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Identification of the reaction products of the purified hyaluronidase from stonefish (*Synanceja horrida*) venom

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Recently we purified to homogeneity hyaluronidase from stonefish (*Synanceja horrida*) venom, for the first time from a marine source [Poh, Yuen, Chung & Khoo (1992) Comp. Biochem. Physiol., in the press]. In the present study the reaction products of the hyaluronidase purified from stonefish venom were analysed. It produced tetra-, hexa-, octa- and deca-saccharides as major end products, but not disaccharides. The structure of the tetrasaccharide product was determined by enzymic analysis, in conjunction with h.p.l.c. and by ^{1}H n.m.r., as GlcAβ1-3GlcNAcβ1-4GlcAβ1-3GlcNAc. Chemical shifts of the structural-reporter-group protons of the constituent monosaccharides for the tetrasaccharide have been assigned. The enzyme did not act on chondroitin sulphate or dermatan sulphate. The results indicate that the stonefish hyaluronidase is an endo-β-*N*-acetylglucosaminidase specific for hyaluronate.

INTRODUCTION

Hyaluronidase is an enzyme which degrades hyaluronate, one of the major connective-tissue constituents in animals. This enzyme is widely distributed in a variety of sources (for reviews, see [1,2]), such as snake venom [3,4], bee venom [5], lizard venom [6], scorpion venom [7], extracts of leech [8], submandibular glands of mammals [9,10], and mammalian testes [11]. It occurs ubiquitously in the lysosomes of mammalian cells. Micro-organisms also produce hyaluronidase.

Hyaluronidase from animal sources has been implicated in many physiological and pathological events. Hyaluronidase has been referred to as 'spreading factor' [12,13]. Hydrolysis of hyaluronate facilitates toxin diffusion into the tissues and potentiates the local haemorrhagic effects of a toxin. Hyaluronidase may thus contribute to systemic envenomation by accelerating venom absorption and diffusion. The high concentration of hyaluronidase in the head of mammalian spermatozoa apparently serves in the process of fertilization by dispersing the granulosa-cell layer embedded in a jelly composed of protein and hyaluronate around the ovum. Lysosomal hyaluronidase has been demonstrated in the metamorphosing tadpole [14] and may play a role in the tissue-remodelling processes. Hyaluronidase has a potential for practical use: it may be used for pretreatment of eggs in artificial fertilization and for treatment of pathological conditions such as myocardial infarction [15].

Recently we purified to homogeneity hyaluronidase from stonefish (*Synanceja horrida*) venom, for the first time from a marine source [16]. The purified enzyme is a 62 kDa glycoprotein with a pI of 9.2. The pH optimum of 6.0 is higher than those for other reported venom hyaluronidases. Intravenous injection of the purified enzyme did not result in the lethal effects of the stonefish venom, and the enzyme is considered to be a spreading factor.

In the present study we identified the reaction products of the stonefish hyaluronidase by enzymic and ^{1}H-n.m.r. analysis. The ^{1}H-n.m.r. spectrum of an isolated hyaluronate oligosaccharide has never, to our knowledge, been reported, although kinetic ^{1}H-n.m.r. studies of the anomer accumulation during the course of testicular-hyaluronidase digestion of hyaluronate has been previously reported [17]. We also assigned chemical shifts of the structural-reporter-group protons of the constituent monosaccharides for the tetrasaccharide product. This led to the unambiguous identification of the reaction product.

