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Cartilage degradation by hyaluronate lyase and chondroitin ABC lyase: a MALDI-TOF mass spectrometric study

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Abstract

Matrix-assisted laser desorption ionization and time-of-flight mass spectrometry (MALDI-TOF MS) has been used to investigate degradation products of two selected polysaccharides of cartilage (chondroitin sulfate and hyaluronic acid). Testicular hyaluronate lyase and chondroitin ABC lyase were used for enzymic digestion of both polysaccharides as well as of cartilage specimens. Polysaccharide solutions and cartilage supernatants were assayed by positive and negative ion MALDI-TOF MS. Especially chondroitin ABC lyase produced high amounts of digestion products (unsaturated di- and tetrasaccharides) from polysaccharides as well as from cartilage, clearly monitored by MALDI-TOF MS. It is concluded that MALDI-TOF MS provides a precise and fast tool for the determination of oligosaccharides since no previous derivatization is required.

Keywords: Testicular hyaluronate lyase; Chondroitin ABC lyase; Cartilage; Acidic polysaccharides; MALDI-TOF mass spectrometry.

1. Introduction

Degenerative joint diseases like rheumatoid arthritis or osteoarthritis are a major cause of disability and early retirement in industrialized countries [1]. In the course of these diseases, a massive degradation of the articular cartilage layer takes place under the influence of reactive oxygen species [2,3] or different enzymes [4,5] released by neutrophilic granulocytes and other cells [6]. The extracellular matrix of cartilage consists of collagen and acidic glycosaminoglycans, which lend the cartilage its swelling and water-binding properties [7]. Mainly the carbohydrates of cartilage, i.e., chondroitin sulfate (CS), keratan sulfate (KS), and hyaluronic acid (HA), seem to be sensitive towards degradation-causing agents [8].

Oligosaccharides of cartilage accumulate in synovial fluids of patients with rheumatic diseases. They are detectable, e.g., by NMR spectroscopy [8] due to their highly mobile *N*-acetyl groups [9]. Unfortunately, NMR spectroscopy suffers from its comparably low sensitivity and, thus, more sensitive methods on the basis of chromatography were developed. For such assays, enzymes are utilized to depolymerize selectively the high-molecular-mass proteoglycans and glycosaminoglycans [10–12].

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In combination with high-performance liquid chromatography (HPLC), streptomyces or testicular hyaluronate lyases [EC 3.2.1.35] were successfully used for the determination of hyaluronic acid in synovial fluids [11,12]. Accordingly, chondroitin ABC or AC lyase was used for the determination of oligomeric carbohydrates in human urine [10].

Chondroitin ABC lyase from *Proteus vulgaris* [EC 4.2.2.4] converts glycosaminoglycans of cartilage into oligosaccharides that contain one terminal $\Delta^{4,5}$-unsaturated glycopyranosyluronic acid absorbing at 232 nm [10]. Testicular hyaluronate lyase is less specific and produces a mixture of different carbohydrates (ranging from the monosaccharides up to the hexasaccharides) which can be separated by HPLC [11,13].

More recently, mass spectrometric methods have also been used in the analysis of such oligosaccharides. Whereas methods like electrospray (ESI) [14], liquid secondary ion mass spectrometry (LSIMS) [15], fast atom bombardment (FAB) [16] or fast atom bombardment tandem mass spectrometry [17] are established tools, the application of MALDI-TOF mass spectrometry is relatively rare in carbohydrate analysis [18]. This is mainly caused by the very low extent of desorption induced by laser irradiation of samples containing highly acidic polysaccharides [19]. Up to now, acidic carbohydrates were mainly analyzed after an artificial (positively charged) peptide has been added [20]. The resulting neutral complexes of protein and carbohydrate desorb easily and can be analysed. However, the applicability of this procedure may be doubtful for biological samples. On the other hand, MALDI-TOF analysis would be most appropriate for the investigation of biological fluids, since this method is most sensitive, requires no extensive separation of carbohydrate mixtures and has a high tolerance towards salt and buffer contaminations.

