Glycoconj J (2009) 26:559–566
DOI 10.1007/s10719-008-9200-2

Novel products in hyaluronan digested by bovine testicular hyaluronidase

Fengchao Chen · Ikuko Kakizaki · Masanori Yamaguchi · Kaoru Kojima · Keiichi Takagaki · Masahiko Endo

Received: 26 July 2008 / Revised: 24 September 2008 / Accepted: 1 October 2008 / Published online: 15 November 2008

Abstract When the products of hyaluronan (HA) digested by bovine testicular hyaluronidase (BTH) were analyzed by high-performance liquid chromatography (HPLC), minor peaks were detected just before the main even-numbered oligosaccharide peaks. The amount of each minor peak was dependent on the reaction conditions for transglycosylation, rather than hydrolysis, by the BTH. Mainly based on HPLC and MS analysis, each minor peak was found to correspond to its oligosaccharide with one *N*-acetyl group removed from the reducing terminal *N*-acetylglucosamine. Enzymatic studies showed that the *N*-deacetylation activity was closely related to reaction temperature, pH, and the concentration of NaCl contained in the buffer, and glycosaminoglycan types and chain lengths of substrates. These findings strongly suggest that the *N*-deacetylation reaction in minor peaks was due to a novel enzyme contaminant in the BTH, *N*-deacetylase, that carries out *N*-deacetylation at the reducing terminal *N*-acetylglucosamine of oligosaccharides and is dependent on HA hydrolysis by BTH.

Keywords Bovine testicular hyaluronidase · Hyaluronan oligosaccharide · *N*-Deacetylase · *N*-Deacetylated oligosaccharide · *N*-Deacetylation

Abbreviations

BTH	Bovine testicular hyaluronidase
Ch	Chondroitin
Ch4S	Chondroitin 4-sulfate
Ch6S	Chondroitin 6-sulfate
HA	Hyaluronan
HPLC	High-performance liquid chromatography
NDST	*N*-Deacetylase/*N*-sulfotransferase
TLC	Thin-layer chromatography

F. Chen (✉) · I. Kakizaki · M. Yamaguchi · K. Kojima · K. Takagaki · M. Endo
Department of Glycotechnology,
Center for Advanced Medical Research,
Hirosaki University Graduate School of Medicine,
5 Zaifu-cho,
Hirosaki 036-8562, Japan
e-mail: seika1@cc.hirosaki-u.ac.jp

Introduction

Hyaluronidase, which acts on hyaluronan (HA), chondroitin (Ch), chondroitin 4-sulfate (Ch4S) and chondroitin 6-sulfate (Ch6S), is distributed widely in animal tissues, especially human testis and liver [1, 2]. The enzyme is endo-β-*N*-acetylhexosaminidase. The final reaction products obtained by digestion with the enzyme are primarily tetra- and hexasaccharides having an *N*-acetylhexosamine residue at the reducing terminus and a glucuronic acid residue at the non-reducing terminus.

Bovine testicular hyaluronidase (BTH) is useful for biochemical and histochemical analysis of glycosaminoglycans, and recently has also become an extremely important tool in glycotechnology [3–8] whereby its transglycosylation reaction can be used together with hydrolysis to reconstruct various glycosaminoglycan chains such as HA and Ch, in a similar way to gene technology. This has opened a new avenue of glycotechnology.

When using commercially marketed BTH for the reconstruction of glycosaminoglycans, we have been aware of some oligosaccharides possessing an unknown structure in the products derived from HA after BTH digestion. These oligosaccharides were not produced under hydrolysis conditions, but during transglycosylation by the enzyme, suggesting that a novel enzyme is present as a contaminant

in commercial BTH, which acts as an *N*-deacetylase of *N*-acetylglucosamine at the reducing terminal of HA oligosaccharides.

Materials and methods

Chemicals

HA (M_r=80,000) was obtained from Kibun Food Chemifa Co. (Tokyo, Japan), and BTH (type I-S, 300–500 U/mg) was purchased from Sigma-Aldrich Corp. (St. Louis, MO). Ch4S (from whale cartilage; M_r=34,000) and Ch6S (from shark cartilage; M_r=64,000) were obtained from Seikagaku Kogyo Co. (Tokyo, Japan). Ch was prepared from Ch4S based on the method of Kantor and Schubert [9]. Other reagents and chemicals were of the highest grade commercially available.

Incubation with BTH

Time course One milligram of HA was dissolved in 100 μl of 0.1 M sodium acetate buffer (pH 6.0) and then 0.2 mg of the BTH was added and incubated at 37°C for 3, 6, 12, 24, 48 and 96 h. The other substrates (1 mg), tetra-, hexa-, octa-, doca- and dodecasaccharides derived from HA [10], Ch, Ch4S and Ch6S, and *N*-acetylglucosamine, were digested with 0.2 mg of the BTH in 100 μl of 0.1 M sodium acetate buffer (pH 6.0) at 37°C for 24 h.

Effect of pH on enzyme reaction One milligram of HA was digested with BTH in 100 μl of 0.1 M sodium acetate buffer with different pH values of 3.0, 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0; 0.1 M sodium phosphate buffer with different pH values of 6.0, 6.5, 7.0, 7.5 and 8.0; and 0.1 M Tris–HCl buffer with different pH values of 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0 at 37°C for 24 h, respectively.