EXPERIMENTAL

Materials

Materials were obtained from the following sources: leech hyaluronidase (Orgelase) (EC 3.2.1.36), chondroitinase ABC (EC 4.2.2.4), whale cartilage chondroitin 4-sulphate, shark cartilage chondroitin 6-sulphate, pig skin dermatan sulphate and BSA from Seikagaku Kogyo Co., Tokyo, Japan; *Ampullaria* (freshwater apple shell) hepatopancreas β-glucuronidase (EC 3.2.1.31) purified to homogeneity [18], from Tokyo Zouki Chemical Co., Tokyo, Japan; sheep testis hyaluronidase (EC 3.2.1.35) and human umbilical-cord hyaluronate (grade I) from Sigma; cetyltrimethylammonium bromide (cetrimide), from Wako Pure Chemical Industries, Osaka, Japan; stonefish hyaluronidase was purified to homogeneity as reported [16]; ^2H_2O (99.95%), from Commissariat a l'Energie Atomique (CEA), Gif-sur-Yvette, France; standard even-numbered oligosaccharides with GlcA (glucuronic acid) at their non-reducing ends were prepared by sheep-testis-hyaluronidase digestion of human umbilical-cord hyaluronate; a standard mixture containing a saturated and an unsaturated disaccharide was prepared by chondroitinase ABC digestion of the above tetrasaccharide.

Hyaluronidase assay

The hyaluronidase activity of the stonefish enzyme was measured by a turbidity assay [19]. A 20 μl [approx. 2 NFU (National Formulary Units)] portion of the enzyme preparation dissolved in 1% BSA/0.08 M-NaCl/0.05 M-NaH_2PO_4, pH 5.5, was incubated with 10 μg of hyaluronate or a substitute in a total volume of 100 μl of 0.25 M-NaCl/0.2 M-sodium acetate buffer, pH 6.0, at 37 °C (1 NFU corresponds to the amount of the

Abbreviations used: 1(2)D, one(two)-dimensional; COSY, correlation spectroscopy; HOHAHA, homonuclear Hartmann–Hahn; MLEV, Malcom Levitt; NFU, National Formulary Unit(s).

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enzyme which hydrolyses 74 μg of hyaluronate/min [16]). The reaction was terminated by adding 200 μl of 2.5% (v/v) cetrimide/2% (w/v) NaOH and the turbidity formed was measured by absorption at 400 nm.

Enzyme digestion

Stonefish-hyaluronidase digestion was conducted by using 102 NFU of the enzyme and 500 μg of hyaluronate in a total volume of 1.3 ml of 0.08% BSA/150 mM-NaCl/50 mM-NaH_2PO_4, pH 6.0, at 37 °C. After 24 h, the reaction was terminated by heating at 100 °C for 1 min. Digestion with sheep testis hyaluronidase (24 NFU) was performed in the same manner, except that the buffer did not contain BSA. Leech-hyaluronidase digestion was performed by incubating 170 μl (357 units) of the enzyme preparation dissolved in 1% BSA/0.08 M-NaCl/0.05 M-NaH_2PO_4, pH 5.5, with 500 μg of hyaluronate in a total volume of 1.3 ml of 60 mM-citrate buffer, pH 6.0, at 37 °C; 1 unit of leech hyaluronidase is defined as the amount of enzyme which produces oligosaccharides corresponding to 1 μg of GlcA at the reducing ends/h. After 25 h, the reaction was terminated by heating at 100 °C for 1 min.

Exo-β-glucuronidase digestion of the tetrasaccharide (6 nmol) was performed using 110 Fishman units of the enzyme in a total volume of 50 μl of 50 mM-sodium acetate buffer, pH 4.5, at 37 °C for 0, 15, 30 and 75 min. The reaction was terminated by heating at 100 °C for 1 min.

Chondroitinase ABC digestion of the tetrasaccharide (3 nmol each) was performed using 10 munits of the enzyme in a total volume of 30 μl of 60 mM-sodium acetate/50 mM-Tris/HCl, pH 8.0, at 37 °C for 5 min. The reaction was terminated by heating at 100 °C for 1 min.

For h.p.l.c. analysis the enzyme digests were treated with Disposil C18 (Nacalai Tesque Inc.) to remove proteins (if necessary), freeze-dried and reconstituted in 16 mM-NaH_2PO_4.

Oligosaccharide analysis

Concentrations of hyaluronate oligosaccharides were determined by the carbazole method, with glucuronic acid as standard [20].

H.p.l.c.

