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High-performance capillary electrophoresis separation of hyaluronan oligosaccharides produced by *Streptomyces hyalurolyticus* hyaluronate lyase

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Abstract

The action of *Streptomyces hyalurolyticus* hyaluronate lyase on hyaluronic acid (HA) determined by high-performance capillary electrophoresis (HPCE) (electrokinetic chromatography with sodium dodecyl sulphate) was examined and compared with the HPLC procedure. By using an uncoated fused-silica capillary tube, 50 μm ID, 65 cm long from the injection point to the detector, the separation of HA species from DP 4 to approx. DP 30 was obtained. The length of the capillary was found to be fundamental for the separation and resolution of the unsaturated HA oligosaccharides. Furthermore, the HPCE separation of HA Δ-tetrasaccharide and Δ-hexasaccharide species produced a greater detection sensitivity (about 20 times greater) than HPLC. Various HA samples were analyzed for the ratio of tetrasaccharide/hexasaccharide (*T*/*H*) after treatment with *S. hyalurolyticus* hyaluronidase. HA samples of extractive origin of different molecular masses showed a *T*/*H* ratio between 1.28 and 1.48 determined by HPCE, with a good correspondence with the HPLC separation. On the contrary, a sample of cross-linked HA resulted in a great discrepancy between the two analytical techniques (greater than 40%). HA of fermentative origin had a *T*/*H* ratio of approx. 1.92–2.00, close to that of HA (approx. 1.80) for the first time extracted and purified from the body of a species of mollusc bivalve, *Mytilus galloprovincialis*. The presence of resistant (less susceptible to enzyme cleavage) site repeating unit in the carbohydrate backbone of HA is discussed in relation with the *T*/*H* values experimentally determined by HPCE.

Keywords: Hyaluronan; Hyaluronic acid; Capillary electrophoresis; HPLC

1. Introduction

Hyaluronan (hyaluronic acid, HA) is a linear homogeneous acid polymer typically found in the connective tissues and body fluids of vertebrates as well as in some bacteria (Fraser, Laurent, & Laurent, 1997). HA is a polysaccharide composed of alternating residues of the monosaccharides D-glucuronic acid (GlcA) and *N*-acetyl-D-glucosamine (GlcNAc) linked by β(1 → 3) bonds in repeating units. Disaccharides are linked to each other by β(1 → 4) bonds. HA is quite distinct from other glycosaminoglycans. All contain sulphate groups and their polysaccharide chains are relatively short (< 100 kDa) (Mao, Thanawiroon, & Linhardt, 2002). On the contrary, the primary structure of HA contains neither sulphate groups nor peptide as it is not covalently bound to proteins. Although it consists of a single polysaccharide chain like other glycosaminoglycans, its molecular mass is usually in the range of millions (Fraser et al., 1997).

HA shows distinctive attributes arising from its extraordinary molecular mass, which underlies its distinctive role in extracellular matrix (Fraser et al., 1997). Furthermore, the rheological properties of HA as a lubricating and shock absorbing agent of high molecular mass provide for its use as an adjunct in ophthalmic surgery and in facilitating manipulation of ocular tissues; moreover, the rationale for the use of HA in the treatment of patients with rheumatoid or osteoarthritis is based on the enhanced lubricating properties of synovial fluid after exogenous supplementation (Goa & Benfield, 1994). HA maintains water balance

Abbreviations: HA, Hyaluronan, Hyaluronic acid; HPCE, High-performance capillary electrophoresis; SAX-HPLC, Strong-anion exchange high-performance liquid chromatography; GlcNAc, *N*-acetyl-glucosamine; GlcA, Glucuronic acid; HexA, D-Hexuronic acid; DP, Degree of polymerization.

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and is important in cellular interactions (Laurent & Fraser, 1992) as it interacts with a variety of different proteins including link protein, CD44 and a family of aggregating proteins, the hyaladherins (Ehnis, Dieterich, Bauer, Lampe, & Schuppan, 1996; Fraser et al., 1997). HA also plays a crucial role during development and differentiation and has many other important cell regulatory activities (Laurent & Fraser, 1992).

