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Separation of capsular polysaccharide K4 and defructosylated K4 derived disaccharides by fluorophore-assisted carbohydrate electrophoresis (FACE)

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Abstract

An inexpensive, fast, simple, sensitive and reproducible fluorophore-assisted carbohydrate electrophoresis (FACE) method is described for the quantitative analysis of microgram amounts of the *Escherichia coli* K4 bacterium capsule polysaccharide and its defructosylated polymer. Following chondroitinase digestion of K4 and its derivative, the two disaccharides, ΔHexAFru-GalNAc for K4 and ΔHexA-GalNAc for defructosylated K4, are fluorotagged by 2-aminoacridone (AMAC) and the products separated on a polyacrylamide gel electrophoresis. A linear relationship was found for the two unsaturated disaccharides over a wide range of concentrations, from 0.5 to 5 μg for the Δ-disaccharide of K4 and from 0.2 to 5 μg for the Δ-disaccharide of K4d. The detection limit was found to be approx. 0.5–1 μg for K4 and 0.1–0.2 μg for K4d. The FACE procedure described is especially useful when many samples need to be analyzed.

Keywords: Glycosaminoglycans; K4 polysaccharide; Chondroitin; FACE

1. Introduction

The *Escherichia coli* K4 bacterium (05:K4:H4) synthesizes a capsule polysaccharide with a carbohydrate backbone identical to chondroitin (Rodriguez, Jann, & Jann, 1988). This polyanion consists of a chondroitin (GlcA (β1->3) GalNAc (β1–>4)n; backbone to which β-fructofuranose units are linked to C-3 of D-glucuronic acid (GlcA) residues. The polysaccharide backbone obtained after hydrolytic removal of the fructose residues can be used as a model substrate for the C-5 epimerase involved in dermatan sulfate biosynthesis (Hannesson, Hagner McWhirter, Tiedemann, Lindahl, & Malmstrom, 1996), and both the GlcA- and the GalNAc-transferases in chondroitin sulfate formation (Lidholt & Fjelstad, 1997). Therefore, defructosylated K4 is useful to gain detailed insight into the mode of action of enzymes. For this purpose native K4 is defructosylated to produce the polysaccharide possessing the chondroitin backbone.

In previous papers, high-performance capillary electrophoresis (HPCE) (Volpi, 2003) and high-performance liquid chromatography (HPLC) and postcolumn derivatization with 2-cyanoacetamide and fluorimetric detection (Volpi, 2004) methods were developed for the determination of disaccharides present in the polysaccharide K4 and its defructosylated product. Following chondroitinase digestion of K4 and its derivative, the two disaccharides, ΔHexAFru-GalNAc for K4 and ΔHexA-GalNAc for K4d, were separated and determined within 20 min in HPCE and approx. 10 min in isocratic strong-anion exchange-HPLC and detection at 230 nm. However, the K4 and K4d unsaturated disaccharides separation requires at the moment complex and expensive equipment, such as HPCE and HPLC.

The free reducing groups of the carbohydrate molecules that are exposed by enzyme cleavage can be derivatized

Abbreviations FACE, fluorophore-assisted carbohydrate electrophoresis; HPCE, high-performance capillary electrophoresis; HPLC, high-performance liquid chromatography; GalNAc, N-acetyl-galactosamine; GlcA, glucuronic acid; Fru, fructose; HexA, D-hexuronic acid; AMAC, 2-aminoacridone; DMSO, dimethylsulfoxide; TEMED, tetramethylethylenediamine; K4d, defructosylated K4 polysaccharide.

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with 2-aminocridone by reductive amination in the presence of cyanoborohydride (Jackson, 1994; Calabro, Benavides, Tammi, Hascall, & Midura, 2000). Initial studies utilized 2-aminopyridine and defined conditions that achieved quantitative derivatization without desulfatation (Plaas, Hascall, & Midura, 1996). The fluorotagging introduces a highly fluorescent label that provides an identical signal for every free reducing group. The fluorotagged producs can be separated on polyacrylamide gels (fluorophore-assisted carbohydrate electrophoresis, FACE) and individual bands scanned for fluorescence. This analytical approach has been utilized for the qualitative and quantitative separation of disaccharides and oligosaccharides of various complex natural polysaccharides derived from specific enzyme digestion, such as plant cell wall polysaccharides (Goubet, Jackson, Deery, & Dupree, 2002), heparin (Calabro et al., 2001), hyaluronan and chondroitin/dermatan sulfate (Calabro et al., 2000), keratan sulfate (Plaas, West, & Midura, 2001).