Oligosaccharides were fractionated and analysed by h.p.l.c. by using an amine-bound silica PA03 column (4.6 mm × 250 mm) (YMC Co., Kyoto, Japan) as described in [21]. Samples were diluted so as to contain 0.5–20 nmol of oligosaccharides in 100–400 μl of 16 mM-NaH_2PO_4. The NaH_2PO_4 concentration was increased using a linear gradient at a flow rate of 1 ml/min from 16 to 530 mM over 60 min to analyse the hyaluronidase digests or from 16 to 300 mM over 45 min to analyse β-glucuronidase or chondroitinase ABC digestion respectively. Eluates were monitored by u.v. absorption at 206 or 210 nm (due mainly to the *N*-acetyl group of GlcNAc). Collected fractions were concentrated and desalted through a column (0.9 cm × 60 cm) of Sephadex G-25 (fine grade).

500 MHz ^{1}H-n.m.r. spectroscopy

Tetrasaccharides obtained by hyaluronidase digestion were repeatedly exchanged in ^2H_2O with intermediate freeze-drying. The 500 MHz ^{1}H-n.m.r. spectra were measured on a Varian VXR-500 at a probe temperature of 26 °C. Chemical shifts (δ) are given relative to sodium 4,4-dimethyl-4-silapentane-1-sulphonate, but were actually measured indirectly relative to acetone (2.225 p.p.m.) in ^2H_2O [22]. The $^1HO^2H$ signal was suppressed by presaturation during 3 s or 1 s for one-dimensional (1D) or two-dimensional (2D) spectra, respectively. For 2D spectra, 128 experiments of 512 data points for a spectrum width of 1500 Hz were measured, and the time-domain data were multiplied with a squared sine-bell or a shifted Gaussian function for 2D correlation spectroscopy (COSY) or 2D homonuclear Hartmann–Hahn (HOHAHA) spectroscopy [23] respectively. The 90° pulse width was 20 μs, and a Malcom Levitt (MLEV)-17 mixing sequence of 90 ms was used for 2D HOHAHA [23a]. The sample amounts used for measurements were 112, 52 and 150 nmol for the tetrasaccharide prepared by digestion with stonefish, leech or sheep testis hyaluronidase respectively.

RESULTS AND DISCUSSION

Hyaluronidases have various substrate specificities and different reaction mechanisms according to which they are divided into three major groups [1]. The first group is called hyaluronoglucosaminidase or testicular-type hyaluronidase (hyaluronate 4-glycanohydrolase, EC 3.2.1.35), which acts as an endo-β-*N*-acetyl-D-hexosaminidase. Lysosomal hyaluronidase of the same specificity as that of testicular hyaluronidase has been purified from human [24] and pig liver [25] and from human placenta [26]. The second group consists of hyaluronoglucuronidase (leech hyaluronidase, hyaluronate 3-glycanohydrolase, EC 3.2.1.36), which acts as an endoglucuronidase on β-1,3-glycosidic linkages between β-1,3-glucuronic acid and *N*-acetyl-D-glucosamine. So far this type of enzyme has only been found in the leech *Hirudo medicinalis*, and it is apparently localized in the salivary glands. This enzyme is specific for hyaluronate and does not act on chondroitin or chondroitin sulphate. The third group consists of microbial hyaluronidases which act on their substrates by β-elimination producing Δ-4,5-