Several procedures for the determination of HA involve chromatographic isolation with subsequent colorimetric reactions (Armand & Reyes, 1983). Other approaches to the problem of HA analysis employ electrophoretic separation (Armstrong & Bell, 2002; Cappelletti, Del Rosso, & Chiarugi, 1979; Lee & Cowman, 1994; Volpi & Maccari, 2002) and SEC-HPLC (size-exclusion chromatography-HPLC) (Kvam, Granese, Flaibani, Zanetti, & Paoletti, 1993). However, the separation of HA from other polyanions present in a preparation might present some difficulties. This problem is considerable when the relative content of glycosaminoglycans is very high. HA can be broken down enzymatically into oligosaccharides. Two distinct types of hyaluronidase are known, a hydrolase of animal origin and an eliminase from bacterial sources (*Propionibacterium*, *Peptostreptococcus*, *Staphylococcus*, *Streptococcus* and *Streptomyces* genera) (Ernst, Langer, Cooney, & Sasisekharan, 1995; Linhardt, Galliher, & Cooney, 1986). These enzymes have been used to study the structure of HA and to prepare HA-derived oligosaccharides for the evaluation of biological activity (McCarthy & Toole, 1989; Nemec, Toole, & Knudson, 1987). The hyaluronidase from *Streptomyces hyalurolyticus* (EC 4.2.2.1) is specific for HA and does not act on the related galactosaminoglycans, chondroitin and chondroitin sulphate. This lyase cleaves → 3)-β-D-GlcNAc(β1 → 4)-β-D-GlcA(1 → linkages through an elimination reaction, yielding 4,5-unsaturated tetrasaccharide and hexasaccharide as final products (Ohya & Kaneko, 1979; Shimada & Matsumura, 1980). The products of the action of this enzyme on HA have been separated by various analytical approaches, such as HPLC (Lauder, Huckerby, & Nieduszynski, 2000; Mahoney, Aplin, Calabro, Hascall, & Day, 2001; Payan et al., 1991; Tawada et al., 2002) and fluorophore-assisted carbohydrate electrophoresis (Calabro et al., 2000; Tawada et al., 2002). The high specificity of the hyaluronidase from *S. hyalurolyticus* has been exploited to characterize HA samples from tissues and biological fluids (Akiyama et al., 1991; Lamari, Katsimpris, Gartaganis, & Karamanos, 1998; Pfeiler, Toyoda, Williams, & Nieman, 2002).

High-performance capillary electrophoresis (HPCE) has recently emerged as a promising analytical technique capable of rapid, high-resolution separation, characterization, and reproducible quantitation of analytes. Although CE has been widely applied to the disaccharide and oligosaccharide compositional analysis of glycosaminoglycans (Linhardt & Pervin, 1996; Pervin, Al-Hakim, & Linhardt, 1994; Tawada et al., 2002), the analysis of the HA-products generated by the action of *S. hyalurolyticus* hyaluronate lyase has not been greatly explored so far. Suzuki, Toyoda, Toida, and Imanari (2001) reported a CE separation of HA–oligosaccharide mixture produced by partial bacterial hyaluronidase digestion with no closer investigation of the technique, and Park, Cho, and Linhardt (1997) illustrated the CE analysis of fluorescently labelled HA-products generated by the enzyme.

This study examines the action of *S. hyalurolyticus* hyaluronate lyase on HA determined by HPCE (electrokinetic chromatography with sodium dodecyl sulphate), and compared with strong-anion exchange high-performance liquid chromatography (SAX-HPLC). The optimized HPCE separation of the lyase products was applied to the characterization of different HA samples used as active drugs and of extractive origin.

2. Materials and methods

2.1. Materials

HA from rooster comb, extractive origin, approx. 2000 kDa, was from Sigma. HA samples pharmaceutical grade were from Bioiberica (Spain, extractive origin, approx. 500–800 kDa), Maruha (Japan, extractive origin, approx. 200–300 kDa), Fidia (Italy, Hyalgan®, extractive origin, 500–730 kDa), Kraeber GMBH (Germany, fermentative origin, 600–700 kDa), Fidia (Italy, fermentative origin and cross-linked derivative, approx. 2000 kDa). Hyaluronate lyase from *S. hyalurolyticus* (E.C. 4.2.2.1) was purchased from Sigma. Papain (E.C. 3.4.22.2) was from Sigma. QAE Sephadex® A-25 anion exchange resin was from Pharmacia Biotech, Uppsala, Sweden. Stains-All (3,3′-dimethyl-9-methyl-4,5,4′,5′-dibenzothiacarbocyanine) was from Sigma.

2.2. Purification of HA from Mytilus galloprovincialis

Adult specimens of the mussel *M. galloprovincialis Lmk.* were collected from rocks in the Adriatic sea around Cattolica (Rimini, RN, Italy) and maintained in the laboratory. After each collection, the molluscs were killed and the shell removed. The body (28.0 g) was defatted by acetone. After centrifugation at 10,000*g* for 10 min and drying at 60 °C for 24 h, the pellet (2.4 g) was solubilized (1 g/20 ml) in 100 mM Na-acetate buffer pH 5.5 containing 5 mM EDTA and 5 mM cysteine. 100 mg of papain were added per gram of tissue and the solution incubated for 24 h at 60 °C in a stirrer. After boiling for 10 min, the mixture was centrifuged at 5000*g* for 15 min, and the pellet treated as reported above. After boiling for 10 min, the mixture was centrifuged at 5000*g* for 15 min, and the supernatants were assembled. Three volumes of ethanol saturated with sodium acetate were added to the pooled supernatants and stored at