Effect of temperature on enzyme reaction One milligram of HA as a substrate was incubated with 0.2 mg of the BTH dissolved in 100 μl of 0.1 M sodium acetate buffer (pH 6.0) at different temperatures of 4°C, 20°C, 30°C, 40°C, 50°C and 60°C for 24 h, respectively.

Effect of NaCl concentration on enzyme reaction One milligram of HA was dissolved in 100 μl of 0.1 M sodium acetate buffer (pH 6.0) containing 0, 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 M NaCl, and incubated with BTH at 37°C for 24 h, respectively.

The reaction was terminated by boiling in a heat block (100°C) for 3 min, and the incubated solutions were centrifuged at 10,000 rpm for 5 min. The supernatant was then filtered with a 0.45 μm cellulose acetate filter (Millipore Corp. Bedford, MA) to remove insoluble proteins, and the filtrates were analyzed by high-performance liquid chromatography (HPLC).

N-Acetylation of minor peaks

N-Acetylation was performed based on the method of Hase *et al*. [11]. Collected minor peak samples (about 100 μg) were dissolved in 30 μl distilled water, and mixed with 8 μl of acetic anhydride and 35 μl of 17% NaOH solution at room temperature. Then the reaction mixture was stirred every 5 min and the procedure was repeated three times. The reaction products were further identified by mass spectroscopy.

Nitrous acid degradation assay

The lyophilized minor peak samples were treated with 0.5 ml of 1.8 M acetic acid containing 0.24 M $NaNO_2$ (pH 3.1) at room temperature for 80 min [12] and the reaction was stopped by adjusting to pH 4.5 by addition of 1 N NaOH solution. The end product was desalted on a PD-10 column [13] and further analyzed by HPLC and mass spectroscopy.

HPLC analysis

HPLC analysis was performed using a Hitachi L-6200 instrument equipped with a UV–vis detector (L-7420, Hitachi Co. Tokyo, Japan). The reaction products were eluted through a polyamine II column (4.6×250 mm, YMC Co. Kyoto, Japan) under the following conditions: condition 1, the column was equilibrated with solution A (50 mM NaH_2PO_4), and the ratio of solution B (246 mM NaH_2PO_4) to solution A was increased linearly to 100% over 100 min after sample injection; condition 2, the column was equilibrated with solution A, and the ratio of solution B to solution A was increased linearly to 50% over 50 min after sample injection; the flow rate was fixed at 1.0 ml/min, and the column temperature was 40°C; the eluates were monitored at a wavelength of 215 nm.

Mass spectroscopy

All mass spectra were obtained on a Sciex API-300 single-quadrupole mass spectrometer (Thornhill, Ontario, Canada) equipped with an atmospheric pressure ionization source [3, 10]. The mass spectrometer was operated in the negative mode; the ion-spray voltage was set at −3,500 V, and the orifice voltage at −55 V. The samples were introduced in 0.1% formic acid/acetonitrile (50:50 by volume). A micro-HPLC syringe pump, JASCO Familic 100 N (Harvard Apparatus Inc., Holiston, MA), was used to deliver the samples at a flow rate of 2 μl/min.

Thin-layer chromatography (TLC) and detection

TLC analysis was performed in vertical glass development chambers. Stock solutions of glucosamine, *N*-acetylglucosamine, and the reaction solution of *N*-acetylglucosamine incubated with inactivated BTH and active BTH were applied to a silica gel plate (Merck, Darmstadt, Germany) manually by means of a micro-syringe in a volume of 2 μl, and developed with 2-propanol–acetic acid–water (7:0.1:2, *v*/*v*) at room temperature. The spots on the chromatograms were detected by immersion in anisaldehyde–sulfuric acid reagent [14] and heated for color development.

Results

Preparation of minor peaks

HA was digested with BTH and the products were analyzed by HPLC as described before. The minor peaks were detected very clearly between every two continuous main peaks, and remained evident as incubation time was prolonged. As shown in Fig. 1, the minor peaks present after 96 h of incubation were minor peak 1 (MP1, before the tetrasaccharide peak), minor peak 2 (MP2, between the tetrasaccharide and hexasaccharide peaks), minor peak 3 (MP3, between the hexasaccharide and octasaccharide peaks), minor peak 4 (MP4, between the octasaccharide and decasaccharide), and minor peak 5 (MP5, between the decasaccharide and dodecasaccharide peaks). As representative cases, MP3, MP4 and MP5 were each collected and purified by HPLC with polyamine II for further studies.