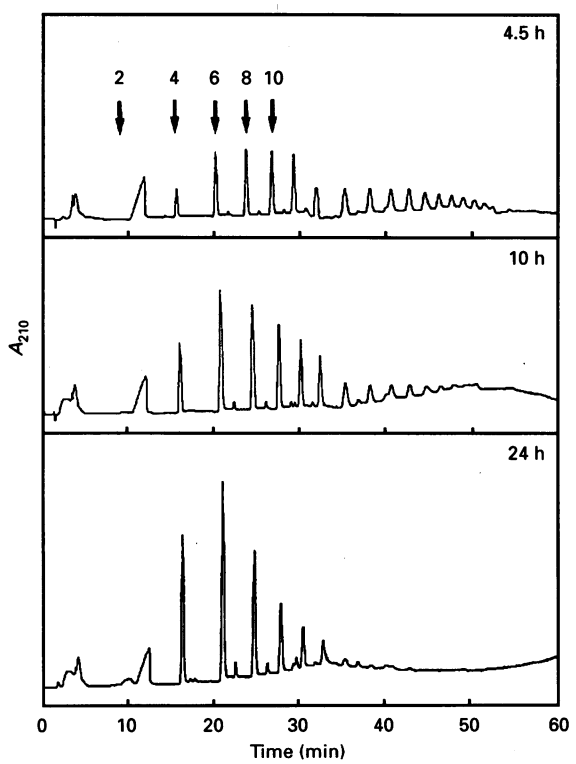

Fig. 1. Digestion of hyaluronate with stonefish hyaluronidase

Hyaluronate was digested with stonefish hyaluronidase, and aliquots corresponding to 45 μg of hyaluronate were withdrawn at 2.5, 4.5, 10 and 24 h and analysed by h.p.l.c. Eluates were monitored by u.v. absorption at 210 nm. Results from 4.5, 10 and 24 h time points are shown. Arrows indicate the elution positions of the standard even-numbered oligosaccharides.

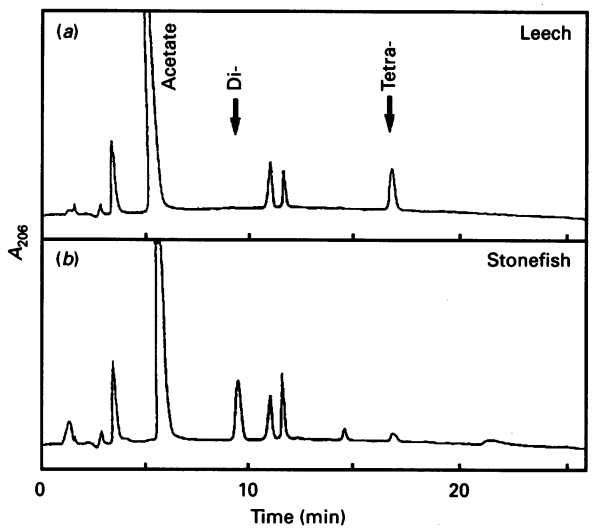

Fig. 2. Chondroitinase ABC digestion of the tetrasaccharide obtained by stonefish or leech hyaluronidase digestion

The tetrasaccharide prepared by digestion with hyaluronidase from leech (*a*) or the stonefish (*b*) was treated with chondroitinase ABC and the incubation mixtures corresponding to 1.5 nmol of the tetrasaccharide were analysed by h.p.l.c. Eluates were monitored by u.v. absorption at 206 nm. Arrows indicate the elution positions of the standard hyaluronate tetrasaccharide and the mixture of a saturated and an unsaturated disaccharide.

unsaturated disaccharides. Thus the enzymes are eliminases and are called hyaluronate lyases (EC 4.2.99.1).

In order to characterize the mechanism of enzymic action of the stonefish hyaluronidase, the reaction products were analysed. Human umbilical-cord hyaluronate was digested with the stonefish hyaluronidase and simultaneously analysed by h.p.l.c. on an amine-bound silica column. In this h.p.l.c. system the elution position of each even-numbered oligosaccharide prepared using the testicular enzyme was not distinguishable from that of the corresponding oligosaccharide obtained by using the leech enzyme. As Fig. 1 shows, the products are even-numbered oligosaccharides and the major end products are tetra-, hexa-, octa- and deca-saccharides. No appreciable disaccharide was formed. The broad peak observed between those of the di- and tetrasaccharide peaks was derived from the incubation buffer, as was shown by a control experiment (results not shown). These results suggest that the enzyme is an endo-type glycosidase. On the basis of the u.v.-absorption spectra of the digest, the enzyme is not an eliminase. It remains to be determined whether the enzyme has a transglycosylation activity [1].