In the present investigation, the action of chondroitin ABC lyase (CSase) and hyaluronate lyase (HAase) on isolated polysaccharides and cartilage specimens is characterized by MALDI-TOF mass spectrometry. It is shown that no previous derivatization of carbohydrates is required to obtain adequate spectra.

2. Results and discussion

Enzymic degradation of polysaccharides.— Fig. 1 shows the MALDI-TOF spectra of a

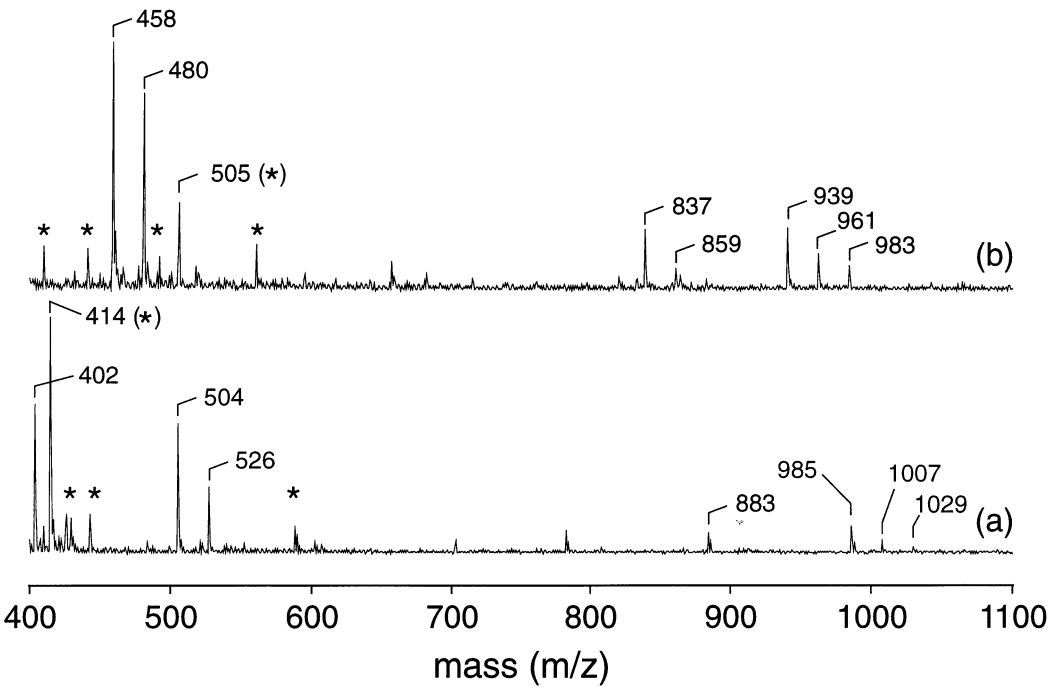

Fig. 1. Positive (a) and negative ion (b) MALDI-TOF mass spectra of a 10 mg mL^{-1} chondroitin sulfate solution digested with 2 U chondroitin ABC lyase for 9 h. Peaks arising from the 2,5-dihydroxybenzoic acid matrix are marked with an asterisk.