+4 °C for 24 h. The precipitate was recovered by centrifugation at 5000*g* for 15 min and dried at 60 °C for 6 h. The dried precipitate (about 715 mg) was dissolved in 10 ml of distilled water by prolonged mixing. After centrifugation at 5000*g* for 15 min, 1 ml of 20% trichloroacetic acid was added to 5 ml of the supernatant. After 2 h at 4 °C, the mixture was centrifuged at 5000*g* for 15 min, and the supernatant recovered and lyophilized. After solubilization in 1 ml of 50 mM NaCl and 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. Glycosaminoglycans were eluted with a linear gradient of NaCl from 50 mM to 1.2 M from 0 to 150 min using a low-pressure liquid chromatography (Biologic LP chromatography system from BioRad) at a flow of 1 ml/min. Fractions of 2 ml were collected and analyzed.

Two volumes of ethanol saturated with sodium acetate were added to the collected fractions corresponding to the HA peak evaluated by uronic acid (Bitter & Muir, 1962) assay and agarose-gel electrophoresis (see below and Volpi & Maccari, 2002), and the polysaccharide precipitated at 4 °C. After centrifugation at 10,000*g* for 10 min, the pellet was dried at 60 °C and solubilized in distilled water for further analysis.

2.3. Enzymatic digestion of HA

Five hundred micrograms of HA from rooster comb by Sigma (10 mg/ml) were treated with 50 mU of hyaluronate lyase from *S. hyalurolyticus* in 500 μl of 20 mM sodium acetate buffer pH 6.0 at 37 °C for varying amounts of time. The enzymatic treatment for HA was stopped at 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0 and 8.0 h by boiling the samples for 1 min.

The enzymatic kinetic was determined by measuring the formation of 4,5-unsaturated GlcUA at 232 nm by spectrophotometric analysis (Fig. 1). Five microliters of samples at different times were mixed with 500 μl of 30 mM HCl and absorbance read at 232 nm.

One hundred micrograms of HA samples from a different supplier (10 mg/ml) were treated with 10 mU of hyaluronate lyase from *S. hyalurolyticus* in 100 μl of 20 mM sodium acetate buffer pH 6.0 at 37 °C for 24 h. The reaction was blocked by boiling the solutions for 1 min.

2.4. Capillary electrophoresis

Capillary electrophoresis was performed on a Beckman HPCE instrument (P/ACE system 5000) equipped with a UV detector set at 230 nm. Separation and analysis by electrokinetic chromatography with sodium dodecyl sulphate were carried out on uncoated fused-silica capillary tubes (50 μm ID, 85 cm total length, 65 cm from the injection point to the detector, and 50 μm ID, 65 cm total length, 45 cm from the injection point to the detector) at

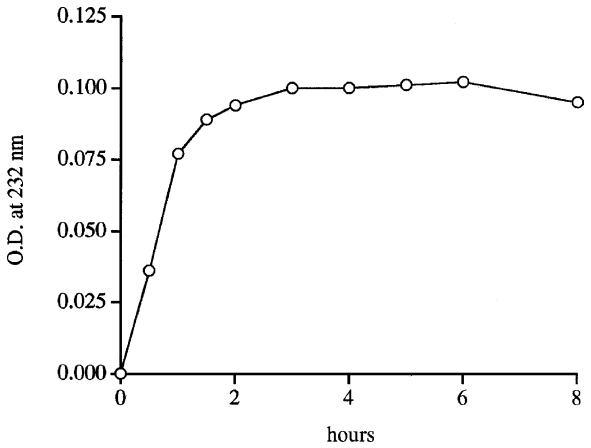

Fig. 1. Enzymatic kinetic determined by measuring at various times the formation of 4,5-unsaturated GlcA of HA, extractive origin from Sigma, treated with *S. hyalurolyticus* hyaluronate lyase. Five microliters of samples were mixed with 500 μl of 30 mM HCl and absorption read at 232 nm.

25 °C. The operating buffer was constituted by disodium hydrogen phosphate (40 mM), sodium tetraborate (10 mM) and SDS (40 mM) buffered at pH 9.0 by the addition of 1 M HCl. The buffer was degassed by vacuum filtration through a 0.2 μm membrane filter, followed by agitation in an ultrasonic bath. Before each run, the capillary tubes were washed with 0.1 M NaOH for 1 min, double distilled water for 5 min, and then conditioned with the operating buffer for 5 min. The samples to be analyzed were injected automatically, using the pressure injection mode, in which the sample is pressurised for 10–30 s. The injection volume can be calculated with the Poiseuille equation as proposed by the manufacturer, giving an estimated volume of 6 nl/s of injection time. The electrophoresis was performed at 20 kV (about 50 μA) using normal polarity. Peak areas were recorded and calculated using the Beckman software system Gold V810.