In order to determine whether the enzyme is an endoglucosaminidase or endoglucuronidase, the tetrasaccharide product (Tetrasaccharide A) was isolated by h.p.l.c. after stonefish-hyaluronidase digestion and was compared with that prepared using sheep testis hyaluronidase (Tetrasaccharide I) or leech hyaluronidase (Tetrasaccharide II). It has been reported that chondroitinase ABC degrades Tetrasaccharide I, but not II [27]. Tetrasaccharide A was degraded with chondroitinase ABC (Fig. 2*b*) as was Tetrasaccharide I (results not shown), yielding a mixture consisting of a saturated and an unsaturated disaccharide, whereas Tetrasaccharide II was resistant to chondroitinase (Fig. 2*a*). In this h.p.l.c. system, the saturated and the unsaturated disaccharide were inseparable, as was checked with standard disaccharides. The peaks eluted at around 3.5, 11 and 12 min are derived from the incubation buffer or the enzyme preparation, as was shown by control experiments (results not

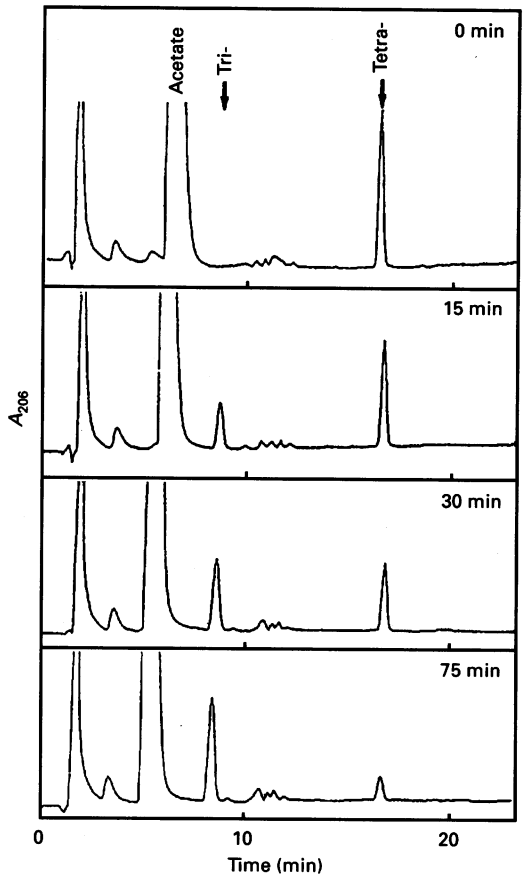

Fig. 3. β-Glucuronidase digestion of the tetrasaccharide obtained by stonefish-hyaluronidase digestion

The isolated tetrasaccharide (6 nmol) prepared by stonefish-hyaluronidase digestion was treated with exo-β-glucuronidase in quadruplicate, and each reaction was terminated at 0, 15, 30 or 75 min and analysed by h.p.l.c. Eluates were monitored by u.v. absorption at 206 nm. Arrows indicate the elution positions of the standard hyaluronate tetrasaccharide and the presumed trisaccharide product respectively.

shown). The results indicate that Tetrasaccharide A has the same structure as that of Tetrasaccharide I, and that the stonefish enzyme is most likely an endo-β-*N*-acetylglucosaminidase.

When incubated with the purified exo-β-glucuronidase, Tetrasaccharide A was digested, yielding a product which has an absorbance at 206 nm similar to that of the parent tetrasaccharide (Fig. 3). The results indicate that Tetrasaccharide A had a GlcA at the non-reducing end and was converted into a trisaccharide and a free GlcA, which has no significant absorption at 206 nm. The peaks eluted at 2 and 3.5 min are derived from the incubation buffer or the enzyme preparation.

