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Glycoblotting-based high throughput protocol for the structural characterization of hyaluronan degradation products during enzymatic fragmentation

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Abstract Increasing interests have been focused on the functional roles of hyaluronan degradation products, namely hyaluronan oligosaccharides, as signal molecules regulating cell growth, differentiation, malignancy, and inflammatory responses. It is clear that molecular size of hyaluronan oligosaccharides might be crucial for defining possible and dynamic roles in supporting and suppressing homeostatic cellular processes. The present paper communicates a facile and efficient approach based on *glycoblotting method* for the characterization of hyaluronan fragments liberated from three different sources of hyaluronan (rooster comb, bovine vitreous humor, and *Streptococcus*) by *in vitro* degradation using two typical hyaluronidases of bovine testicular (EC 3.2.1.35) and *Streptomyces hyalurolyticus* (EC 4.2.2.1). It was demonstrated that glycoblotting method allows for high throughput and quantitative analysis of hyaluronan fragments within a wide dynamic range (1~1,000 pmole) when 5 μg of hyaluronan digests were applied for this enrichment protocol. Molecular size and distribution of hyaluronan fragments were proved to be influenced strongly by conditions and hyaluronidases employed while source of hyaluronan did not affect the degradation profiles. Strikingly, the present method uncovered the existence of the smallest and unusual hyaluronan degradation fragments such as a disaccharide GlcAβ1-3GlcNAc during the digestion by bovine hyaluronidase and a trisaccharide GlcAβ1-3GlcNAcβ1-4GlcA derivative by *Streptomyces* hyaluronidase. Bovine testis hyaluronidases afforded hyaluronan tetra- and hexasaccharides as major products. On the other hand, it was demonstrated that *Streptomyces* hyaluronidase can produce odd number fragments from three to nine sugar residues while even number fragments from four to fourteen sugar residues were major products.

Keywords Hyaluronan metabolism · Hyaluronidases · Hyaluronan oligosaccharides · Glycoblotting method · High throughput glycomics · Mass spectrometry

Abbreviations

HA	hyaluronan (hyaluronic acid)
HAase	hyaluronidase
GlcNAc	*N*-acetyl-D-glucosamine
GlcA	D-glucuronic acid
HexNAc	*N*- acetyl-D-hexosamine
HexA	D-hexuronic acid
UA	uronic acid
MS	mass spectrometry
HPLC	high-performance liquid chromatography
CE	capillary electrophoresis
MALDI-TOF	matrix-assisted laser desorption/ ionization-time of flight
ESI	electro-spray ionization
MS/MS	tandem MS
DHB	2,5-dihydroxybenzoic acid

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MTT	3-methyl-1-*p*-tolyltriazene
aoWR	*N*-aminooxy tryptophanyl arginine
HA_n	GlcA(β1-3GlcNAcβ1-4 GlcA)$_n$ β1-3GlcNAc, n=0~4
HA_{2n+1}^{AA}	GlcA(β1-3GlcNAcβ1-4 GlcA)$_n$, n=1~4
HA_{2n+1}^{NN}	GlcNAc(β1-4GlcAβ1-3GlcNAc)$_n$, n=1~4
ΔHA(2n+2)	ΔUA(β1-3GlcNAcβ1-4GlcA)$_n$ -β1-3GlcNAc, n=0~8

Introduction

Hyaluronan (HA), an anionic linear polysaccharide composed of repeating disaccharides of GlcA and GlcNAc: (4GlcAβ1-3GlcNAcβ1)$_n$, is known to exhibit an indispensable function in maintenance of tissue architecture by providing cells with structural frameworks as an abundant member of the extracellular matrices due to the remarkable hydrodynamic characteristics, especially in terms of its high viscosity and its ability to retain water [1]. Although this characteristic feature is one of the common physiological roles of HA in adult organisms, HA also functions as signal molecules that control cellular behavior during embryonic development, healing processes, inflammatory response, and cancer development [1–6]. It is thought that molecular size of HA degradation products, namely HA oligosaccharides, might be essential for defining possible and dynamic roles in supporting and suppressing homeostatic cellular processes [7–10]. It is also documented that HA and hyaluronidases (HAases) are often overproduced in many types of human tumor [11]. Observations that overexpression of HAases suppresses the growth of colon and breast carcinomas in xenografts [12], and that one of the HAases seems to be a tumor suppressor [13], should support a role for HA in tumor progression. However, it was also suggested that overexpression of hyaluronidases can promote, rather than suppress, tumor progression [14]. Moreover, expression levels of HAases appear to be increased in cases of some malignant tumors such as bladder, prostate, colorectal, and brain cancers [1]. Interestingly, it was demonstrated that HA oligosaccharides show angiogenic property while native HA polymers are known to have anti-angiogenic nature [7]. Furthermore, fragments derived from epithelial cell surface HA might initiate inflammatory responses in acute lung injury through the interaction with Toll like receptors 2 and 4 (TLR-2 and 4) [8]. These results strongly suggest the importance of homeostatic balance of the production and degradation of HA not only in tumor cells but in general epithelial cells behavior. It is clear that both of HA and the degradation products of HA participate in transducing signals in proliferating and migrating cells. Considering that HA oligosaccharides of defined molecular weight and chemical formula have attractive potentials as novel therapeutic reagents, a reliable method for the identification of molecular size, chemical structures of the terminus, and distribution of such HA fragments is particularly important for the insight into the relationship between dynamic HA metabolism and its multiple physiological functions regulated significantly by an enzymatic degradation reaction involving several HAases. Despite of the extensive efforts for the structural characterization of HA fragments on the basis of various mass spectrometry-based approaches such as CE/ESI-MS [15], LC-ESI-MS [16–18], or LC+MALDI-TOFMS [19], there is no standardized protocol allowing for facile and quantitative analysis of HA oligosaccharide fragments produced by HAases. It should be noted that tedious and time-consuming separation processes based on several chromatographic methods as well as negatively charged nature of HA oligosaccharides made definitely high throughput and quantitative assay during digestion by HAase difficult.