In this paper, native K4 and its defructosylated product were quantitatively separated at microgram-level as unsaturated disaccharides produced after treatment with chondroitin ABC lyase by means of FACE. This electrophoretic protocol for the separation of unsaturated disaccharides derived from K4 and its defructosylated product is inexpensive, fast, simple, sensitive and reproducible for the quantitative analysis of low amounts of these polyanions, also taking into account their practical uses.

2. Materials and methods

2.1. Materials

The K4 polysaccharide purified from *Escherichia coli* U1-41 (05:K4:H4, Freiburg collection number 2616) (Rodriguez et al., 1988) was a kind gift from Prof. Klaus Jann (Max-Planck-Institut für Immunbiologie, Freiburg, Germany). Chondroitin ABC lyase from *Proteus vulgaris* [E.C. 4.2.2.4] was from Sigma. Ion-exchange resin AG 501-X8(D), dry mesh size 20–50 and wet bead size 300–1.180 μm, was from Bio-Rad. 2-Aminoacridone (AMAC, >98%), glacial acetic acid, dimethylsulfoxide (DMSO, 99.9%), sodium cyanoborohydride (95%) were from Aldrich-Sigma. Polyacrylamide (>99%), N,N′-methylenebisacrylamide (>99%), N,N,N′,N′-tetramethylethylenediamine (TEMED, 99%) and ammonium persulfate (98%) were purchased by Aldrich-Sigma. All the other reagents were analytical grade.

2.2. Preparation of the defructosylated polysaccharide

The non-sulfated fructosylated K4 was defructosylated by acid treatment at pH 3.0, according to Rodriguez et al. (Rodriguez et al., 1988) and to previous papers (Volpi, 2003; Volpi, 2004). 50 ml of the K4 polysaccharide (100 mg) in distilled water were stirred at room temperature with about 100 mg ion-exchange resin AG 501-X8(D) for approx. 24 h. After removal of the resin by filtration, the mixture was neutralized with sodium hydroxide and dyalized against water for two days. The dialysate was lyophilized to yield defructosylated K4.

2.3. Enzymatic digestion of K4 and defructosylated K4

About 2 μg of K4 or K4d samples were treated with 5 mU of chondroitinase ABC (chondroitin ABC lyase) in 20 μl of 50 mM ammonium acetate/sodium acetate buffer pH 8.0 at 37 °C for 5 h. The reaction was blocked by boiling the solutions for 1 min.

The identity of the two unsaturated disaccharides and the progression of the enzymatic reactions were confirmed by electrospray ionization mass (ESI-MS) (Fig. 1) by using an Agilent 1100 VL series (Agilent Technologies, Inc.). The electrospray interface was set in negative ionization mode with the capillary voltage at 3,500 V and a source of temperature of 325 °C in full scan spectra (200–2200 Da, 10 full scans/s). Direct injection was performed at 5 μl/min and nitrogen was used as a drying (4 l/min) and nebulizing gas (12 p.s.i.). Software versions were 4.0 LC/MSD trap control 4.2 and Data Analysis 2.2 (Agilent Technologies, Inc.).

2.4. Derivatization procedure

Derivatization of K4 (ΔHexAFru-GalNAc) and K4d (ΔHexA-GalNAc)-derived Δ-disaccharides with AMAC was performed as described by Jackson (Jackson, 1991) and modified by Kitagawa et al. (Kitagawa, Kinoshita, & Sugahara, 1995) and Lamari et al. (Lamari, Theocharis, Hjerpe, & Karamanos, 1999). Particularly, 100 to 5,000 ng of each Δ-disaccharide was lyophilized in a microcentrifuge tube at room temperature. A 5 μl volume of a 0.1 M AMAC solution in glacial acetic acid-DMSO (3:17, v/v) and 5 μl of a freshly prepared solution of 1 M sodium cyanoborohydride in water were added to each sample and then mixtures were centrifuged in a microfuge at 11,000×*g* for 3 min. Derivatization was performed by incubating the aliquots at 45 °C for 4 h. Finally, 15 μl of 50% v/v DMSO was added in the samples and aliquots were taken for FACE analysis.