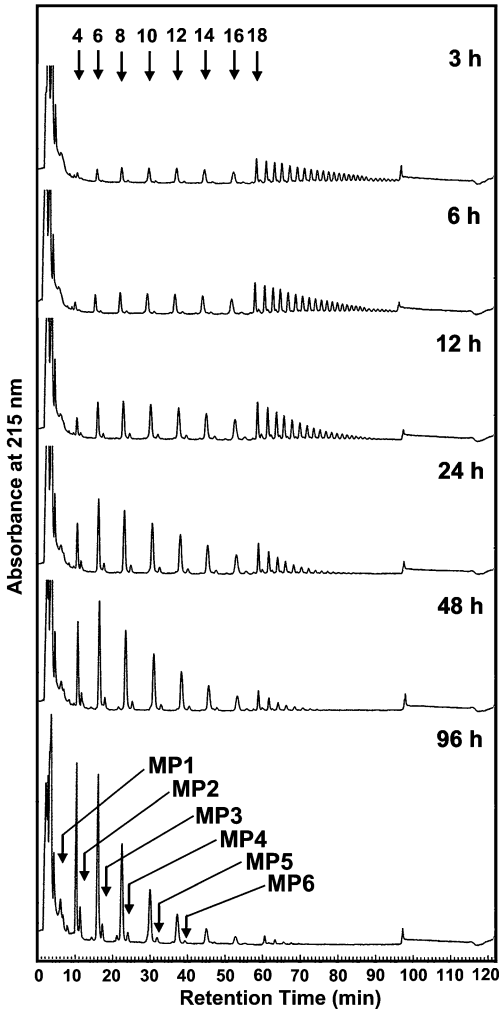

Fig. 1 Time course of HPLC chromatograms of hyaluronan digested by BTH. HA was digested by BTH in 0.1 M sodium acetate buffer (pH 6.0) at 37°C for different times. The digested solution was analyzed by HPLC on a polyamine II column (4.6×250 mm) under condition 1 described in "Materials and methods". *Arrows numbered 4–18* indicate the elution position of HA oligosaccharide standards, and MP1, MP2, MP3, MP4, MP5 and MP6 indicate the respective minor peaks of each oligosaccharide

Mass spectroscopy

The purified samples of MP3, MP4 and MP5 were subjected to mass spectroscopy, which showed characteristic multiply-charged ions produced by proton abstraction [10], each sample having more than one characteristic ion species (Fig. 2a). The major ion species and the computed molecular mass of each minor peak are shown in Table 1. Compared to the molecular mass of octasaccharide (M_r 1535.6), decasaccharide (M_r 1915.0) and dodecasaccharide (M_r 2294.4), respectively, MP3, MP4 and MP5 were each about M_r 43 smaller (*i.e.* the molecular mass of an acetyl radical). Therefore it was thought MP3, MP4 and MP5 were each produced by the removal of a single acetyl group from octasaccharide, decasaccharide and dodecasaccharide, respectively. To verify this possibility, *N*-acetylation of MP5 was carried out, and the product was analyzed again by ion-spray mass spectrometry (Fig. 2b). Based on the spectral peak of $[M\text{-}2H]^{2-}$ at *m*/*z* 1146.2, and $[M\text{-}3H]^{3-}$ at *m*/*z* 763.4, the molecular mass of acetylated MP5 was computed to be 2293.9, *i.e.* about equal to the molecular mass of dodecasaccharide. Therefore, it was concluded that MP3, MP4 and MP5 were simply octasaccharide, decasaccharide and dodecasaccharide, each with one acetyl group removed.

Nitrous acid degradation of minor peaks

To further identify the site from which the acetyl groups of MP3, MP4 and MP5 were removed, MP5 was subjected to nitrous acid degradation, and the reaction products were subjected to HPLC with polyamine II. As a result, only one

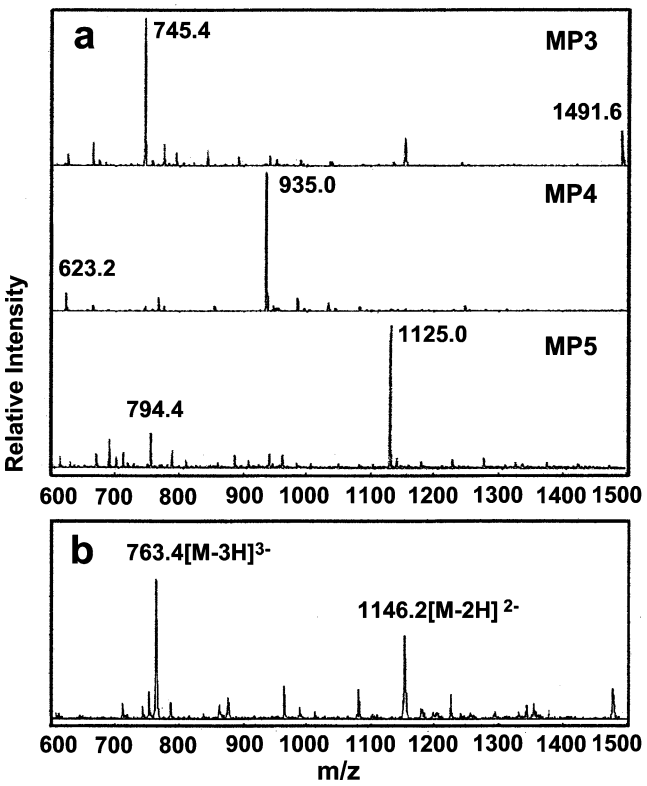

Fig. 2 MS spectra of minor peaks (**a**) and acetylated MP5 (**b**). Each purified minor peak was subjected to ion-spray mass spectrometry (**a**). Details of the conditions used for mass spectrometry are described in "Materials and methods". The *N*-acetylation of MP5 was performed as described in "Materials and methods", and then the reaction solution was further analyzed by mass spectroscopy (**b**)