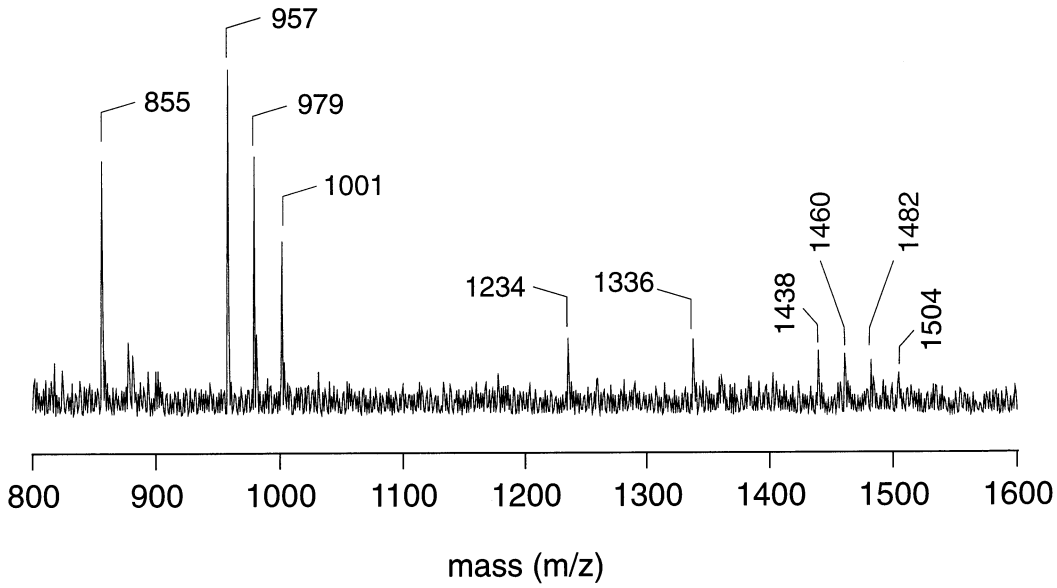

Fig. 2. Negative ion MALDI-TOF mass spectrum of a 10 mg mL^{-1} chondroitin sulfate solution digested with 2 U hyaluronidase for 9 h.

chondroitin sulfate solution upon digestion with chondroitin ABC lyase (for 9 h). The peak at m/z 458 in the negative ion spectrum (Fig. 1(b)) corresponds to the presence of M–2Na + H and that at m/z 480 to M–Na, both indicating the presence of singly charged disaccharides. M indicates the molecular weight of the neutral disaccharide [2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-ene-pyranosyluronic acid)-4,6-di-*O*-sulfo-D-galactose] completely saturated with sodium ions. The corresponding molecules can also be observed in the positive mode (Fig. 1(b)). Two peaks at m/z 504 and 526 correspond to M + H and M + Na, respectively.

Both spectra also exhibit less intense peaks according to higher-molecular-weight products. There are relatively intense peaks (about one third of the height of the basis peak) at m/z 837 and 859 as well as at m/z 939, 961 and 983 in the negative ion spectrum. The last three peaks are caused by the corresponding unsaturated tetrasaccharide (M–3Na + 2H; M–2Na + H; M–Na). This unsaturated tetrasaccharide ($M_W = 1006$) is also detectable in the positive ion mode but with lower sensitivity. Additionally, the required charge compensation with two sodium ions shifts all molecular weights by 46 Da. Unsaturated hexamers and octamers of chondroitin sulfate were detectable as neither positive nor negative ions. The sole degradation product of the tetra- as well as the disaccharide is formed by the loss of the sulfate group ($\Delta = 102$) leading to peaks at m/z 837 (Fig. 1(b)) and 883 as well as 402 (Fig. 1(a)). For means of comparison, the conversion of chondroitin sulfate into the corresponding unsaturated products was also monitored by ^{1}H and ^{13}C NMR spectroscopy. Both spectra were in agreement with previously published data [21,22] and indicated clearly the presence of the unsaturated disaccharide, whereas the corresponding tetrasaccharide could not be detected (data not shown). This clearly shows the, by far, higher sensitivity of MALDI-TOF compared with NMR spectroscopy.

Degradation products are also detectable when chondroitin sulfate is digested with HAase. Here, analogous products as in the case of CSase are observed, but no water elimination takes place [13]. Unfortunately, we failed to record the positive ion spectrum, but the negative ions could be easily detected (Fig. 2). The spectrum clearly shows the presence of the tetra- (m/z 957, M–3Na + 2H; 979, M–2Na + H and 1001, M–Na) and the hexasaccharide (m/z 1438, M–4Na + 3H; 1460, M–3Na + 2H; 1482, M–2Na + H and 1504, M–Na). The ratio between the proto-

nated and sodiated forms of these oligosaccharides clearly depends on the ion distribution of the buffer and can thus be easily controlled (data not shown). Astonishingly, no mono- and disaccharides of chondroitin sulfate were detectable, although one would expect such digestion products.