2.5. Strong-anion exchange high-performance liquid chromatography

High-performance liquid chromatography equipment was from Jasco (pump mod. PU-1580, UV detector mod. UV-1570, Rheodyne injector equipped with a 100 μl loop, software Jasco-Borwin rel. 1.5). The unsaturated oligosaccharides generated from HA samples treated with hyaluronate lyase were analyzed by strong-anion exchange (SAX)-HPLC separation using a 150 × 4.6 mm^2 stainless-steel column spherisorb 5-SAX (5 μm, trimethylammoniopropyl groups $Si-CH_2-CH_2-CH_2-N^+(CH_3)_3$ in Cl^- form, from Phase Separations Limited, Deeside Industrial Park, Deeside Clwyd, UK) and detection at 232 nm. Isocratic separation was performed using 50 mM NaCl pH 4.00 for 5 min followed by a linear gradient from 5 to 60 min of 50 mM NaCl to 1.2 M NaCl pH 4.00, at a flow rate of 1.5 ml/min.

2.6. Submarine agarose-gel electrophoresis (Lee & Cowman, 1994)

Agarose-gel was cast and electrophoresed using the Bio-Rad Sub-Cell GT horizontal electrophoresis unit. A 1.2% agarose-gel was prepared by dissolving 2.16 g agarose in 180 ml of TBE buffer (100 mM Tris, 100 mM borate, 10 mM EDTA, pH 8.2). The solution was poured onto a 20 cm long, 15.0 cm wide gel tray, to produce a 5 mm thick layer of gel. A 15-tooth well-forming comb was used to create the wells. The electrophoresis unit was filled with TBE buffer, thereby creating an approximately 5-mm deep layer of buffer above the gel. Samples of approximately 100 μg HA in 10 μl were mixed with 10 μl 2 M sucrose in TBE containing 2 μl of a 0.02% solution of bromophenol blue. Electrophoresis was carried out at room temperature for 5 h with a constant voltage of 100 V yielding a current of approximately 50 mA.

Immediately after the run, the gel was placed in a solution containing 0.005% Stains-All in 50% ethanol. The gel was stained overnight under light-protective cover at room temperature. For destaining, the gel was transferred to distilled water for approx. 2 h upon exposure to ambient room light.

3. Results

The kinetic of HA degradation by *S. hyalurolyticus* hyaluronidase measured at 232 nm is shown in Fig. 1. Under the experimental conditions adopted, the reaction was apparently complete after approx. 3 h.

The products of the enzymatic treatment at different times were evaluated by SAX-HPLC (Fig. 2A–D). After 30 min, a series of HA oligosaccharides, from DP 4 to approx. 30, having the structure of $[\Delta HexA(\beta 1 \rightarrow 3)GlcNAc]_n$, were obtained (Fig. 2A). These oligosaccharides were detected at 232 nm by the presence of the double bond at the non-reducing end uronate. After 60 min, Ha oligosaccharides were from DP 4 to approx. 14 (Fig. 2B). After 3 h, oligosaccharides from DP 4 to 8 were detected (Fig. 2C), while after 8 h, the enzymatic reaction produced almost completely tetrasaccharide and hexasaccharide species (Fig. 2D).

The unsaturated uniform-size HA oligosaccharides tested by SAX-HPLC were successfully separated by HPCE on uncoated fused-silica capillary tubes, 50 μm ID, 65 cm long from the injection point to the detector, by using an electrokinetic chromatography method with sodium dodecyl sulphate (Fig. 3A–D). Fig. 3A (and Fig. 3Abis