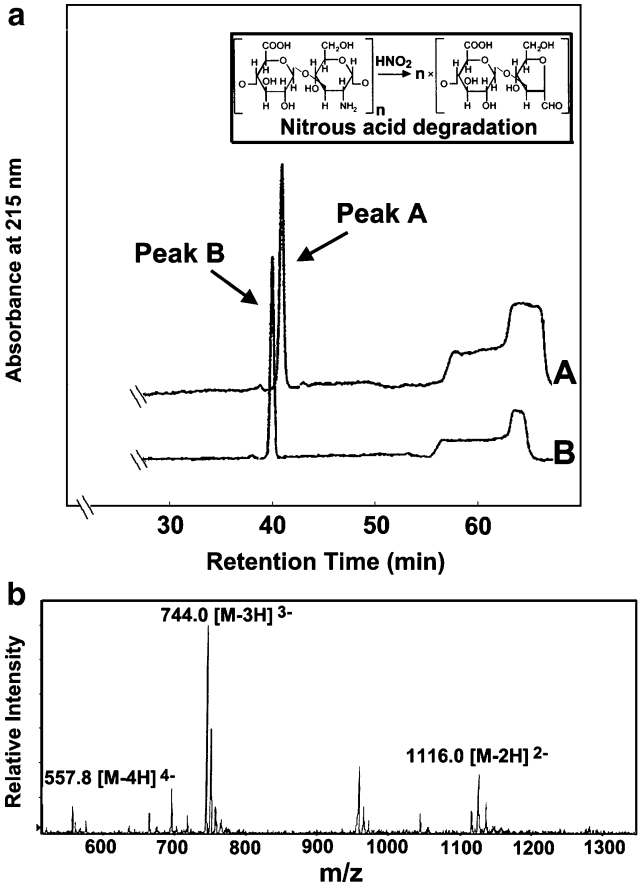

Fig. 3 HPLC chromatogram of nitrous acid degradation of MP5 (**a**) and mass spectroscopy of MP5 treated with nitrous acid (**b**). The minor peak MP5 was treated with nitrous acid under methods described in "Materials and methods", and the reaction products were subjected to HPLC on a Polyamine II column. **A** Without nitrous acid; **B** treated with nitrous acid; peaks A and B indicate *N*-deacetylated dodecasaccharide and dodecasaccharide containing 2,5-anhydromannose, respectively (**a**). The reaction product (peak B) of minor peak MP5 treated with nitrous acid was further analyzed by mass spectroscopy (**b**)

new peak (Fig. 3a, peak B) was detected and eluted earlier than the original peak (peak A) for the sample untreated with nitrous acid in the HPLC chromatogram. This new peak B was further analyzed by mass spectroscopy (Fig. 3b). Based on the spectral peaks of $[M\text{-}4H]^{4-}$ at *m/z* 557.8, $[M\text{-}3H]^{3-}$ at *m/z* 744.0 and $[M\text{-}2H]^{2-}$ at *m/z* 1116.0, the molecular mass of peak B was computed to be 2,235.2, which was coincident with the molecular mass of dodecasaccharide with 2, 5-anhydromannose at the reducing terminal. Therefore, it seemed likely that only one acetyl group was removed from *N*-acetylglucosamine at the reducing terminal of dodecasaccharide (Fig. 4).

In addition, MP3 and MP4 were also analyzed with nitrous acid degradation and HPLC. As a result, MP3 and MP4 were identified as octasaccharide and decasaccharide respectively, with *N*-unsubstituted glucosamine residue only at the reducing terminus (data not shown).

Table 1 Major observed ion species of minor peaks

Minor peaks	*m/z*			Estimated mass
	$[M\text{-}H]^-$	$[M\text{-}2H]^{2-}$	$[M\text{-}3H]^{3-}$	
MP3	1,491.6	745.4		1,492.8
MP4	1,870.6	935.0	623.2	1,872.1
MP5		1,125.0	749.4	2,251.7

Minor peak production in relation to reaction time

The time course of the HA hydrolysis reaction was investigated in 0.1 M sodium acetate buffer (pH 6.0) at 37°C. It was observed that the minor peaks of smaller size increased along with the corresponding main peaks as incubation time was prolonged, and became maximal at 96 h of incubation (Fig. 1). However, when HA was digested by BTH (type IV-S, 750–1,500 U/mg) and sheep testicular hyaluronidase (type V-S, 3,000–15,000 U/mg), minor peaks with each main peak were very small in BTH (type IV-S) digestion and no minor peaks were detected in sheep testicular hyaluronidase digestion (data not shown).