A loss of one sulfate group could be detected in the case of the tetrasaccharide (855, $\Delta = 102$) as well as for the hexasaccharide (1336), whereas only two sulfate residues were lost from the hexasaccharide (1234). Products containing no more sulfate groups at all were not detected. This indicates that at least one sulfate group has to be present to be able to detect the corresponding ion in the negative ion mode since the sulfate group mainly determines the negative charge density. We assume that the high negative charge density in chondroitin sulfate prevents the acquisition of positive ion spectra. On the other hand, in marked contrast to chondroitin sulfate, in the case of the less acidic hyaluronic acid, both kinds of spectra can be obtained after digestion with hyaluronate lyase. However, both spectra differ considerably (Fig. 3). Whereas the corresponding tetrasaccharide ($M_W = 820$) is detectable in the negative ion spectrum (m/z 775; M−2Na + H, Fig. 3(b)) as well as in the positive ion spectrum (m/z 799; M−Na + 2H, Fig. 3(a)), two further products are only detectable in the negative ion spectrum (Fig. 3(b)). There are intense peaks at m/z 1154 (M−3Na + 2H) according to the hexasaccharide ($M_W = 1221$) and at 1533 (M−4Na + 3H) according to the octasaccharide ($M_W = 1622$) of HA. This clearly indicates once again the higher sensitivity of the negative mode for the detection of acidic carbohydrates. Astonishingly, no reaction was observed when hyaluronic acid was treated with CSase [10].

Enzymic degradation of cartilage.—Principially, the same products as obtained upon treatment of the isolated polysaccharides can be found in the supernatants of cartilage. Negative and positive ion spectra measured after chondroitin ABC lyase treatment (Fig. 4(a,c)), respectively, closely resemble the spectra of pure, digested chondroitin sulfate and indicate the formation of the unsaturated disaccharide ($M_W = 503$) and the tetrasaccharide ($M_W = 1006$). However, the higher sodium content in cartilage samples in comparison with isolated polysaccharides favours the formation of ions containing high amounts of sodium. This is clearly visible in Fig. 4(a), where five different forms of the unsaturated tetrasaccharide can be observed ($m/z = 939$, 961, 983, 1005 and 1027). Unfortunately, the small peaks at 656 and 682 cannot yet be assigned.

The negative ion spectrum obtained upon hyaluronidase digestion of cartilage (Fig. 4(b))

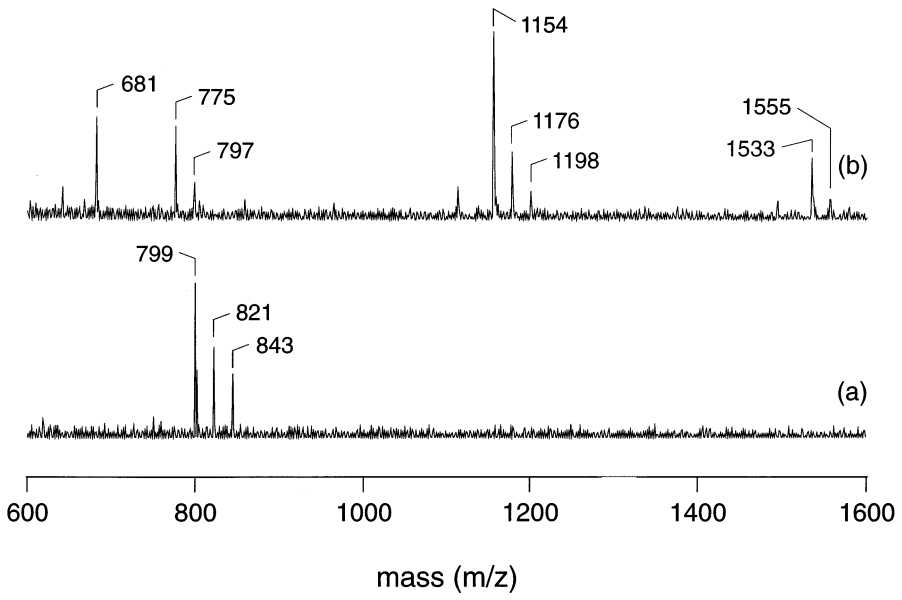

Fig. 3. Positive (a) and negative ion (b) MALDI-TOF mass spectra of a 10 mg mL^{-1} hyaluronic acid solution digested with 2 U hyaluronidase for 9 h. The spectrum was recorded in 2,5-dihydroxybenzoic acid matrix after a 1:10 dilution of the carbohydrate solution.