The tetrasaccharides prepared using hyaluronidases from the above three sources were analysed by ^{1}H-n.m.r. (Fig. 4). The spectrum of Tetrasaccharide A (Fig. 4*a*) is different from that of Tetrasaccharide II (Fig. 4*c*), but is indistinguishable from that of Tetrasaccharide I (Fig. 4*b*). Chemical shifts of the structural-reporter-group protons [22,28] resonating out of the bulk region (δ 3.5–3.9 p.p.m.) of the constituent monosaccharides for Tetrasaccharide A have been assigned by using 2D COSY (results not shown), as well as by 2D HOHAHA (Fig. 5) methods, and the n.m.r. data are presented in Table 1. The anomeric signal at δ 5.157 p.p.m. is characteristic of GlcNAc-*1* H-1 of the α-anomer, whereas the GlcNAc-*1* H-1 signal of the β-anomer is hidden under the ^{1}HO^{2}H signal [17], but was revealed in 2D HOHAHA

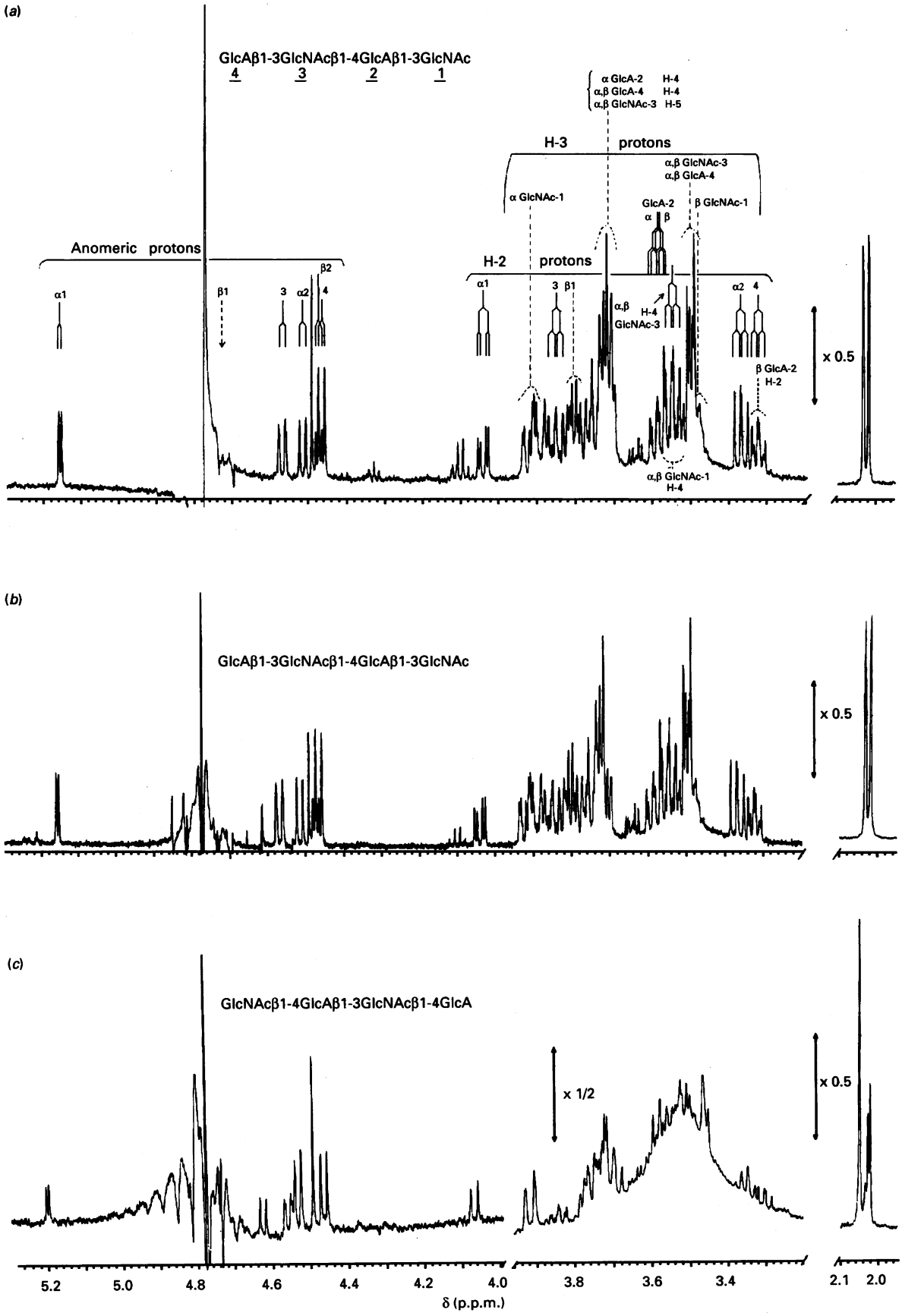

Fig. 4. 500 MHz ^{1}H-n.m.r. spectra of the hyaluronate tetrasaccharides