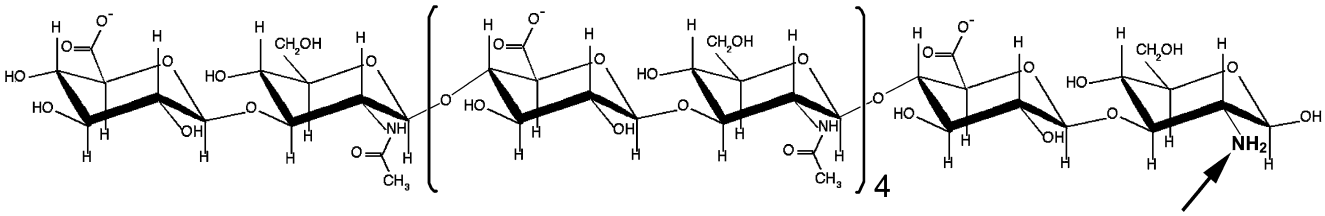

Fig. 4 Expected structure of MP5. Diagram showing the expected structure of *N*-deacetylated dodecasaccharide (MP5), the *arrow* indicating the site of *N*-deacetylated free amino group

Effect of pH on minor peak production

HA was incubated with BTH in 0.1 M sodium acetate buffer within a pH range of 3.0–6.0, in 0.1 M sodium phosphate buffer within pH range of 6.0–8.0, and in 0.1 M Tris–HCl buffer within a pH range of 6.0–9.0 at 37°C for 24 h. No minor peaks were observed at pH 3.0 on the HPLC chromatogram, while minor peaks began to be detected at pH 3.5 The production of MP3 (*N*-deacetylated octasaccharide) became maximal at pH 5.0, while the production of MP4 (*N*-deacetylated decasaccharide) and MP5 (*N*-deacetylated dodecasaccharide) became maximal at pH 6.0 (Fig. 5a). On the other hand, the minor peaks evident in 0.1 M sodium phosphate buffer and in 0.1 M Tris–HCl buffer were much fewer and smaller than those in 0.1 M sodium acetate buffer, even at the same pH value of 6.0 (Fig. 5a).

Effect of temperature on minor peak production

HA as a substrate was dissolved in 0.1 M sodium acetate buffer (pH 6.0) and digested with BTH at different temperatures of 4°C, 20°C, 30°C, 40°C, 50°C and 60°C for 24 h. The digestion products were then analyzed by