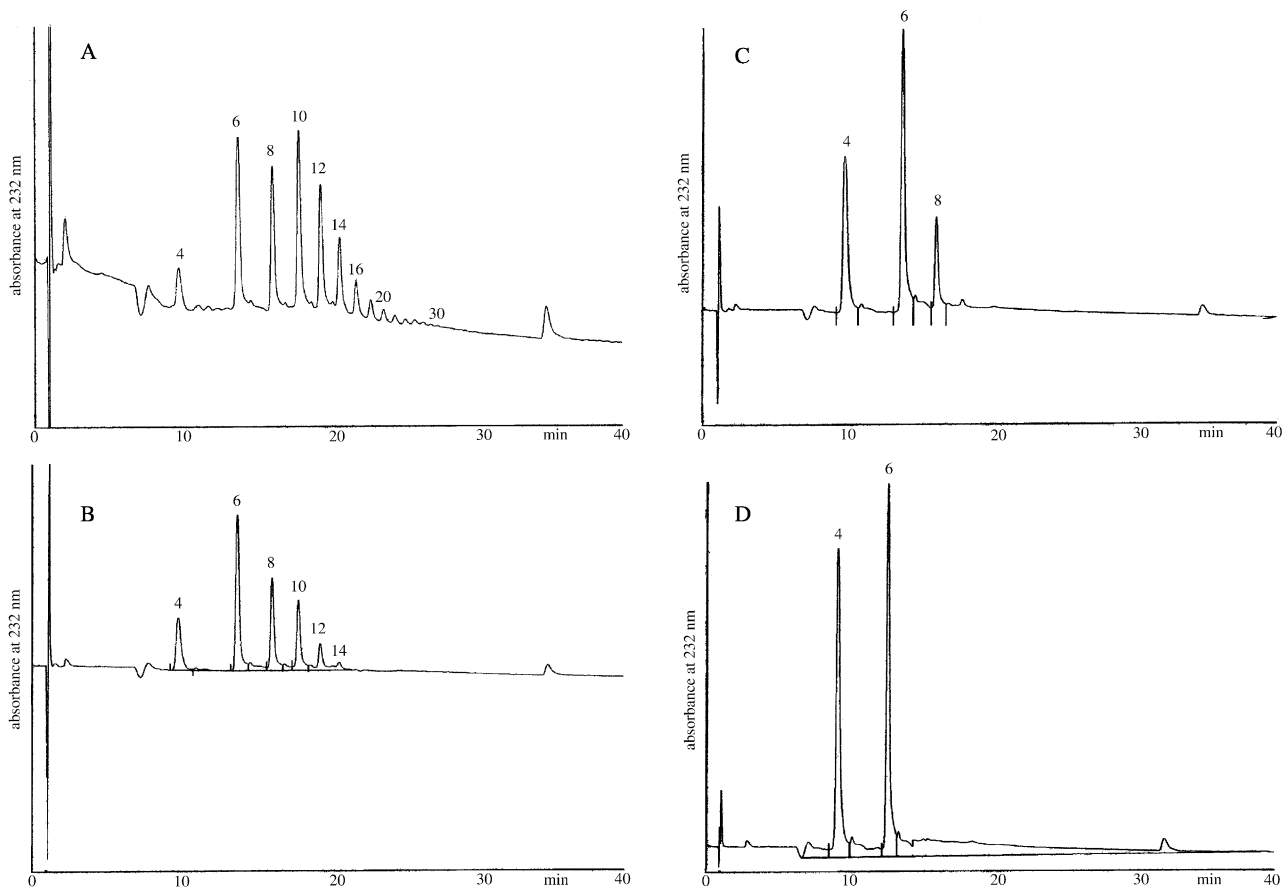

Fig. 2. SAX-HPLC chromatograms of HA oligosaccharides, having the structure of $[\Delta HexA(\beta 1 \rightarrow 3)GlcNAc]_n$, produced after various treatment times with *S. hyalurolyticus* hyaluronate lyase. (A) After 30 min; (B) after 60 min; (C) after 3 h; (D) after 8 h. The DP of the HA oligosaccharides is reported.