The tetrasaccharides prepared by digestion with hyaluronidase from the stonefish (*a*), sheep testis (*b*) and leech (*c*) were analysed by 500 MHz ^{1}H-n.m.r. The numbers and letters in the spectra refer to the corresponding residues in the structure. The anomerization effects resulted in two sets of proton signals for GlcNAc-*1* and GlcA-*2*, but not for GlcNAc-*3* and GlcA-*4*.

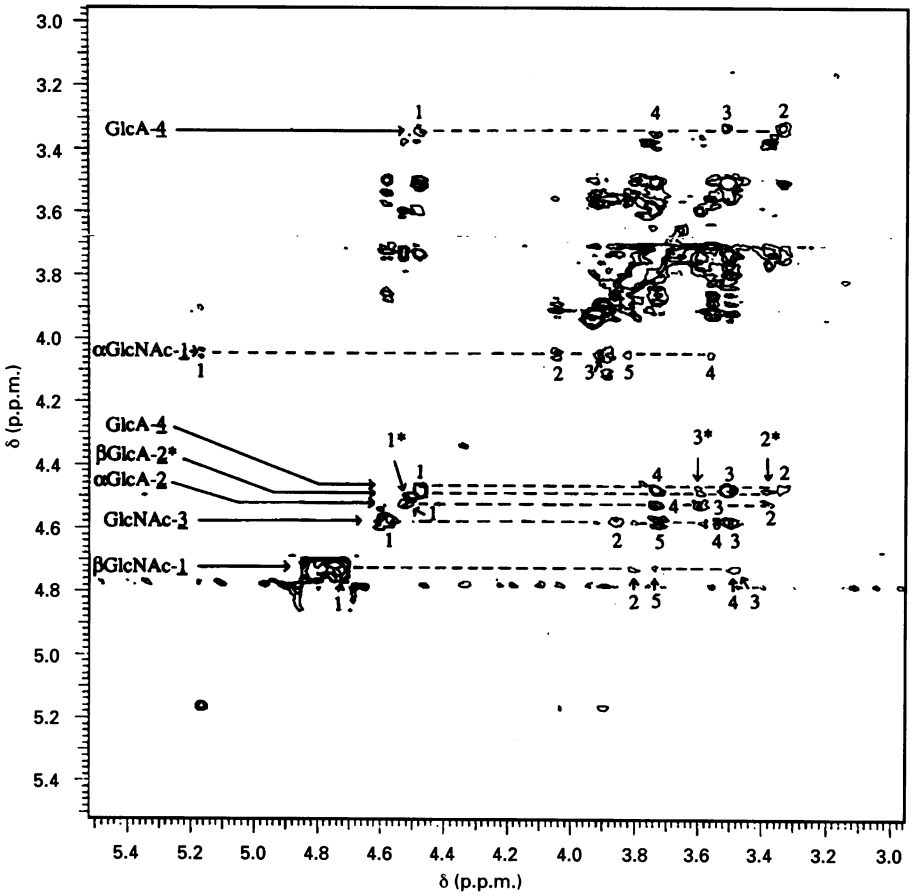

Fig. 5. 2D HOHAHA spectrum of the hyaluronidase tetrasaccharide

The 2D HOHAHA spectrum of the tetrasaccharide resulting from stonefish-hyaluronidase digestion was recorded at 500 MHz in ^2H_2O at 26 °C. In the Figure the assignment of the component residues is indicated on the cross sections (----). The numbers near cross-peaks in the Figure refer to the protons of the scalar coupling network belonging to a diagonal peak in the anomeric region. Signals belonging to GlcA-*2* of the β-anomer are indicated by asterisks to differentiate them from others on closely located lines. Two sets of signals corresponding to α- and β-anomers were observed for GlcNAc-*1* and GlcA-*2*, but not for GlcNAc-*3* and GlcA-*4*.