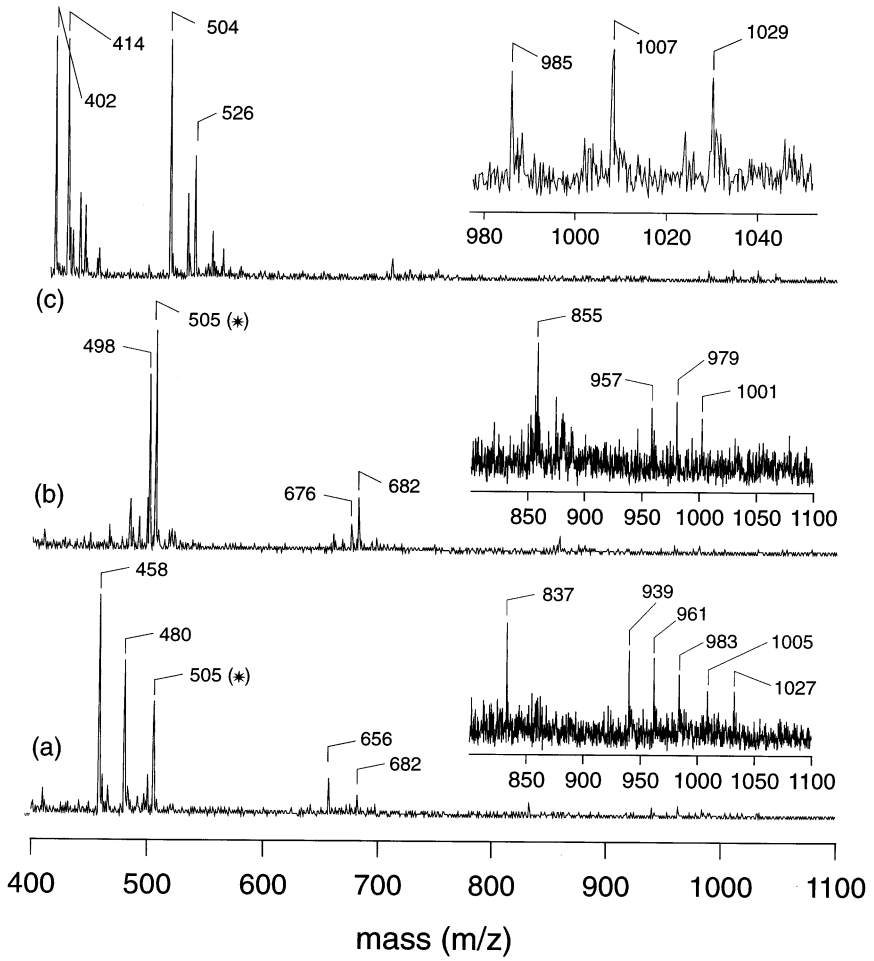

Fig. 4. MALDI-TOF mass spectra of bovine nasal cartilage upon digestion with chondroitin ABC lyase (a,c) and hyaluronidase (b). Spectra (a) and (b) were recorded in the negative mode, and spectrum (c) in the positive mode. Peaks arising from the 2,5-dihydroxybenzoic acid matrix are marked with an asterisk.

also closely resembles the spectrum upon hyaluronidase digestion of chondroitin sulfate. No degradation products of HA are detectable since cartilage contains only very low amounts of hyaluronic acid in comparison to chondroitin sulfate [7,23]. However, there is one marked difference between cartilage degradation and the degradation of isolated polysaccharides: whereas in solutions of chondroitin sulfate treated with HAase mainly the tetra- and hexamer occurred, the intense peak at m/z 498 in cartilage supernatants most likely corresponds to the disaccharide of chondroitin sulfate (M–Na). Finally, positive ion spectra could only be recorded in the case of chondroitin ABC lyase treatment. Here, the same peaks as observed with pure chondroitin sulfate are detectable (cf. Fig. 1(a)).