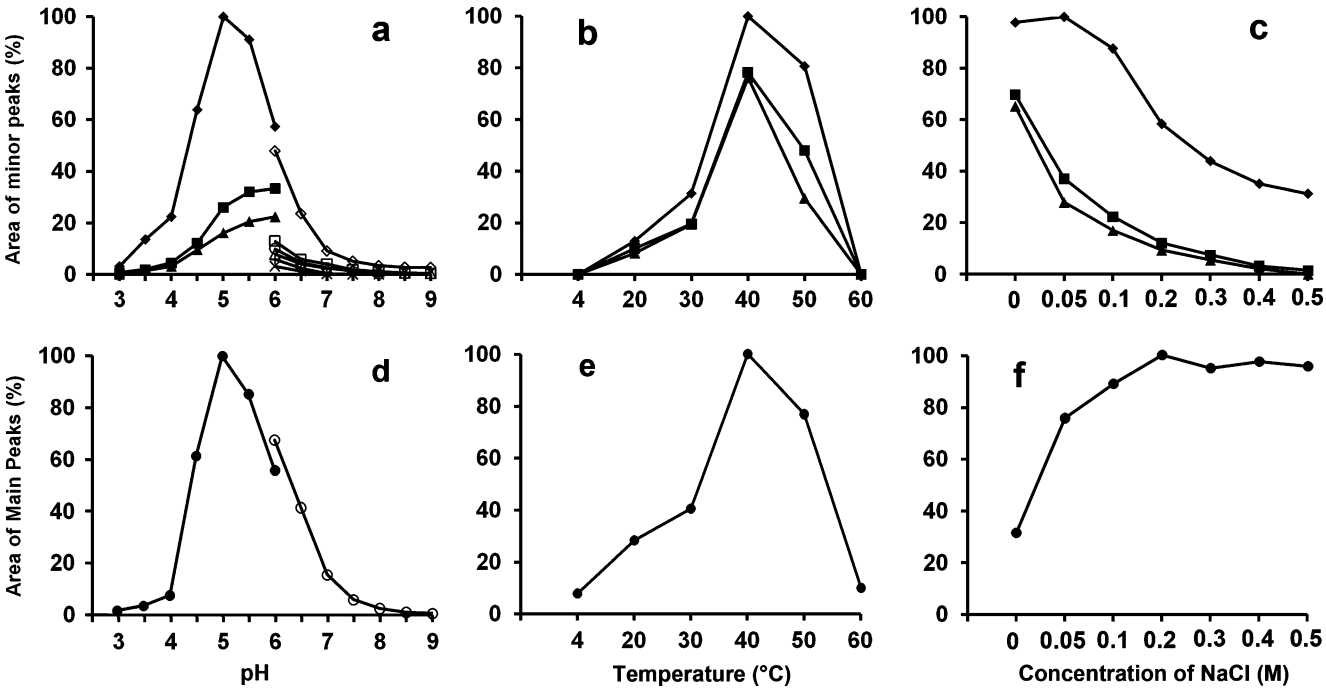

Fig. 5 Effects of pH, temperature and NaCl concentration on the generation of minor peaks. HA was digested under various reaction conditions as described in "Materials and methods" and the reaction products were subjected to HPLC on a polyamine II column (4.6× 250 mm). The *upper panels* show the effects of pH (**a**), temperature (**b**) and NaCl concentration (**c**) on *N*-deacetylation activity, respectively. *filled diamond*, MP3; *filled square*, MP4; *filled triangle*, MP5 in 0.1 M sodium acetate buffer, and *open diamond*, *open square*, and *open triangle* indicate corresponding minor peaks in 0.1 M Tris–HCl buffer, and *circle*, *plus sign*, and *ex* indicate corresponding minor peaks in 0.1 M sodium phosphate buffer, respectively. The *lower panels* show the effects of pH (**d**), temperature (**e**) and NaCl concentration (**f**) on BTH activity, respectively. The total products of tetrasaccharide and hexasaccharide were used to plot BTH activity curve, because tetrasaccharide and hexasaccharide are main final products of HA digested by BTH. *Filled circle*, BTH activities in 0.1 M sodium acetate buffer. *Open circle*, BTH activities in 0.1 M Tris–HCl buffer. Each point represents the average of three experiments, and the maximum area of peak was considered as 100%

HPLC. The minor peaks began to be detected from 20°C, became maximal at 40°C before decreasing, and were not detectable at 60°C (Fig. 5b).

Effect of NaCl concentration on minor peak production

The effects of NaCl concentration on production of the minor peaks were investigated at various concentrations of NaCl in 0.1 M sodium acetate buffer (pH 6.0) at 37°C for 24 h. The results of HPLC analysis showed that the minor peaks decreased sharply with increasing NaCl concentration, although MP3 (*N*-deacetylated octasaccharide) increased slightly in 0.05 M NaCl and then decreased sharply. MP4 (*N*-deacetylated decasaccharide) and MP5 (*N*-deacetylated dodecasaccharide) were very small from 0.2 M NaCl and were hardly evident in 0.5 M NaCl, although MP3 (*N*-deacetylated octasaccharide) was still evident in 0.5 M NaCl (Fig. 5c). These data indicated that minor peak production was inhibited by increasing NaCl concentration.

Substrate specificity of *N*-deacetylation

N-Acetylglucosamine was incubated with BTH and the reaction solution was analyzed by TLC. Compared with glucosamine, no new band was generated on the chromatogram at a position corresponding to that of a control sample of glucosamine (data not shown). This suggested that the *N*-acetyl group in the monosaccharide was not removed from *N*-acetylglucosamine by BTH.

The size preference of substrates was examined in the optimal conditions for minor peak generation. As a result, minor peaks were detected in the BTH reaction products of HA octasaccharide and larger oligosaccharides, however no minor peaks were detected in the incubation of BTH with HA tetrasaccharide and hexasaccharide by HPLC analysis (data not shown).

When Ch, Ch4S, Ch6S and their oligosaccharides were each digested by BTH, the HPLC and MS data of products revealed no evidence to indicate that *N*-deacetylation had occurred in any of the reactions.

Discussion

Based on the results of mass spectroscopy and HPLC of MP3, MP4 and MP5, it was suggested that each minor peak was produced by removal of only one *N*-acetyl group from octasaccharide, decasaccharide and dodecasaccharide, respectively (Fig. 2 and Table 1). Further study using nitrous acid degradation (Fig. 3a) and mass spectroscopy (Fig. 3b) of MP5 demonstrated that an *N*-acetyl group had been removed from *N*-acetylglucosamine at the reducing terminal of the oligosaccharide (Fig. 4).

When a hexosamine residue in a glycosaminoglycan oligosaccharide, which has a free amino or *N*-sulfate group, is treated with nitrous acid, the hexosaminide is cleaved and the hexosamine is converted to 2, 5-anhydro-D-mannose or talose as a reducing terminal [15]. The glycosaminoglycan oligosaccharide bearing the hexosamine residue within the chain is depolymerized by chemical degradation. If the *N*-deacetylated glucosamine is situated within the glycosaminoglycan chain, the glycosaminoglycan chain will be cleaved into two oligosaccharides by nitrous acid degradation. In this study, only one evident peak was detected in the HPLC chromatogram after nitrous acid degradation of the minor peak (Fig. 3a), suggesting that only the *N*-acetyl group at the reducing terminal was removed. This was further corroborated by mass spectroscopy (Fig. 3b), which indicated that the molecular mass of the new peak B produced from MP5 (*N*-deacetylated dodecasaccharide) by nitrous acid treatment was exactly the same as that of the dodecasaccharide with 2, 5-anhydro-D-mannose at the reducing terminal. Obviously, *N*-deacetylation occurred in the incubation of HA with BTH. Thus it was concluded that all the minor peaks were oligosaccharides bearing *N*-unsubstituted glucosamine at the reducing terminal.