The present study demonstrates the feasibility of *glycoblotting method*, a technology developed for high throughput and quantitative glycomics of glycoproteins and glycosphingolipids [20–29], in the characterization of HA fragments liberated from three different sources of hyaluronan (rooster comb, bovine vitreous humor, and *Streptococcus*) by *in vitro* degradation using two typical hyaluronidases of bovine testicular (EC 3.2.1.35) and *Streptomyces hyalurolyticus* (EC 4.2.2.1).

Experimental section

Materials and methods

3-Methyl-1-*p*-tolyltriazene (MTT) was obtained from Tokyo Chemical Industry Co. (Tokyo, Japan). 2,5-Dihydroxybenzoic acid (DHB) was purchased from Wako Pure Chemical Industries Ltd. (Tokyo, Japan). Glycoblotting beads (BlotGlycoH™) and *N*-aminooxy tryptophanyl arginine (ao-WR) were purchased from Sumitomo Bakelite, Co. (Tokyo, Japan). Rooster Comb, bovine vitreous humor, and *Streptococcus* HA were respectively obtained from Wako Pure Chemical Industries Ltd. (Tokyo, Japan), Tokyo Chemical Industry (Tokyo, Japan), and Funakoshi Chemicals (Tokyo, Japan). HAases from bovine testicular (EC 3.2.1.35) and *Streptomyces hyalurolyticus* (EC 4.2.2.1) were supplied by Aldrich Chemical Co. (Milwaukee, WI). HA tetrasaccharide (hyaluronan oligosaccharide 4mer sodium salt, HA4) and HA dodecasaccharide (hyaluronan oligosaccharide 12mer sodium salt, HA12) were purchased from Iduron Ltd. (Manchester, UK). Cellohexaose was from SEIKAGAKU CORPORATION (Tokyo, Japan). Solvents and other reagents