2.5. Fluorophore-assisted carbohydrate electrophoresis (FACE)

A Miniprotean II cell vertical slab gel electrophoresis apparatus (Bio-Rad) was used. The stock buffer solutions were Tris-borate (pH 8.8; 1.5 M) and Tris-HCl (pH 8.8; 1.5 M). Acrylamide solution T 50%/C 7.5% for the resolving gel and T 50%/C 15% for the stacking gel were used as stock solutions [%T refers to the total concentration (w/v) of acrylamide monomer (i.e. acrylamide plus methylenebisacrylamide); % C refers to the concentration (w/v) of cross-linker relative to the total monomer]. A 10 ml volume of T

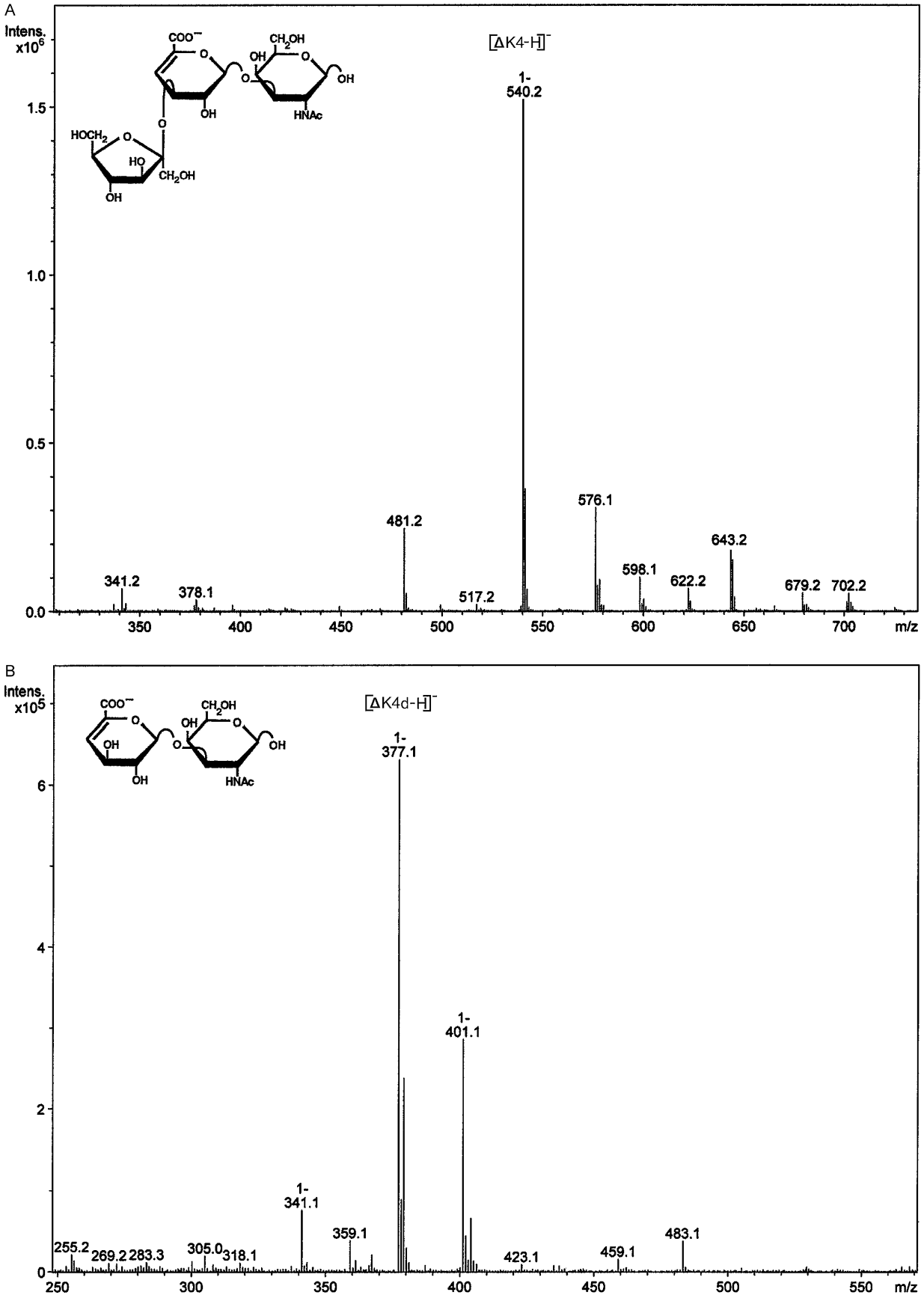

Fig. 1. Electrospray ionization mass (ESI-MS) spectra of ΔHexAFru-GalNAc disaccharide of K4 and ΔHexA-GalNAc disaccharide of defructosylated K4. The electrospray interface was set in negative ionization mode with the capillary voltage at 3,500 V and a source of temperature of 325 °C in full scan spectra (200–2200 Da, 10 full scans/s). Direct injection was performed at 5 μl/min and nitrogen was used as a drying (4 l/min) and nebulizing gas (12 p.s.i.).

