA(Fru) derived from the loss of a GalNAc monosaccharide, and at *m/z* 628.2 for the unsaturated difructosylated oligomer ΔHexA(Fru)-GalNAc-GlcA(Fru)-GalNAc-GlcA generated by the loss of the Fru-GalNAc unit [14, 16–18]. The peak at 11.2 min was easily identified as the unsaturated 8-mer species ΔHexA(Fru)-GalNAc-[GlcA(Fru)-GalNAc]$_3$ (named D in Fig. 2a) by the presence of the main ion with a charge state of −2 at *m/z* 1082.3 (Fig. 4d) corresponding to the four-fructosylated octasaccharide having a molecular mass of 2,166.0. Finally, the ESI-MS spectrum of the peak at the retention time of 13.7 min (species named E in Fig. 2a) is shown in Fig. 4e, having a main ion with a charge state of −2 at *m/z* 1,352.3, belonging to the fully fructosylated deca-

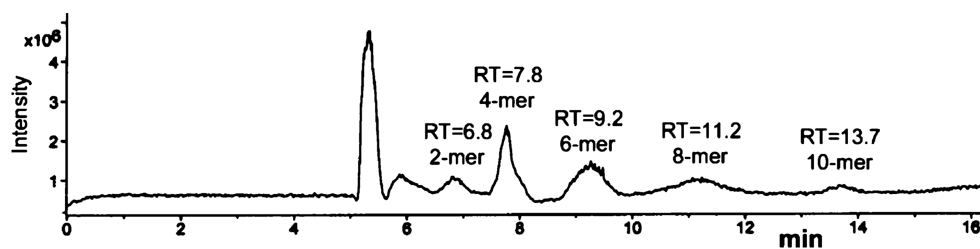

Fig. 3 Total ion chromatograms (TIC) of K4 oligosaccharides produced by means of chondroitin C lyase up to 10-mers separated by means of on-line RPIP-HPLC-ESI-MS

saccharide ΔHexA(Fru)-GalNAc-[GlcA(Fru)-GalNAc]$_4$ possessing a molecular mass of 2,707.5.

After purification by preparative SAX-HPLC, the oligosaccharides obtained by chondroitinase C treatment were submitted to exhaustive degradation with chondroitinase ABC. The species named A in Fig. 2a and having the structure of the unsaturated non-fructosylated disaccharide ΔHexA-GalNAc was, obviously, not degraded by the enzyme. In contrast, the unsaturated oligomers named B, C and D in Fig. 2a (the unsaturated decasaccharide E was not recovered by the preparative purification due to its very low content in the oligosaccharides mixture) were totally degraded by chondroitinase