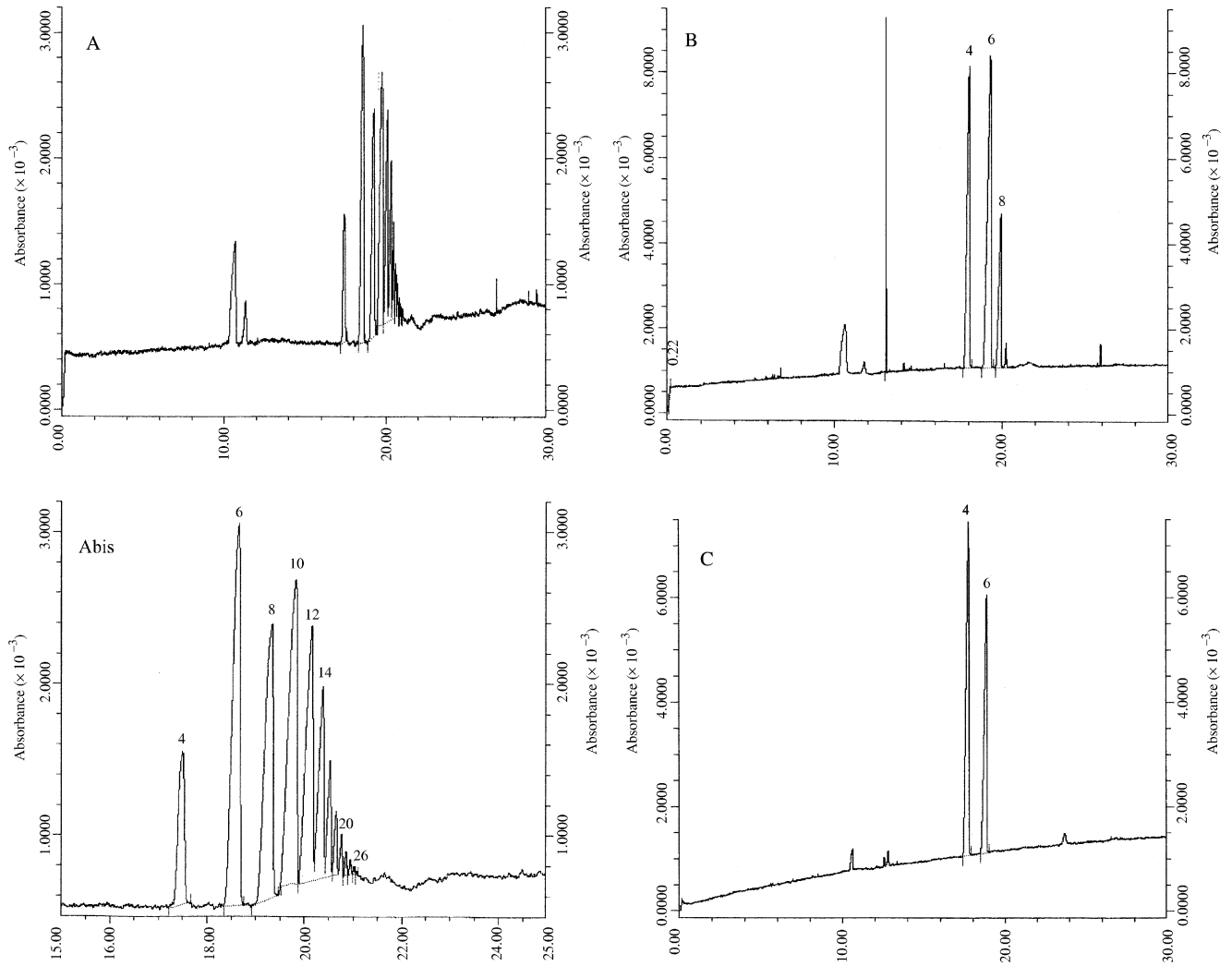

Fig. 3. HPCE electrophoregrams of HA oligosaccharides, having the structure of $[\Delta HexA(\beta 1 \rightarrow 3)GlcNAc]_n$, produced after various treatment times with *S. hyalurolyticus* hyaluronate lyase. (A) After 30 min; (Abis) as in A expanded in the region from 15 to 25 min. (B) After 3 h; (C) after 8 h. The DP of the HA oligosaccharides is reported.

expanded in the region from 15 to 25 min) shows the separation of HA species from DP 4 to approx. 26 obtained after 30 min of enzymatic treatment. Fig. 3B shows the HPCE separation of oligosaccharides from DP 4 to 8 produced after 3 h of hyaluronate lyase treatment, while after 8 h, the enzymatic reaction produced almost completely tetrasaccharide and hexasaccharide species (Fig. 3C), as already detected by SAX-HPLC (Fig. 2D), distinctly separated by HPCE.

We found that the length of the capillary was fundamental for the separation of unsaturated HA species, as a fused-silica capillary tube having a length of 45 cm from the injection point to the detector produced a strong decrease in the oligosaccharide resolution (Fig. 4). This was also marked by the strong decrease in the retention times (approx. 18 min for the HA tetrasaccharide separated by using the 65 cm long capillary and approx. 5 min for the 45 cm long capillary).

After defining the optimal HPCE separation conditions for the HA oligosaccharides, various HA samples were analyzed for the ratio of tetrasaccharide/hexasaccharide (*T*/*H*) after treatment with *S. hyalurolyticus* hyaluronidase. The results were compared with those obtained by SAX-HPLC. Rooster comb HA from Sigma shows a *T*/*H* ratio of approx. 1.28 by HPCE (Fig. 3C) and of 1.14 by HPLC (Fig. 2D, Table 1). The *T*/*H* ratio values of different pharmaceutical grade HA samples determined by HPCE and HPLC are shown in Table 1. HA from the body of *M. galloprovincialis* was extracted and purified by proteolysis, anion exchange chromatography and precipitation with organic solvents. HA was identified by the uronic acid test (Bitter & Muir, 1962), agarose-gel electrophoresis stained with toluidine blue/Stains-All for non-sulphated polyanions (Volpi & Maccari, 2002) and by submarine agarose-gel electrophoresis (Lee & Cowman, 1994) (see below). This sample of HA shows a *T*/*H*