25%/C 3.75% resolving gel solution, in Tris-HCI (pH 8.8; 0. 375 M) was prepared and degassed. A 5 μl volume of TEMED and 50 μl of a freshty prepared 10% (w/v) ammonium persulfate were added. The solution was mixed rapidly and then placed between the glass plates, avoiding the air bubbles. The non-polymerized gel was overlaid with butanol. The resolving gel surface was rinsed with stacking gel buffer (Tris-HCI diluted from the stock solution). A 5 ml of T 5%/C 1.5% acrylamide stacking gel in Tris-HCl (pH 8.8; 0.36 M) was prepared, followed by an addition of 5 μl of TEMED and 50 μl of 10% ammonium persulfate. Immediately the solution was poured on the top of the resolving gel and the well-forming comb was inserted. The height of the stacking gel was 5 mm.

Immediately before the electrophoresis, the wells were rinsed with electrophoresis buffer and a pre-run of the gel was performed at 400 V for 10 min at 4 °C. A 5 μl volume of each sample was loaded in each well. Electrophoresis was done at 400 V for 60 min at 4 °C.

The gels were illuminated with UV light (365 nm) from a Transilluminator and imaged with a CCD camera. Quantitative analysis was performed with a densitometer composed of a Macintosh IIsi computer interfaced with Microtek Color Scanner from Microtek International Inc., Hsinchu, Taiwan. The gels were scanned and saved in gray scale. Image processing and analysis program, Ver. 1.41 from Jet Propulsion Lab., NASA, Florida, U.S.A., was used for densitometry.

3. Results and discussion

As reported in previous papers (Volpi, 2003; Volpi, 2004), the determination of K4 and its defructosylated product is based on analyses of Δ-disaccharides (ΔHexAFru-GalNAc for K4 and ΔHexA-GalNAc for defructosylated K4) produced by digestion with chondroitinase ABC, so the separation of these two unsaturated disaccharides is therefore essential for the analysis of these polyanions. This separation was performed by HPLC or HPCE, in view of the fact that these analytical approaches are widely used to analyze other similar polysaccharides such as hyaluronic acid, chondroitin sulfate, dermatan sulfate (Imanari, Toida, Koshiishi, & Toyoda, 1996; Mao, Thanawiroon, & Linhardt, 2002). Due to the presence of the double bond in position $\Delta_{4,5}$-hexuronic acid, these two disaccharides are generally quantitatively determined by a photometric detection at 230 nm.

The aim of this study is to develop an electrophoretic method able to detect microgram amounts of the natural polysaccharide K4 and its defructosylated product by analysing their unsaturated disaccharides (ΔHexAFru-GalNAc for K4 and ΔHexA-GalNAc for defructosylated K4). After treatment with chondroitinase ABC, the produced unsaturated disaccharides (see Fig. 1) were derivatized with AMAC as reported in the Experimental section and by Calabro et al. (Calabro et al., 2000) (Fig. 2) and separated by means of polyacrylamide gel electrophoresis. As illustrated in Fig. 3, the ΔHexAFru-GalNAc disaccharide for K4 (2 μg) is separated with a mobility of approx. 1.44×10^{-3} cm/A/min while the defructosylated ΔHexA-GalNAc unsaturated disaccharide for K4d (2 μg) shows a mobility of approx. 1.89×10^{-3} cm/A/min. Furthermore, a good resolution of the two disaccharides was obtained to monitor the progression of the defructosylation process. Other bands were detected with a greater mobility of the unsaturated disaccharides belonging to the reagents used.