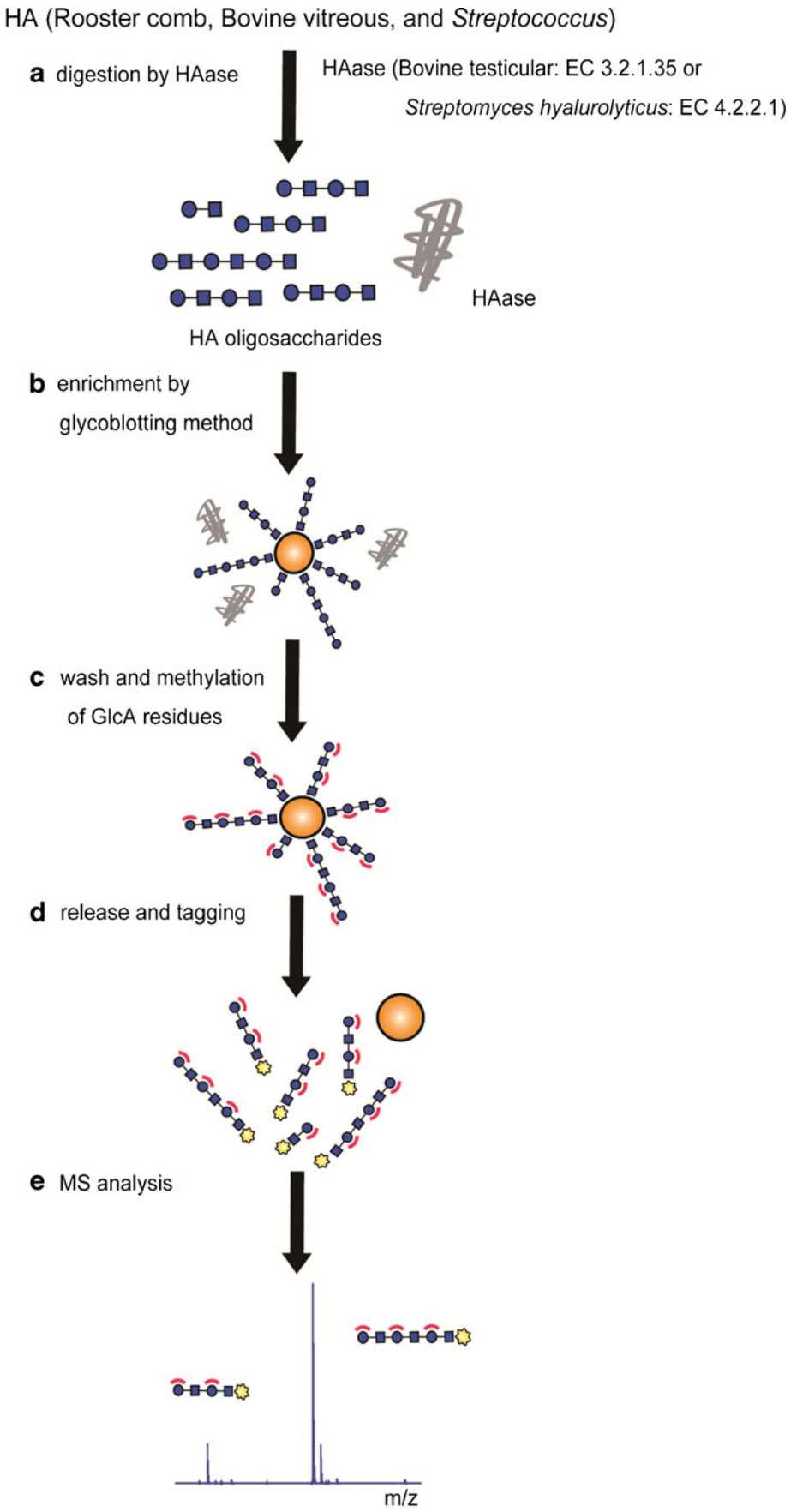
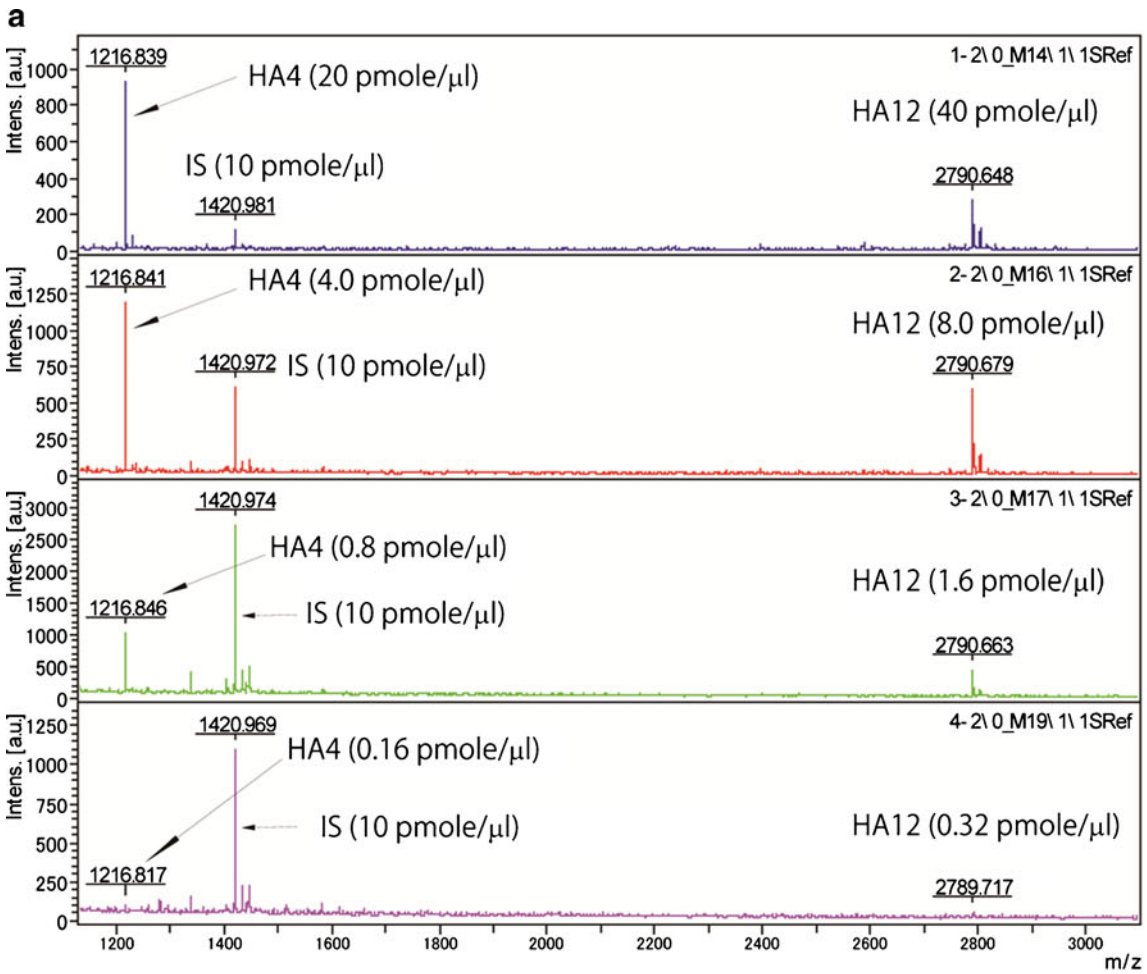
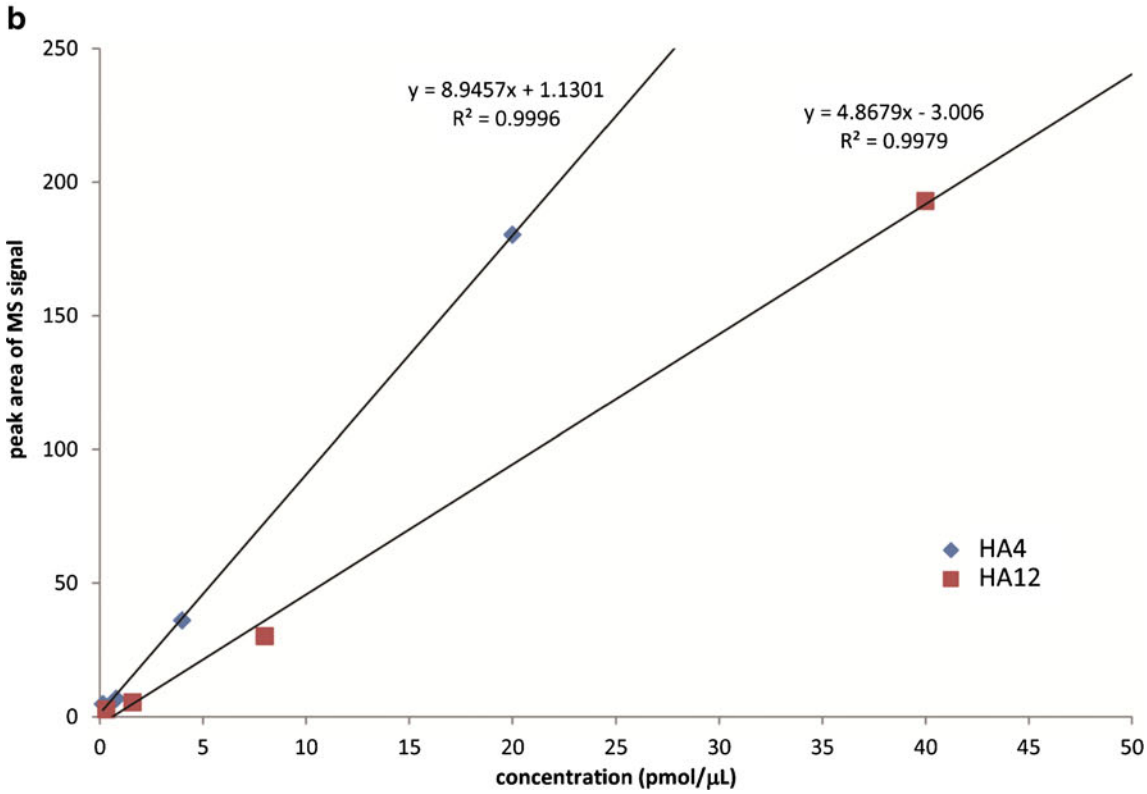