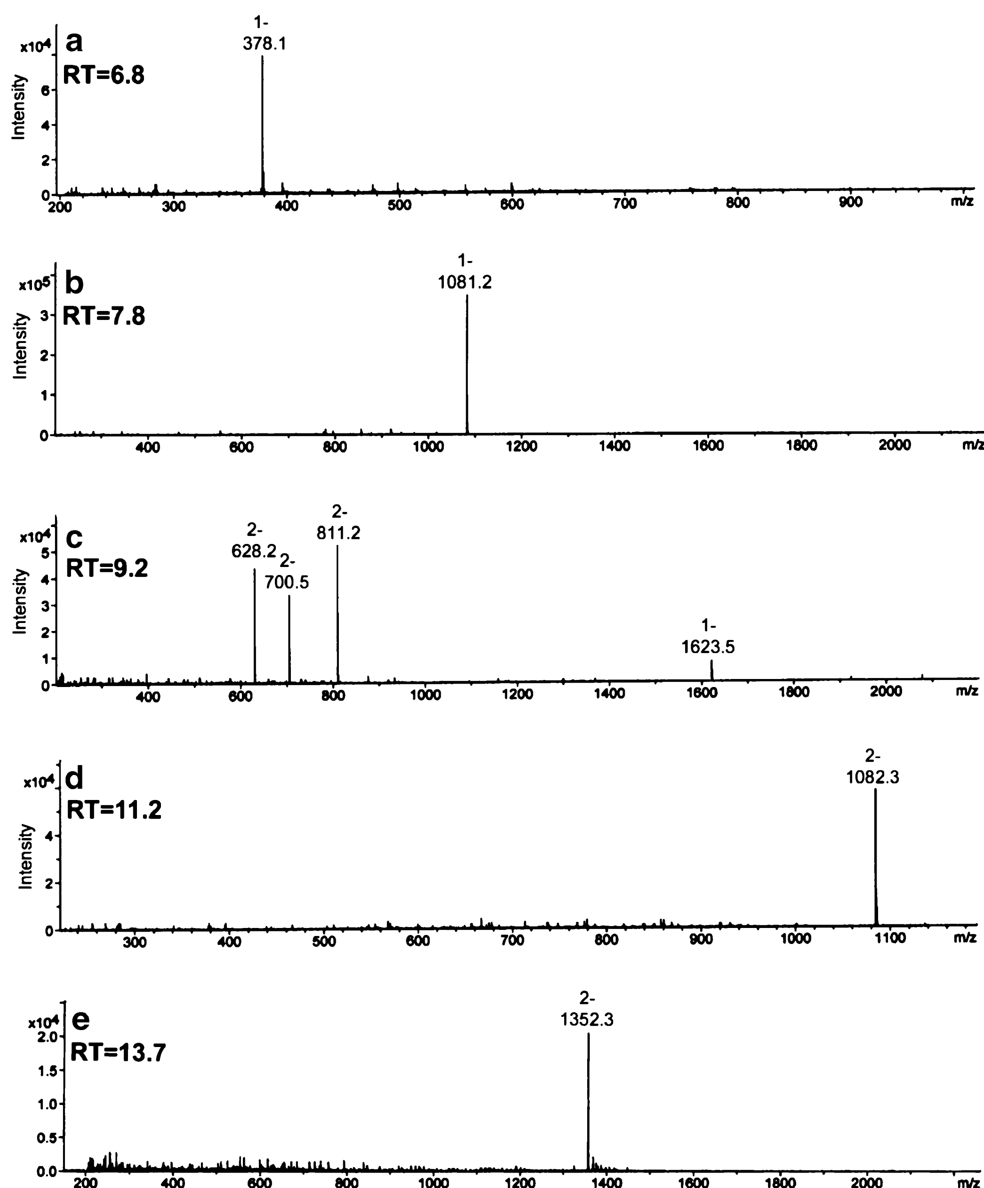

Fig. 4 The ESI-MS spectra in the negative mode of each single K4 oligosaccharide species produced by chondroitinase C and separated by means of RPIP-HPLC from 2-mers to 10-mers (from **a** to **e**)