The production of minor peaks through *N*-deacetylation of the oligosaccharides was closely related to the conditions of the enzymatic reaction. Firstly, when HA, as substrate, was incubated with BTH, the optimum pH for *N*-deacetylation was 6.0 (Fig. 5a). On the other hand, the optimum pH for the activities of BTH was about 5.0 (Fig. 5d) [16–18], and that of the transglycosylation reaction is 7.0 [4].

The *N*-deacetylation of the oligosaccharides was also dependent on temperature, the optimum reaction occurring at 40°C (Fig. 5b), and BTH activities also had a temperature optimum of 40°C (Fig. 5e).

In this study, production of the minor peaks was suppressed by increasing the NaCl concentration in the incubation medium (Fig. 5c), while BTH activities were increased with the increasing of NaCl concentration (Fig. 5f). It is known that the hydrolytic activity of testicular hyaluronidase depend upon the presence of electrolytes such as LiCl, NaCl, KCl, CsCl, $NaNO_3$ and Na_2SO_4 [17], NaCl concentration being especially important, with a concentration of 0.2 M for optimal enzyme activity [17]. On the other hand, the transglycosylation activity of testicular hyaluronidase is inhibited by NaCl in the incubation medium [4].

N-Deacetylated oligosaccharides, which were obtained as minor peaks in this study, have not been observed previously just because the presence of NaCl under hydrolysis conditions suppresses the *N*-deacetylation. Under the conditions of hyaluronidase transglycosylation, *N*-deacetylation of HA-oligosaccharides was observed in this study. Therefore, it was obvious that the *N*-deacetylation

responsible for production of the minor peaks was due to an enzymic, rather than a chemical reaction.

When HA was digested by BTH, minor peaks were detected with each even-numbered oligosaccharide peak and increased along with prolonging incubation time (Fig. 1). However no minor peaks were detected, when tetra- and hexasaccharide as substrates were each digested by BTH, while minor peaks were detected in HPLC chromatogram when HA octasaccharide or larger oligosaccharides as substrates were digested by BTH (data not shown). It was thought that the production of minor peaks of tetra- and hexasaccharide perhaps depends on the further depolymerization of octasaccharide and larger HA-oligosaccharides. It is well known that BTH cleaves the (β1-4) *N*-acetylhexosaminide linkage in HA, yielding even-numbered oligosaccharide units with *N*-acetylglusosamine at the reducing terminal, and with tetrasaccharides and hexasaccharides as the major end products [2]. Although the hexasaccharide is the well-known minimum substrate of BTH [19], the hydrolysis efficiency for hexasaccharide is much lower than for the octasaccharide [20]. Taken together, the findings suggest that *N*-deacetylation occurs in octasaccharide and larger HA-oligosaccharides during the hydrolysis of HA by BTH.

Although several bacterial strains contain an enzyme capable of hydrolyzing the amide bond of *N*-acetylglucosamine [21], until now no report has indicated the presence of *N*-acetylglucosamine *N*-deacetylase acting on a reducing terminal of HA oligosaccharides in animal tissues. Although a recombinant form of *N*-deacetylase/*N*-sulfotransferase (NDST), a key enzyme in the biosynthesis of heparin [22–24], has been tested for its effect on heparosan, the results suggested that the *N*-deacetylase acts on the *N*-acetyl group of *N*-acetylglucosamine in a random manner [22]. The *N*-deacetylation of HA oligosaccharides was different from that of NDST. Therefore, it was strongly suggested that the *N*-deacetylation accompanying HA hydrolysis by BTH was due to a new *N*-deacetylase that removed the acetyl group from *N*-acetylglucosamine at the reducing terminal of HA-oligosaccharides. This new enzyme is expected to play an important role in the remodeling of new bioactive oligosaccharides in many physiological and pathological processes.

Acknowledgments This work was supported by Grants-in Aid (Nos. 15370041 and 17770106) for Scientific Research from the Ministry of Education, culture, Sports, Science, and Technology of Japan and Cooperation for Innovative Technology and Advanced Research in Evolution Area (CITY AREA).