By using K4 (Fig. 4A) and defructosylated K4 (Fig. 5A) at various concentrations and the densitometric analysis (Figs. 4B and 5B), the sensitivity and linearity of the method was tested. A linear relationship (correlation coefficient greater than about 0.96) was found for the two unsaturated disaccharides over a wide range of concentrations, from 0.5 to 5 μg for the Δ-disaccharide of K4 (Fig. 6) and from 0.2 to 5 μg for the Δ-disaccharide of K4d (Fig. 6). However, as

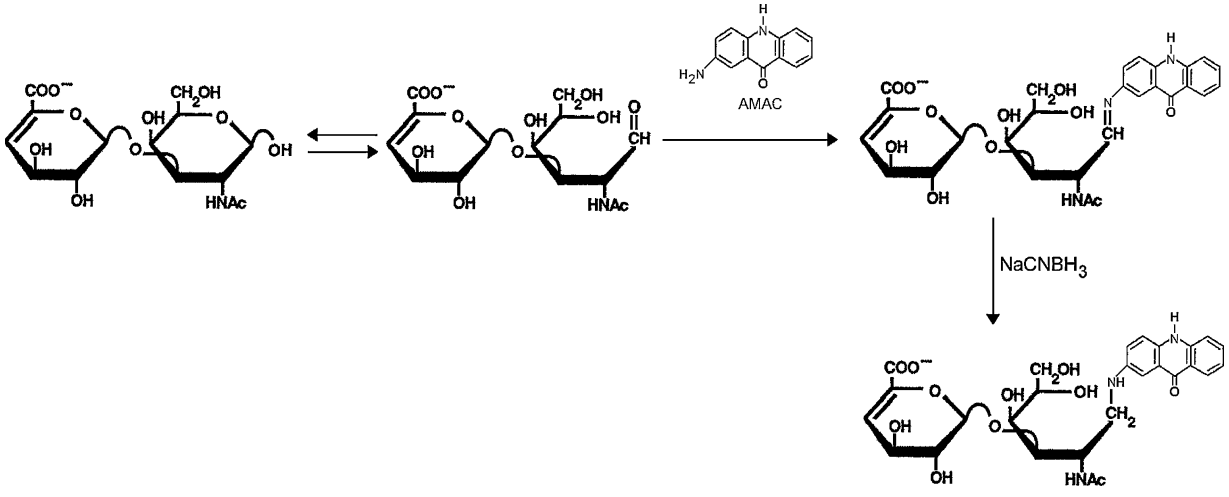

Fig. 2. Fluorophore derivatization of ΔHexA-GalNAc disaccharide of defructosylated K4 for FACE analysis.

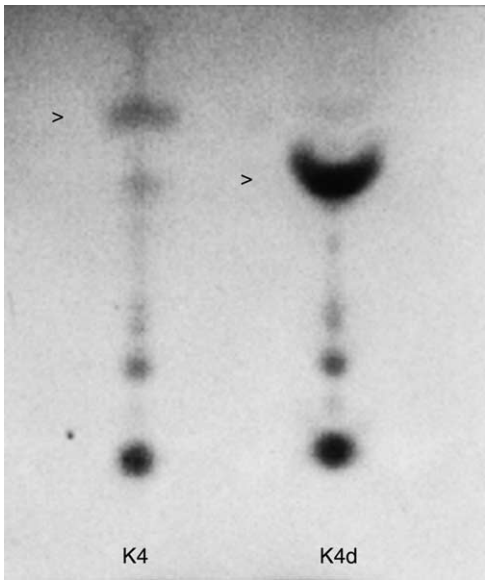

Fig. 3. FACE analysis of K4 (2 μg) and K4d (2 μg) Δ-disaccharides (indicated with arrows) derivatized with AMAC as described in Experimental section.