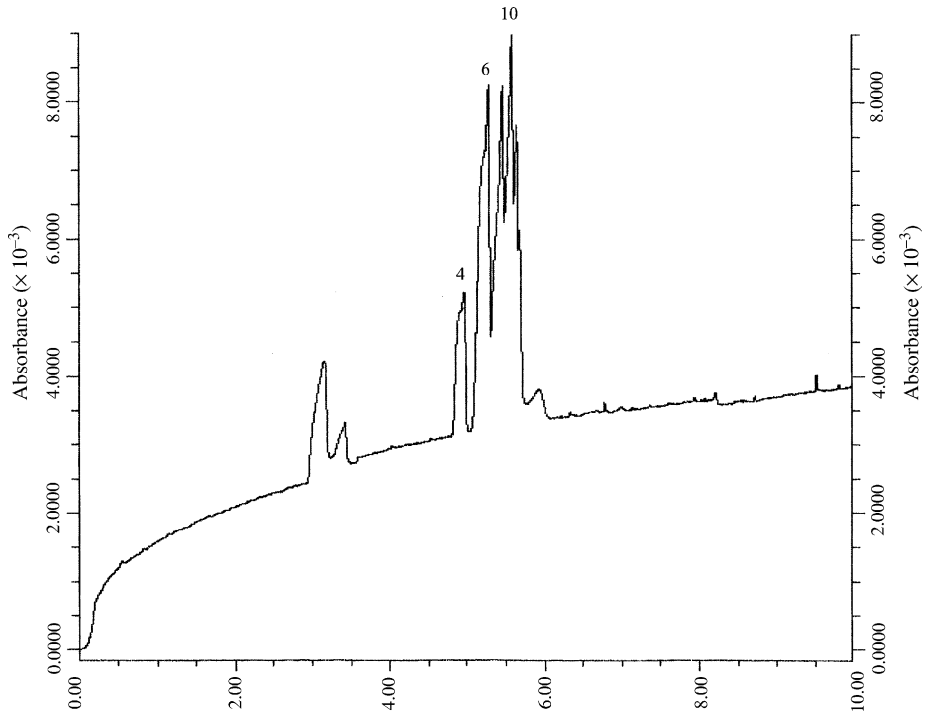

Fig. 4. HPCE electrophoregrams of HA oligosaccharides produced after 30 min of treatment with *S. hyalurolyticus* hyaluronate lyase obtained by using a capillary tube of 65 cm total length, 45 cm from the injection point to the detector. The DP of the HA oligosaccharides is reported.

ratio of approx. 1.82 by HPCE and of 1.77 by HPLC (Table 1).

The various HA samples were also analyzed by submarine agarose-gel electrophoresis (Lee & Cowman, 1994) to demonstrate differences in the molecular mass distribution (Fig. 5). As expected, notable variations were found for the molecular masses of the HA samples, with the cross-linked HA showing the greatest molecular mass (approx. 2000 kDa). The molecular mass of HA from *M. galloprovincialis* was found to be about 150–200 kDa (Fig. 5), also determined by high-performance size-exclusion chromatography (not shown).

The HPCE separation of HA tetrasaccharide and hexasaccharide species produced a greater detection sensitivity (about 20 times greater) than HPLC. In fact, the detection limits, estimated as the quantity of HA Δ-tetrasaccharide and Δ-hexasaccharide producing a peak signal twice the volume of the baseline signal, were to be approx. 0.10 μg of HA for HPCE and approx. 2 μg of HA for HPLC.

4. Discussion

Suzuki et al. (2001) reported a CE separation of HA–oligosaccharide mixture produced by partial bacterial hyaluronidase digestion with no closer examination of the technique and, as a consequence, the possibility of applying this method to the analysis of HA samples. Park et al. (1997) illustrated the CE analysis of fluorescently

Table 1
Ratio values of Δ-tetrasaccharide/Δ-hexasaccharide of various HA samples (the molecular mass in kDa is indicated) treated with *S. hyalurolyticus* hyaluronate lyase and quantified by HPCE and HPLC

HA sample	kDa	HPCE	HPLC
Extractive origin, Sigma	2000	1.28 ± 0.11	1.14 ± 0.09
Extractive origin, Bioiberica	500–800	1.47 ± 0.09	1.45 ± 0.10
Extractive origin, Maruha	200–300	1.48 ± 0.12	1.54 ± 0.09
Hyalgan®, Fidia	500–730	1.43 ± 0.13	1.51 ± 0.15
Cross-linked, Fidia	2000	1.22 ± 0.10	1.73 ± 0.16
Fermentative origin, Kraeber GMBH	600–700	1.92 ± 0.21	2.00 ± 0.22
M. galloprovincialis	150–200	1.82 ± 0.16	1.77 ± 0.14

The variation coefficients for three different analyses are reported.