References

1. Kreil, G.: Hyaluronidases: a group of neglected enzymes. Protein Sci. **9**, 1666–1669 (1995)
2. Csoka, T.B., Frost, G.I., Stern, R.: Hyaluronidases in tissue invasion. Invasion Metastasis **17**, 297–311 (1997)
3. Takagaki, K., Nakamura, T., Izumi, J., Saitoh, H., Endo, M., Kojima, K., Kato, I., Majima, M.: Characterization of hydrolysis and transglycosylation by bovine testicular hyaluronidase using ion-spray mass spectrometry. Biochemistry **33**, 6503–6507 (1994). doi:10.1021/bi00187a017
4. Saitoh, H., Takagaki, K., Majima, M., Nakamura, T., Matsuki, A., Kasai, M., Narita, H., Endo, M.: Enzymic reconstruction of glycosaminoglycan oligosaccharide chains using the transglycosylation reaction of bovine testicular hyaluronidase. J. Biol. Chem. **270**, 3741–3747 (1995). doi:10.1074/jbc.270.8.3741
5. Takagaki, K., Munakata, H., Majima, M., Endo, M.: Enzymatic reconstruction of a hybrid glycosaminoglycan containing 6-sulfated, 4-sulfated, and unsulfated *N*-acetylgalactosamine. Biochem. Biophys. Res. Commun. **258**, 741–744 (1999). doi:10.1006/bbrc.1999.0697
6. Takagaki, K., Munakata, H., Kakizaki, I., Majima, M., Endo, M.: Enzymatic reconstruction of dermatan sulfate. Biochem. Biophys. Res. Commun. **270**, 588–593 (2000). doi:10.1006/bbrc.2000.2452
7. Takagaki, K., Munakata, H., Majima, M., Kakizaki, I., Endo, M.: Chimeric glycosaminoglycan oligosaccharides synthesized by enzymatic reconstruction and their use in substrate specificity determination of *Streptococcus* hyaluronidase. J. Biochem. **127**, 695–702 (2000)
8. Iwafune, M., Kakizaki, I., Yukawa, M., Kudo, D., Ota, S., Endo, M., Takagaki, K.: Reconstruction of glycosaminoglycan chains in decorin. Biochem. Biophys. Res. Commun. **297**, 1167–1170 (2002). doi:10.1016/S0006-291X(02)02350-1
9. Kantor, T.G., Schubert, M.: A method for the desulfation of chondroitin sulfate. J. Am. Chem. Soc. **79**, 152–153 (1957). doi:10.1021/ja01558a040
10. Takagaki, K., Kojima, K., Majima, M., Nakamura, T., Kato, I., Endo, M.: Ion-spray mass spectrometric analysis of glycosaminoglycan oligosaccharides. Glycoconj. J. **9**, 174–179 (1992). doi:10.1007/BF00731162
11. Hase, S., Tsuji, Y., Matsushima, Y.: Structural studies on a glucose-containing polysaccharide obtained from *Micrococcus lysodeikticus* cell walls. J. Biochem. **72**, 1549–1555 (1972)
12. Lindahl, U., Backstrom, G., Jansson, L., Hallen, A.: Biosynthesis of heparin. II. Formation of sulfamino groups. J. Biol. Chem. **248**, 7234–7241 (1973)
13. Vives, R.R., Pye, D.A., Salmivirta, M., Hopwood, J.J., Lindahl, U., Gallagher, J.T.: Sequence analysis of heparan sulphate and heparin oligosaccharides. Biochem. J. **339**, 767–773 (1999). doi:10.1042/0264-6021:3390767
14. Lee, D.E., Lillibridge, C.B.: A method for qualitative identification of sugars and semiquantitative determination of lactose content suitable for a variety of foods. Am. J. Clin. Nutr. **29**, 428–440 (1976)
15. Shively, J.E., Conrad, H.E.: Formation of anhydrosugars in the chemical depolymerization of heparin. Biochemistry **15**, 3932–3942 (1976). doi:10.1021/bi00663a005
16. Highsmith, S., Garvin, J.H., Garvin, J.R., Chipman, D.M.: Mechanism of action of bovine testicular hyaluronidase. Mapping of the active site. J. Biol. Chem. **250**, 7473–7480 (1975)
17. Gorham, S.D., Olavesen, A.H., Dodgson, K.S.: Effect of ionic strength and pH on the properties of purified bovine BTH. Connect. Tissue Res. **3**, 17–25 (1975). doi:10.3109/03008207509152337
18. Afify, A.M., Stern, M., Guntenhoner, M., Stern, R.: Purification and characterization of human serum hyaluronidase. Arch. Biochem. Biophys. **305**, 434–441 (1993). doi:10.1006/abbi.1993.1443

19. Cramer, J.A., Bailey, L.C., Bailey, C.A., Miller, R.T.: Kinetic and mechanistic studies with bovine testicular hyaluronidase. Biochim. Biophys. Acta **1200**, 315–321 (1994)
20. Hofinger, E.S., Bernhardt, G., Buschauer, A.: Kinetics of Hyal-1 and PH-20 hyaluronidases: Comparison of minimal substrates and analysis of the transglycosylation reaction. Glycobiology **17**, 963–971 (2007). doi:10.1093/glycob/cwm070
21. Roseman, S.: Glucosamine metabolism. I. *N*-acetylglucosamine deacetylase. J. Biol. Chem. **226**, 115–124 (1957)
22. Duncan, M.B., Liu, M., Fox, C., Liu, J.: Characterization of the *N*-deacetylase domain from the heparan sulfate *N*-deacetylase/*N*-sulfotransferase 2. Biochem. Biophys. Res. Commun. **339**, 1232–1237 (2006)
23. Berninsone, P., Hirschberg, C.B.: Heparan sulfate/heparin *N*-deacetylase/*N*-sulfotransferase. The *N*-sulfotransferase activity domain is at the carboxyl half of the holoenzyme. J. Biol. Chem. **273**, 25556–25559 (1998). doi:10.1074/jbc.273.40.25556
24. Orellana, A., Hirschberg, C.B., Wei, Z., Swiedler, S.J., Ishihara, M.: Molecular cloning and expression of a glycosaminoglycan *N*-acetylglucosaminyl *N*-deacetylase/*N*-sulfotransferase from a heparin-producing cell line. J. Biol. Chem. **269**, 2270–2276 (1994)