Table 1. Chemical shifts of structural-reporter-group protons of the constituent monosaccharides for the tetrasaccharide prepared by stonefish hyaluronidase digestion

Chemical shifts are given in p.p.m. downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulphonate, but were actually measured indirectly relative to acetone (δ 2.225 p.p.m.) in ^2H_2O at 26 °C.

	Chemical shift (δ) of reporter group (p.p.m.)							
	H-1	H-2	H-3	H-4	H-5	H-6	H-6′	*N*-Ac
GlcNAc-*1*	α5.157	α4.040	α3.90	α3.55	αn.d.*	αn.d.	αn.d.	α2.017†
	β4.73	β3.80	βn.d.	β3.55	βn.d.	βn.d.	βn.d.	β2.017†
GlcA-*2*	α4.516	α3.368	α3.593	α3.72	αn.d.	–‡	–	–
	β4.474	β3.32	β3.587	βn.d.	β3.76	–	–	–
GlcNAc-*3*	4.569	3.851	3.50	3.55	3.72	n.d.	n.d.	2.033†
GlcA-*4*	4.466	3.322	3.50	3.72	3.77	–	–	–

* n.d., not determined.
† Chemical shifts of the acetyl protons of GlcNAc-*1* and *3* may be interchanged.
‡ Not occurring.

and 2D COSY (results not shown) spectra. The anomerization effect resulted in resonances at δ 4.516 p.p.m. (α H-1) and at δ 4.474 p.p.m. (β H-1) of the second constituent, GlcA-*2*, respectively. A close inspection of the spectrum also indicates the slight anomerization effect on the third constituent, GlcNAc-*3*, at δ 4.569 p.p.m. (α, β H-1). However, no such anomerization effects are observable on GlcA-*4* H-1 (δ 4.466 p.p.m.). H-2 proton signals at δ 3.3–3.4 p.p.m. are the structural reporter groups of GlcA, whereas an α H-2 proton signal at δ 4.040 p.p.m. in the structural-reporter-group region and β H-2 proton signals at δ 3.80–3.85 p.p.m. in the bulk region are characteristic of GlcNAc. The connectivities of these H-2 signals with the

remaining anomeric signals at δ 4.569 and 4.466 p.p.m. in the 2D HOHAHA spectrum led to the assignment of GlcNAc-*3* H-1 and GlcA-*4* H-1, respectively. The assignment of the structural-reporter-group protons led to the unambiguous identification of the following structure for Tetrasaccharide A, which is identical with that of Tetrasaccharide I:

GlcAβ1-3GlcNAcβ1-4GlcAβ1-3GlcNAc

4 *3* *2* *1*

Testicular hyaluronidase exhibits enzymic activity not only towards hyaluronate but also towards chondroitin, chondroitin 4-sulphate, chondroitin 6-sulphate and towards the chondroitin sulphate-like portion of dermatan sulphate (cf. [2]). In contrast, the stonefish enzyme did not show activity towards chondroitin sulphate or dermatan sulphate, as determined by the turbidity assay (results not shown) and is specific for hyaluronate. This enzyme is unique in that it is specific for hyaluronate in spite of the mechanism of its enzymic action being of the testicular type. Thus the enzyme should be useful for specific degradation of hyaluronate. It should be noted that, although leech hyaluronidase and *Streptomyces* hyaluronidase [29] are also specific for hyaluronate, their mechanism of action is different from that of the stonefish enzyme.

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