Fig. 1 General protocol for the glycoblotting-based high throughput analysis of HA fragments


a
1216.839
HA4 (20 pmole/μl)
IS (10 pmole/μl)
1420.981
HA12 (40 pmole/μl)
2790.648
1-2\0_M14\1\1SRef
1216.841
HA4 (4.0 pmole/μl)
1420.972
IS (10 pmole/μl)
HA12 (8.0 pmole/μl)
2790.679
2-2\0_M16\1\1SRef
1420.974
HA4 (0.8 pmole/μl)
1216.846
IS (10 pmole/μl)
HA12 (1.6 pmole/μl)
2790.663
3-2\0_M17\1\1SRef
1420.969
HA4 (0.16 pmole/μl)
IS (10 pmole/μl)
1216.817
HA12 (0.32 pmole/μl)
2789.717
4-2\0_M19\1\1SRef
Intens. [a.u.]
m/z


b
y = 8.9457x + 1.1301
R² = 0.9996
y = 4.8679x - 3.006
R² = 0.9979
HA4
HA12
peak area of MS signal
concentration (pmol/μL)

◀ **Fig. 2** Quantitative analysis of HA oligosaccharides by glycoblotting method. MALDI-TOFMS of HA_4 (0.16~20 pmole/μl) and HA_{12} (0.32 ~40 pmole/μl) used as authentic samples purchased from Iduron Ltd, Paterson Institute (**a**) and their calibration curves (**b**). IS means an internal standard (10 pmole/μl of cellohexaose) spiked before glycoblotting procedure

were purchased from Aldrich Chemical Co. (Milwaukee, WI), Tokyo Chemical Industry (Tokyo, Japan), and Wako Pure Chemical Industries Ltd. (Tokyo, Japan) unless otherwise noted. MALDI-TOF and MALDI-TOF/TOFMS analyses were performed by Ultraflex II (Bruker Daltonics, Bremen, Germany) in reflector, positive ion mode, typically totaling 200 x10 shots, and controlled by the Flexcontrol 3.0 software package (Bruker Daltonics, Bremen, Germany) according to the general protocols reported in our previous reports [20–29].

Glycoblotting-based protocol for the characterization of HA fragments during HAase digestion

[Step 1] Digestion of HAs by HAases (Fig. 1a): HA (50 μg) was dissolved at a concentration of 5.0 mg/ml (total volume: 15 μl) in digest buffer (100 mM phosphate buffer, 0.2 M NaCl, adjusted to pH 5.3 with H_2O) and bovine testicular HAase (0.62 mg; 500 U or 0.062 mg; 50 U in 15 μl digest buffer) or *Streptomyces* HAase (2 U or 0.2 U in 15 μl digest buffer) was added, and the solution was incubated at 37 °C for bovine testicular HAase or 60 °C for *Streptomyces* HAase. The enzymatic reaction was stopped by boiling for 20 min to deactivate HAase, centrifuged at 10,000 rpm for 30 min, and the supernatant was lyophilized, and stored at −20 °C. HAase digest obtained from 50 μg of HA was dissolved in 100 μl of H_2O and 10 μl of the solution was used for the subsequent enrichment experiments.

[Step 2] Enrichment and tagging of HA oligosaccharides (Fig. 1b~d): Aliquots of BlotGlycoH beads (250 μl) in a 10 mg/ml suspension with water were placed into a well of a MultiScreen Solvinert filter plate (Millipore, Billerica, MA), and the water was removed by vacuum. The sample mixtures (10 μl) containing HA fragments were applied to the well and 10 μl of 0.3 mM cellohexaose solution was also added to the same well as an internal standard, followed by the addition of 180 μl of 2 % acetic acid in acetonitrile. The plate was incubated at 80 °C for 45 min to dryness in a thermostat to capture whole HA oligosaccharides in the sample mixtures onto glycoblotting beads *via* stable hydrazone bonds. The plate was washed with 200 μl of water and 1 % triethylamine in methanol. Each washing step was performed twice. Unreacted hydrazide functional groups on beads were capped by incubation