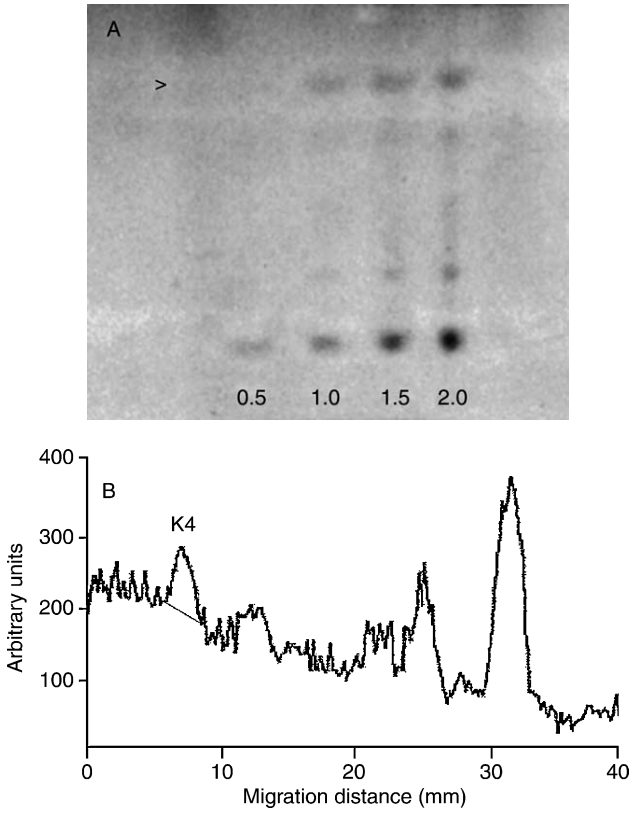

Fig. 4. (A) FACE analysis of increasing amounts of K4 (from 0.5 to 2 μg) and (B) Densitometric profile of ΔHexAFru-GalNAc disaccharide of K4 (1 μg) derivatized with AMAC and separated by using FACE.

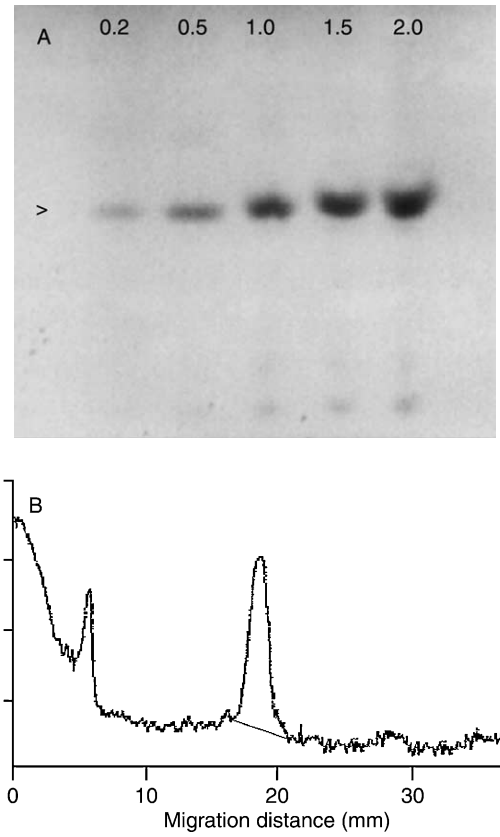

Fig. 5. (A) FACE analysis of increasing amounts of K4d (from 0.2 to 2 μg) and (B) Densitometric profile of ΔHexA-GalNAc disaccharide of K4d (1 μg) derivatized with AMAC and separated by using FACE.

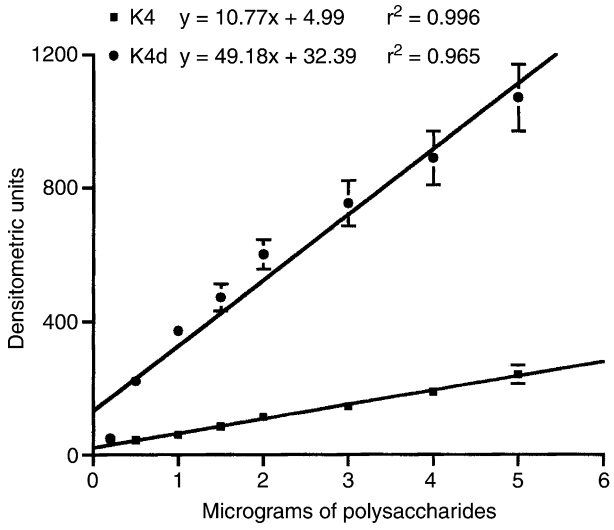

Fig. 6. Calibration curves for the analysis of disaccharides ΔHexAFru-GalNAc of K4 and ΔHexA-GalNAc of defructosylated K4 showing densitometric analysis as a function of the concentration of Δ-disaccharides in μg. The equations and the correlation coefficients are reported.

also evident by comparing the Fig. 4A and B, the fluorotagged K4 Δ-disaccharide shows poor fluorescence intensity compared with the unsaturated disaccharides of K4d, approx 25% of the parental defructosylated disaccharides when calculated at 5 μg (approx. the same result was obtained on molar bases). Furthermore, the detection limit was found to be approx. 0.5–1 μg for K4 and 0.1–0.2 μg for K4d (Fig. 6). The poor fluorescence intensity found for