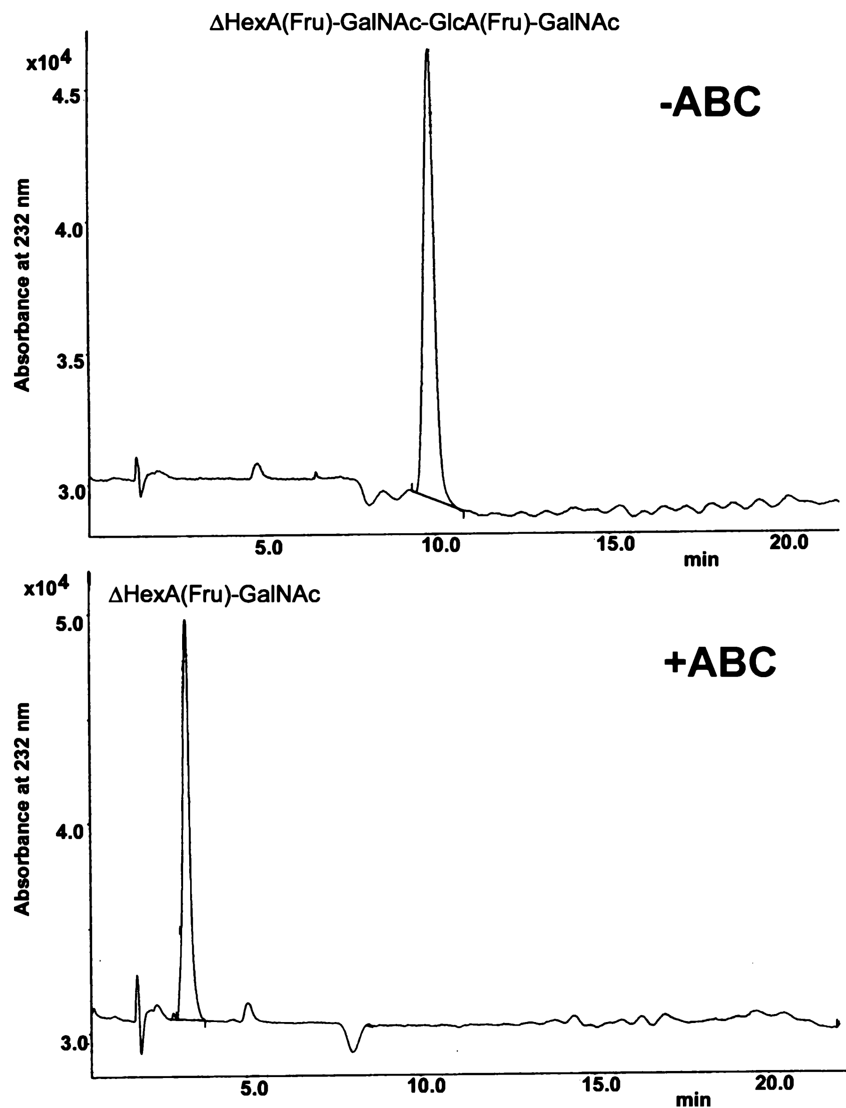

Fig. 5 Strong anion-exchange HPLC at 232 nm of the single purified K4 unsaturated tetrasaccharide produced by chondroitinase C (the species named B in the Fig. 2a) in the presence (+ABC) and absence (−ABC) of chondroitinase ABC treatment

ABC producing only one kind of disaccharide species, the unsaturated ΔHexA(Fru)-GalNAc (Fig. 5 as example), confirming that the sequence of these oligomers is composed of repeating fructosylated disaccharides, according to the mass evidence.

In this study, the incapacity of chondroitin C lyase to act on fructosylated sequences inside a partially fructosylated polysaccharide having the chondroitin backbone structure, the *E. coli* K4 polymer, was demonstrated using different analytical approaches. Chondroitinase C produces various unsaturated oligosaccharides by acting on an approx. 27%-fructosylated K4 polymer but only one kind of disaccharide on fully defructosylated K4 polyanion, ΔHexA-GalNAc. The RPIP-HPLC directly coupled with ESI-MS confirmed the capacity of this enzyme to act on non-sulfated sequences inside a chondroitin polysaccharide [1], but also the structure of the non-digested sequences as oligosaccharides bearing a fructose for each glucuronic acid unit. In fact, these purified oligosaccharide species produced only one kind of disaccharide, the unsaturated fructosylated ΔHexA(Fru)-GalNAc, after treatment with chondroitinase ABC. These results clearly indicate that chondroitinase C cleaved the innermost glucuronic acid-*N*-acetylgalactosamine linkage without affecting the 1,4 glycosidic linkage between fructosylated glucuronic acid and *N*-acetylgalactosamine residues confirming that the 3-*O*-fructosylation of the GlcA residue renders the polysaccharide resistant to the enzyme action.

This novel specific activity of chondroitinase C, very different from that of chondroitinase ABC capable of cleaving the fructosylated sequences of K4 [3, 4 and see Fig. 1], was also useful for the production of discrete microgram amounts of unsaturated fully fructosylated oligomers, from 4- to 10-mers, for possible further studies and applications. Furhermore, this enzymatic approach was also able to quantify the fructose content inside the K4 polymer in good accordance with previous methods [4]. Finally, this study demonstrates

the presence of clusters of fructosylated sequences, differing from each other for the presence of one disaccharidic unit, inside the K4 polymer.

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