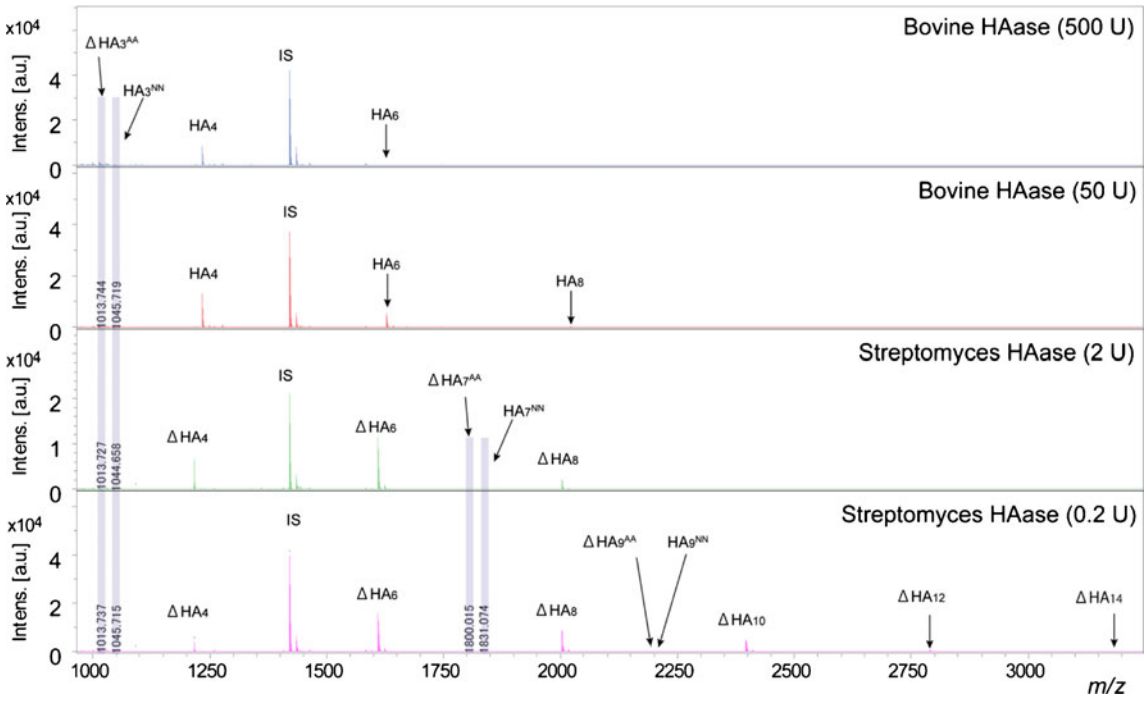


Fig. 3 MALDITOF-MS spectra of rooster comb HA degradation products enriched by glycoblotting method. IS means the signal due to aoWR-labeled cellohexaose used as an internal standard for the quantitation. All signals (*m/z*), relative intensities and estimated compositions/structures were summarized in Table 1

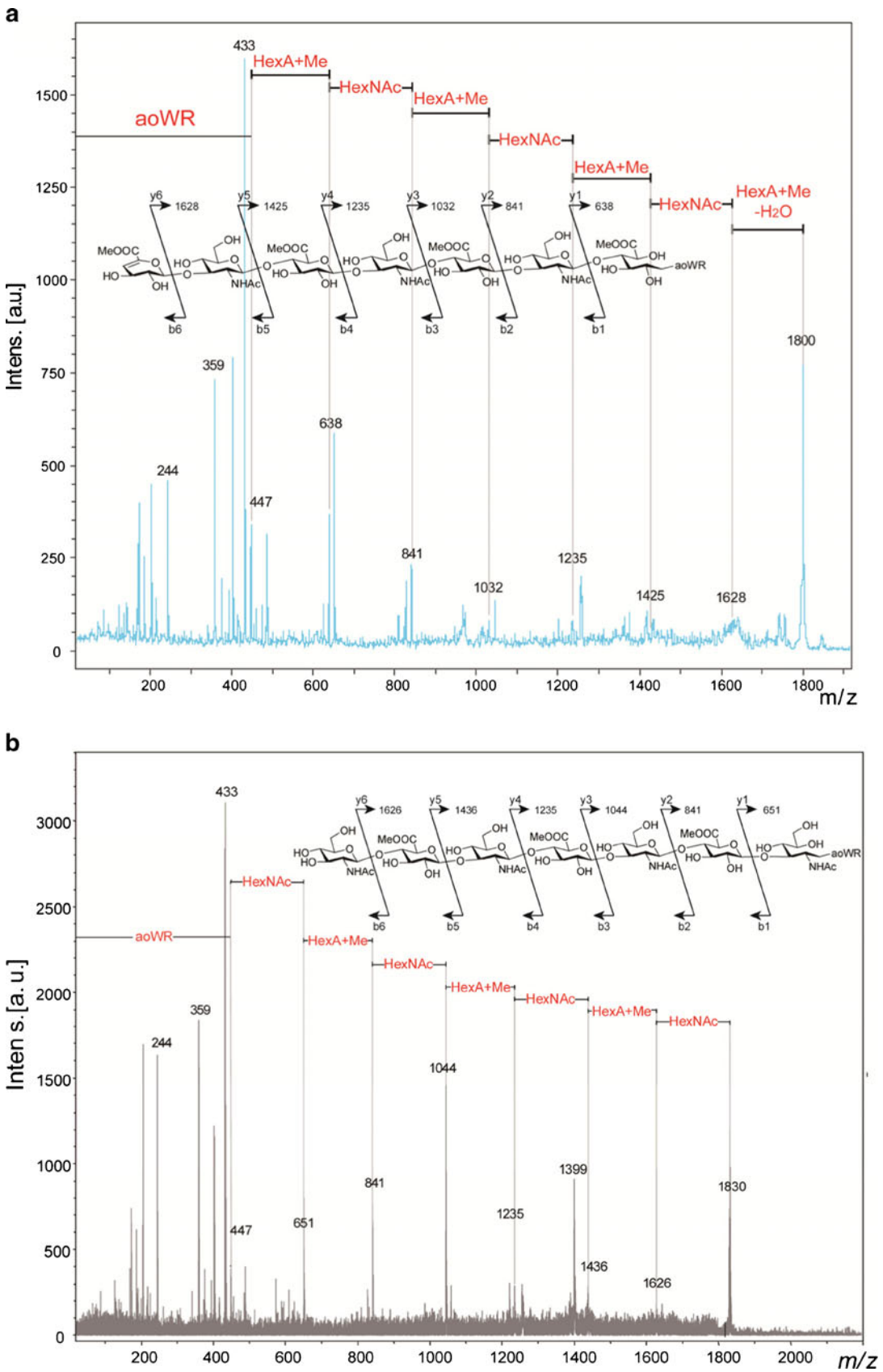


Fig. 4 MS/MS spectra of ΔHA_7^{AA} (**a**) and HA_7^{NN} (**b**) liberated from rooster comb HA by treating with *Streptomyces* HAase (0.2 U)