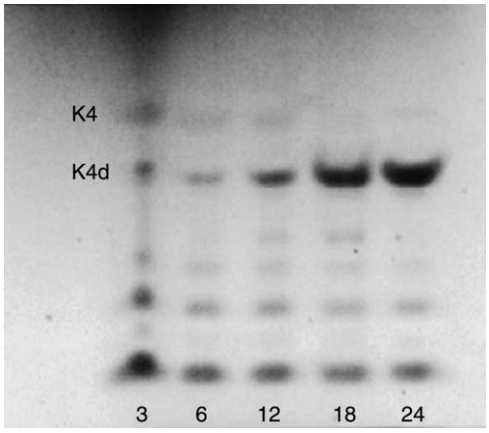

Fig. 7. FACE analysis of ΔHexAFru-GalNAc of K4 (2 μg) and ΔHexA-GalNAc of defructosylated K4 obtained at different times of the defructosylation process under drastic reaction conditions. The time of the defructosylation process is reported in hours.

the disaccharide ΔHexAFru-GalNAc was unexpected as the AMAC derivatization process provides an identical signal for every free reducing group. In fact, the AMAC fluorotag produced the same molar fluorescence value for variously non-sulfated and sulfated unsaturated disaccharides with a free reducing terminus (Calabro et al., 2000). By considering that chondroitinase digestion produces equimolar disaccharide amount for K4 and K4d (Volpi, 2003; Volpi, 2004), the poor capacity of AMAC to fluorotag the disaccharide ΔHexAFru-GalNAc in comparison with the defructosylated ΔHexA-GalNAc disaccharide was probably due to the steric inderance effect of the fructose.

Fig. 7 illustrates the FACE of the two species during the defructosylation process of K4 at different times. Even if the ΔHexAFru-GalNAc disaccharide of K4 showed poor fluorescence intensity, as previously reported, however it was possible to quantitatively follow the defructosylation process by measuring the appearance of the ΔHexA-GalNAc at different times by means of the calibration curve previously determined. After 3 h, defructosylated K4 was produced in a percentage of approx. 19% (Fig. 7), approx. 37% after 6 h, 59% after 12 h, and approx., 83% after 18 h. After 24 h the K4 was totally defructosylated. These data agree with those obtained by using HPLC and HPCE analyses (Volpi, 2003). According to Rodriguez et al. (Rodriguez et al., 1988), the total release of fructose from K4 was obtained under drastic conditions such as in the presence of autohydrolysis at pH of about 3.0. Under these experimental conditions, the K4 was totally defructosylated after about 24 h without fragmentation of the polymer (Rodriguez et al., 1988; Volpi, 2003).

Actually, the progress of the defructosylation process of K4 is evaluated by measuring the amount of fructose still bound to polysaccharide chains by using: thin-layer chromatography on cellulose plates after hydrolysis in trifluoroacetic acid; gas chromatography after hydrolysis and reduction with sodium borohydride; and an enzymatic approach after hydrolysis in trifluoroacetic acid. These methods all require the release of fructose for further qualitative and quantitative analyses. The HPCE (Volpi, 2003) and HPLC (Volpi, 2004) approaches to unsaturated disaccharides derived from K4 and its defructosylated product are rapid, sensitive and reproducible techniques to analyze these polyanions quantitatively. However, these analyses require complex and expensive equipments. The FACE analysis of AMAC fluorotagged disaccharides could be of importance for the qualitative and quantitative determination of K4 and K4d during the purification and preparation processes, also considering the importance of these polymers as useful tools to study the structure-activity relationship and biosynthesis process of this class of complex natural polymers.

3.1. Conclusions

We have developed an inexpensive, fast, simple, sensitive and reproducible technique for the quantitative analysis of microgram amounts of the *Escherichia coli* K4 bacterium capsule polysaccharide and its defructosylated polymer. This protocol takes advantage of bacterial eliminases, such as chondroitinase ABC, which are specific for chondroitin/dermatan sulfate. These enzymes generate specific oligosaccharide products with free reducing aldehydes of known chemistry that were previously blocked in the intact chains. These newly generated reducing aldehydes are then quantitatively derivatized with the fluorophore, 2-aminoacridone (AMAC), by a Schiff's base reaction which is stabilized by reduction with cyanoborohydride. This provides for detection in the microgram or less range after separation by fluorophore-assisted carbohydrate electrophoresis (FACE). The procedure is especially useful when many samples need to be analyzed.

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