Summarizing, we conclude that MALDI-TOF spectroscopy offers the possibility to detect fast and sensitively enzymic degradation products of isolated polysaccharides as well as cartilage supernatants. The main advantage of the MALDI procedure described in this paper is that no previous derivatization (e.g., with semicarbazide [24]) of carbohydrates is needed. Nevertheless, further attempts are necessary to improve the homogeneity of the analyte/matrix mixture since up to now only a qualitative (but no quantitative) analysis of MALDI-TOF spectra is possible. It is our next aim to seek out the applicability of this new mass spectrometric method for the analysis of body fluids, especially pathologically changed synovial fluids.

3. Experimental

Chemicals.—All chemicals, solvents and matrix compounds were obtained in highest

Table 1
Molecular weights of cleavage products of chondroitin sulfate and hyaluronic acid upon digestion with CSase and HAase [a]

MW of oligomer	Hyaluronate [-2-acetamido-2-deoxy-3-*O*-(β-D-glucopyranosyluronic acid)-D-glucose-4-*O*-]$_n$	Chondroitin sulfate [-2-acetamido-2-deoxy-3-*O*-(β-D-glucopyranosyluronic acid)-4,6-di-*O*-sulpho-D-galactose-4-*O*-]$_n$	
	HAase	HAase	CSase
$n=2$	n.o.	521	503
$n=4$	820	1024	1006
$n=6$	1221	1527	n.o.
$n=8$	1622	n.o.	n.o.

[a] Molecular masses were calculated for the mainly abundant isotopes ^{1}H, ^{12}C, ^{14}N, ^{16}O and ^{32}S (n.o., not observed in mass spectra). The indicated molecular weights refer to the completely sodiated molecules.

commercially available purity from Fluka Feinchemikalien GmbH (Neu-Ulm, Germany). Testicular hyaluronate lyase (HAase) and chondroitin ABC lyase (CSase) from *Proteus vulgaris* as well as their substrates (chondroitin sulfate and hyaluronic acid from bovine trachea) were purchased as lyophylisates by Fluka and used without further purification.

Cartilage preparation and incubation conditions.—Bovine nasal cartilage was obtained from juvenile animals within a few hours after slaughter. To minimize biological variability the cartilage was frozen in liquid nitrogen and minced in a portland mortar. For each sample 100 mg of the homogenized cartilage tissue and 2.0 mL buffer (10 mmol/L phosphate, pH 7.4) containing 2 U of the corresponding enzyme were mixed and incubated at 37°C. After incubation, the samples were centrifuged for 10 min to remove debris and insoluble material. In the case of the pure polysaccharides, a 10 mg mL^{-1} solution was used and treated with the same amount of enzyme. Supernatants of cartilage and polysaccharide solutions were diluted 1:10 for MALDI-TOF analysis.

NMR measurements.—NMR measurements were performed for means of comparison and to estimate the extent of fragmentation. Detailed NMR parameters are given in Ref. [25].

Mass spectrometry.—MALDI-TOF spectra were acquired on a Voyager Biospectrometry workstation (PerSeptive Biosystems, Framingham, MA, USA). The system utilizes a pulsed nitrogen laser, emitting at 337 nm. The extraction voltage used was 20 kV. For all spectra, 128 scans were acquired using a reflectron and a low-mass gate at 350 Da. The laser strength was kept about ten percent over threshold setting to obtain the best signal-to-noise ratio. For all samples a 10 mg mL^{-1} 2,5-dihydroxybenzoic acid solution in water containing 0.1% trifluoroacetic acid was used as matrix. All carbohydrate solutions were directly applied to the sample plate as 1 μL droplets, followed by the addition of one drop (1 μL) matrix solution. Mixtures were dried rapidly under a moderate, warm stream of air. Table 1 gives an overview of the expected enzymic degradation products of hyaluronic acid and chondroitin sulfate under the influence of hyaluronate lyase (HAase) and chondroitin ABC lyase (CSase). All molecular weights are calculated assuming that all negative charges (carboxylate and sulfate groups) are completely compensated by sodium ions.

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