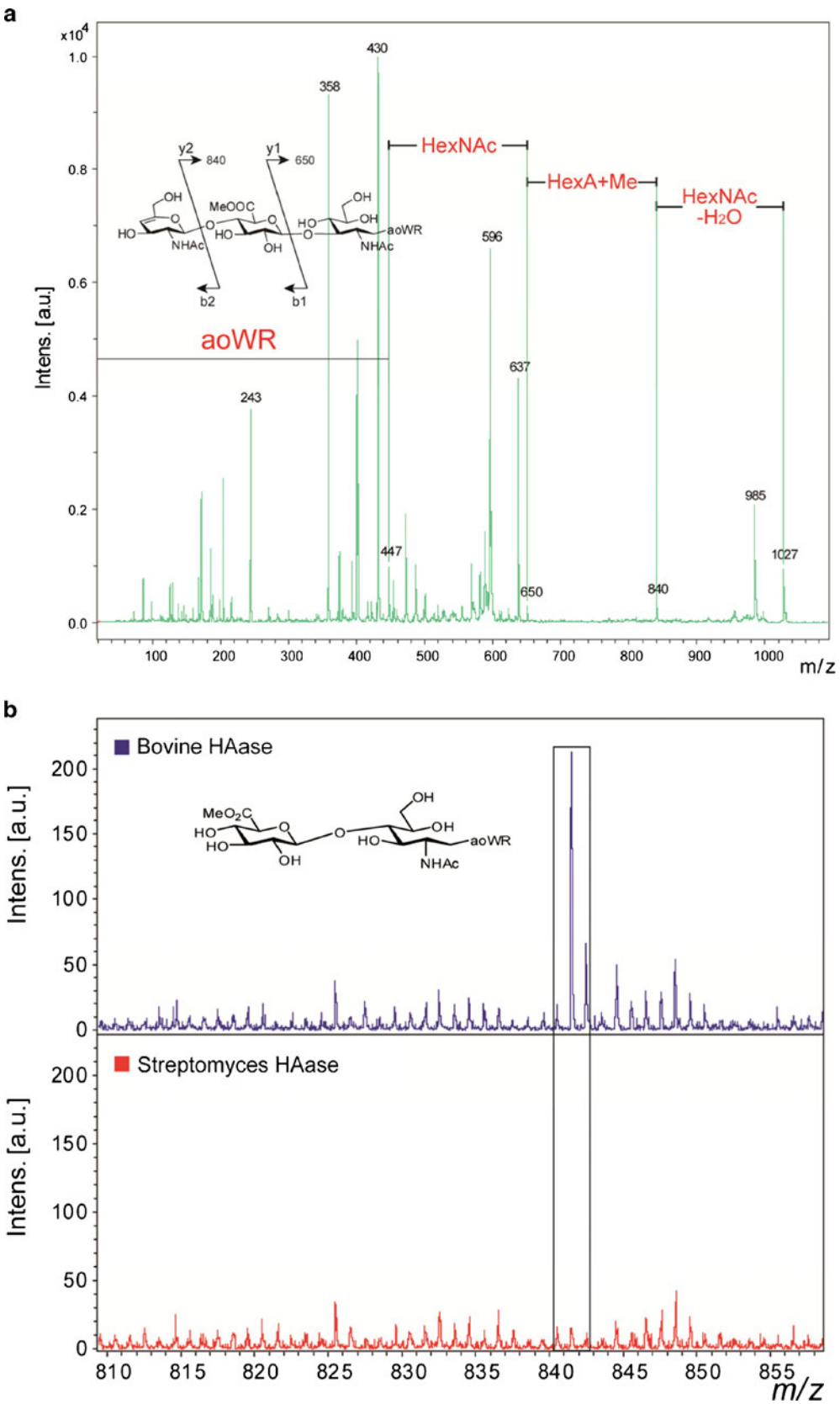


Fig. 5 Existence of unusual HA fragments demonstrated by the present method. **a** GlcNAcβ1-4GlcAβ1-3GlcNAc (ΔHA_3^{NN}) generated during digestion by bovine testis HAase, **b** GlcAβ1-3GlcNAc (HA_2, *m/z* 841.50) detected specifically during the digestion by bovine testis hyaluronidases and, (c) GlcAβ1-3GlcNAc β1-4GlcA (ΔHA_3^{AA}) generated by *Streptomyces* HAase