Fig. 5. Submarine agarose-gel electrophoresis of (1) HA from Bioiberica (approx. 500–800 kDa); (2) HA from Maruha (approx. 200–300 kDa); (3) HA from Kraeber GMBH (600–700 kDa); (4) HA from Fidia (Hyalgan®, approx. 500–730 kDa); (5) cross-linked HA from Fidia (approx. 2000 kDa); (6) HA from *M. galloprovincialis* (approx. 150–200 kDa).

labelled HA-products generated by the action of hyaluronate lyase from *S. hyalurolyticus*. As can be clearly seen, the uniform-size HA oligosaccharides were further chemically modified by the introduction of the fluorescently labelled molecule. In this paper, we have explored the possibility of separating uniform-size HA oligosaccharides, generated by the *S. hyalurolyticus* hyaluronidase activity, with no further chemical modification of the final products by HPCE using a UV detection at 230 nm due to the formation of the 4,5-unsaturated derivatives. Besides, this approach was applied to the analysis of several HA samples of pharmaceutical grade and from tissues and results were compared with those obtained by HPLC.

The length of the capillary was found to be fundamental for the separation of unsaturated HA species. A decrease of approx. 20 cm in the length of the fused-silica tube produced a strong decrease in the HA oligosaccharide species retention times and in their resolution. Nevertheless, we found a greater detection sensitivity (about 20 times greater) of HPCE than HPLC, as already observed for other polysaccharides (Karamanos, Axelsson, Vanky, Tzanakakis, & Hjerpe, 1995; Volpi, 2003). Under these conditions, HPCE was suitable for a rapid and sensitive qualitative and quantitative separation of HA unsaturated products generated by the action of *S. hyalurolyticus* hyaluronate lyase.

Various HA samples of extractive origin were analyzed by HPCE for the *T*/*H* ratio formed by the action of the *S. hyalurolyticus* hyaluronidase and the results were compared with the HPLC results. These values were found to be between 1.28 and 1.48 by HPCE with a good correspondence with the HPLC (differences lower than approx. 11%), and these data were independent of the molecular masses of the extractive origin HA samples. On the contrary, cross-linked HA produced a great discrepancy between the two analytical techniques (greater than 40%) probably due to the HPLC separation showing an asymmetrical distribution of the two peaks of the tetrasaccharide and hexasaccharide species (not shown). Cross-linked HA is produced by chemical cross-linking strategies that produce a more mechanically and chemically robust material possessing improved viscoelastic properties (Gamini et al., 2002; Tomihata & Ikada, 1997) and increased molecular mass. In addition, we proved the possibility of analyzing cross-linked HA derivatives by using bacterial hyaluronidase and HPCE, and this approach was found better than HPLC. HA of fermentative origin has a *T*/*H* ratio of approx. 1.92–2.00, greater than the values calculated for the extractive HA samples. Furthermore, this HA shows a *T*/*H* ratio close to that of HA purified from the body of the mollusc *M. galloprovincialis* (approx. 1.80), and this was independent of their molecular masses.

S. hyalurolyticus hyaluronate lyase acts on HA through a random endolytic action pattern (Park et al., 1997). Using computer simulation models, a final *T*/*H* ratio of 2.0 was predicted (Park et al., 1997; Shimada & Matsumura, 1980), a value found to be greater compared to experimentally observed ratios of approx 1.0–1.2 (Park et al., 1997; Shimada & Matsumura, 1980). According to Park et al. (1997), this suggests the presence of resistant (less susceptible to enzyme cleavage) site repeating unit. Since HA has a uniform primary sequence, these resistant sites might be ascribed to restricted enzyme access based on secondary or higher order structural motifs (Park et al., 1997). As a consequence, HA of fermentative origin and from *M. galloprovincialis*, showing a *T*/*H* ratio close to 2.0, could possess a lower percentage of the resistant sites to the action of the enzyme compared with other HA samples. This property could have a relevant effect on the HA biological

activities. In fact, HA binds to chondrocytes derived from the Swarm rat chondrosarcoma with high affinity (McCarthy & Toole, 1989), and the chondrocyte-binding site recognizes five disaccharide repeats of HA. Interestingly, this also corresponds to the same length as the spacing of lyase resistant sites (Park et al., 1997). Thus, motifs of five or six disaccharide units may represent biologically relevant protein binding sites in HA.

Glycosaminoglycans abound in vertebrate tissues and invertebrate species are also a rich source of polysaccharides with novel structures (Albano & Mourao, 1986; Cassaro & Dietrich, 1977; Vieira & Mourao, 1988). Sulphated polysaccharides, such as chondroitin sulphate, dermatan sulphate, heparan sulphate, heparin, acharan sulphate (Kim et al., 1996), have also been isolated and characterized from different families of molluscs, but, to our knowledge, no HA was found in molluscs or in other invertebrates in spite of its various fundamental biological properties and functions. In this paper, HA was for the first time extracted from the species of mollusc bivalve *M. galloprovincialis* and characterized for its capacity to be degraded by bacterial hyaluronate lyase.

The results of this study confirm that the use of hyaluronate lyase in conjunction with HPCE might offer a new approach to understanding possible differences in various samples of HA, probably related to order structures of this polyanion, and also to analyze its presence qualitatively and quantitatively in biological tissues and fluids.

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