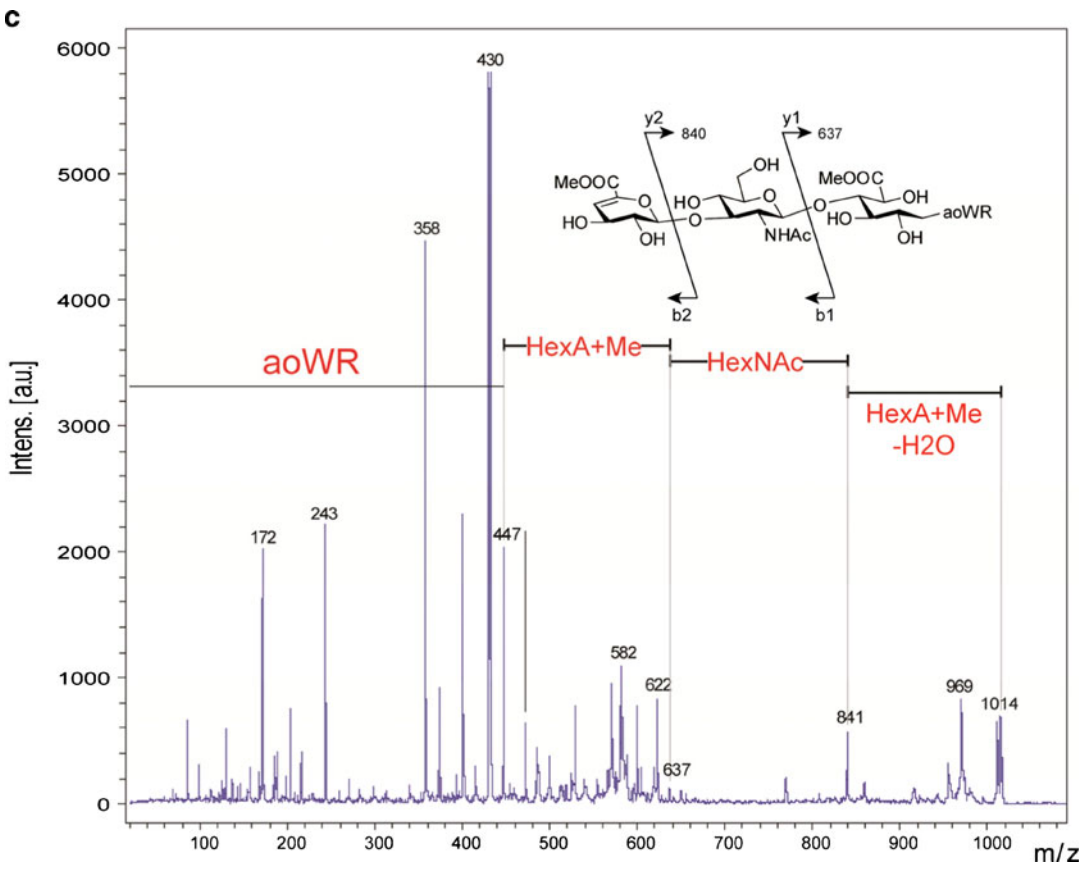


Fig. 5 (continued)

with 10 % acetic anhydride in methanol at 25 °C for 30 min. The solution was removed by vacuum, and then the beads were washed twice with 200 µl of 10 mM HCl, methanol, and dioxane, successively. Carboxyl groups in GlcA residues of HA oligosaccharides were converted into methyl ester on beads with 100 mM MTT in dioxane at 60 °C for 60 min to dryness. Then the beads were serially washed twice with 200 µl of dioxane, water, methanol and water. The HA oligosaccharides captured on beads were subjected to the *trans*-iminization reaction with 20 µl of 20 mM aoWR, followed by the treatment with 180 µl of 2 % acetic acid in acetonitrile for 45 min at 80 °C. The aoWR-labeled HA oligosaccharides were eluted by treatment with 100 µl of water. These procedures were performed three times for individual trial and subjected to the mass spectrometric analysis.

[Step 3] MALDI-TOFMS (Fig. 1e): The recovered aoWR-labeled HA oligosaccharides (n=3) were diluted by 10 times and mixed with an equivalent volume of the DHB solution consisting of 10 mg/ml in 30 % acetonitrile on an MYP 384 target plate (polished steel TF, Bruker Daltonics, Bremen, Germany), and dried under vacuum to afford crystals of the analytes. Then the analytes were subjected to MALDI-TOFMS and MALDI-TOF/TOFMS measurements. Quantitation of individual HA fragments related to an internal standard (the peak intensity due to aoWR-labeled cellohexaose) was represented as an average of three MALDI-TOFMS data.

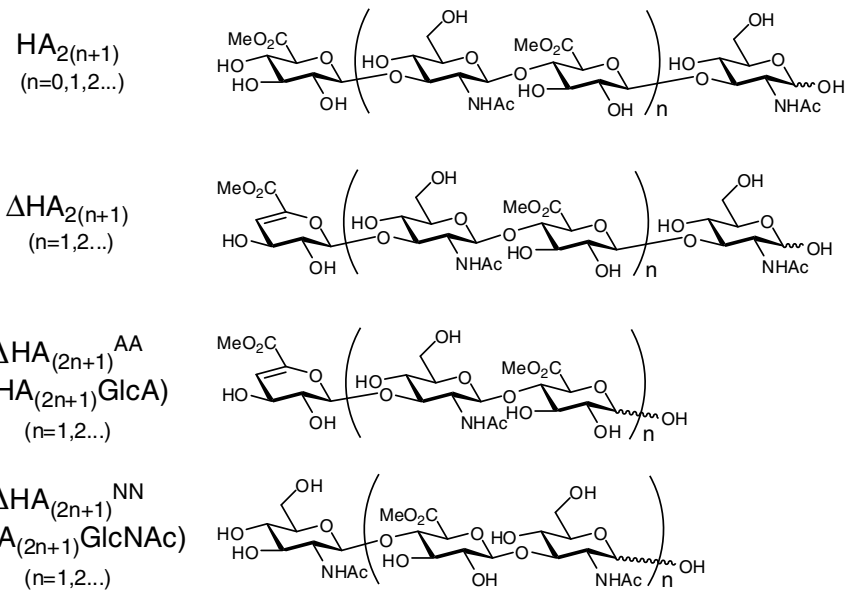
Result and discussion

Figure 1 indicates a general protocol for the characterization of HA degradation products under *in vitro* enzymatic digestion in the presence of two typical HAases from bovine testicular (EC 3.2.1.35) and *Streptomyces hyalurolyticus* (EC 4.2.2.1). As described in the experimental section, this protocol was designed basically according to the glycoblotting method established for the enrichment analysis of total *N*-glycans from human serum glycoproteins [23, 24, 26], cellular glycoproteins and glycosphingolipids [25, 29, 30],

and other complex biological materials [27]. We hypothesized that simple esterification of carboxyl groups of GlcA residues by MTT (Fig. 1c) as well as bead-based enrichment process (Fig. 1b) should greatly facilitate MS-based characterization of HA oligosaccharides generated during enzymatic hydrolysis/elimination because anionic nature of HA oligosaccharides has made sensitive and reliable mass spectrometric analyses under common positive ion mode

Table 1 Summary of hyaluronan degradation fragments uncovered by glycoblotting method

m/z	R.I.	Compositions	Assignments
841.50	0.3	$(HexA+Me)_1(HexNAc)_1$	HA_2
1013.72	0.5	$(\Delta HexA+Me)_1(HexA+Me)_1(HexNAc)_1$	ΔHA_3^{AA}
1045.71	1.1	$(HexA+Me)_1(HexNAc)_2$	HA_3^{NN}
1216.72	11.8	$(\Delta HexA+Me)_1(HexA+Me)_1(HexNAc)_2$	ΔHA_4
1234.73	40.8	$(HexA+Me)_2(HexNAc)_2$	HA_4
1406.80	0.3	$(\Delta HexA+Me)_1(HexA+Me)_2(HexNAc)_2$	ΔHA_5^{AA}
1437.97	0.7	$(HexA+Me)_2(HexNAc)_3$	HA_5^{NN}
1420.84	100.0	$(Glc)_6$	IS
1609.95	36.4	$(\Delta HexA+Me)_1(HexA+Me)_2(HexNAc)_3$	ΔHA_6
1627.96	22.1	$(HexA+Me)_2(HexNAc)_2$	HA_6
1800.02	0.4	$(\Delta HexA+Me)_1(HexA+Me)_3(HexNAc)_3$	ΔHA_7^{AA}
1831.09	0.5	$(HexA+Me)_3(HexNAc)_4$	HA_7^{NN}
2003.14	7.3	$(\Delta HexA+Me)_1(HexA+Me)_3(HexNAc)_4$	ΔHA_8
2021.15	0.8	$(HexA+Me)_4(HexNAc)_4$	HA_8
2193.16	0.3	$(\Delta HexA+Me)_1(HexA+Me)_4(HexNAc)_4$	ΔHA_9^{AA}
2224.26	0.4	$(HexA+Me)_4(HexNAc)_5$	HA_9^{NN}
2396.35	18.3	$(\Delta HexA+Me)_1(HexA+Me)_4(HexNAc)_5$	ΔHA_{10}
2414.38	0.1	$(HexA+Me)_5(HexNAc)_5$	HA_{10}
2789.59	14.7	$(\Delta HexA+Me)_1(HexA+Me)_5(HexNAc)_6$	ΔHA_{12}
3182.94	5.6	$(\Delta HexA+Me)_1(HexA+Me)_6(HexNAc)_7$	ΔHA_{14}
3576.27	1.2	$(\Delta HexA+Me)_1(HexA+Me)_7(HexNAc)_8$	ΔHA_{16}
3969.68	0.4	$(\Delta HexA+Me)_1(HexA+Me)_8(HexNAc)_9$	ΔHA_{18}



Abbreviation representing for HA degradation fragments was described according to the definition reported by Blundell *et al* [32]

difficult. Although MS measurements under a negative ion mode may allow for the structural characterization of anionic substances such as *N*-glycans bearing sialic acid residues, it is likely that lower sensitivity usually reduces quality of the MS spectra and higher energy required for the ionization process often induces serious deletion of the terminal sialic acid residues from oligosaccharides [22]. Therefore, it is considered that conversion of the anionic GlcA residues in HA oligosaccharides into the protected neutral sugar derivative will greatly facilitate the ionization under lower energy and permit consequently highly enhanced sensitivity in the structural characterization without any loss due to the decomposition during MS measurements. To assess this hypothesis, we examined the feasibility of this protocol for the quantitation of authentic HA samples (commercially available HA4 and HA12). As shown in Fig. 2a and b, the glycoblotting method allowed for the quantitation of the different size of HA fragments in the wide range of concentration while the intensity of ion signals in MALDI-TOFMS appeared to depend significantly on the molecular size of HA fragments. In other words, the absolute quantitation of HA fragments can be performed by MALDI-TOFMS using calibration curves prepared for the individual HA oligosaccharides. In addition, it is likely that the internal standard spiked prior to the glycoblotting should facilitate the relative quantitation of complex HA fragments generated from HA polymers during hydrolytic action by some HAases in various conditions.

As anticipated, the present protocol allowed for rapid and efficient enrichment analysis of HA oligosaccharides generated by HAases. Figure 3 shows MALDI-TOFMS of the degradation products from rooster comb HA by treating with bovine testis and *Streptomyces* HAases (As for the results using bovine vitreous HA and *Streptococcus* HA as substrates, see, Supporting Information Figure S1~S8). It was demonstrated that glycoblotting method allows for high throughput and quantitative analysis of hyaluronan fragments within a wide dynamic range (1~1000 pmole) when 5 μg of hyaluronan digests were applied for this enrichment protocol. It is clear that molecular size and distribution of the degradation products were depending strongly on the amounts (unit) of both bovine testis and *Streptomyces* HAases used while chemical structures at non-reducing sugar residues differed from each other due to the mechanisms whether hydrolytic (bovine testicular, EC 3.2.1.35) or elimination (*Streptomyces hyalurolyticus*, EC 4.2.2.1) enzyme, respectively. Merit of the present approach in the structural characterization is evident from the typical MS/MS spectra that exhibit highly sensitive and informative fragment ions even in cases for the low abundant precursor ions observed at *m/z* 1800.02 and 1831.09 corresponding to ΔHA_7^{AA} and HA_7^{NN} liberated from rooster comb HA by treating with *Streptomyces* HAase (0.2 U) as shown in Fig. 4. This result indicates that *Streptomyces* HAase known as an elimination enzyme might act as hydrolytic HAase concurrently or some contamination of other hydrolytic enzymes. Alternatively, MS/MS analysis of a signal at *m/z* 1027 suggested that bovine testis HAase may produce a faint amount of the trisaccharide GlcNAcβ1-4GlcAβ1-3GlcNAc (ΔHA_3^{NN}) that had never been reported previously (Fig. 5a). Surprisingly, the present method uncovered the existence of the smallest disaccharide GlcAβ1-3GlcNAc (HA_2, *m/z* 841.50) during the digestion by bovine testis hyaluronidases and unusual trisaccharide GlcAβ1-3GlcNAc β1-4GlcA (ΔHA_3^{AA}, *m/z* 1013.72) in the presence of *Streptomyces* HAase (Fig. 4b and c), respectively. Table 1 summarized for major mass signals (*m/z*) detected, relative intensities comparing with the signal intensity at *m/z* 1420.84 due to the internal

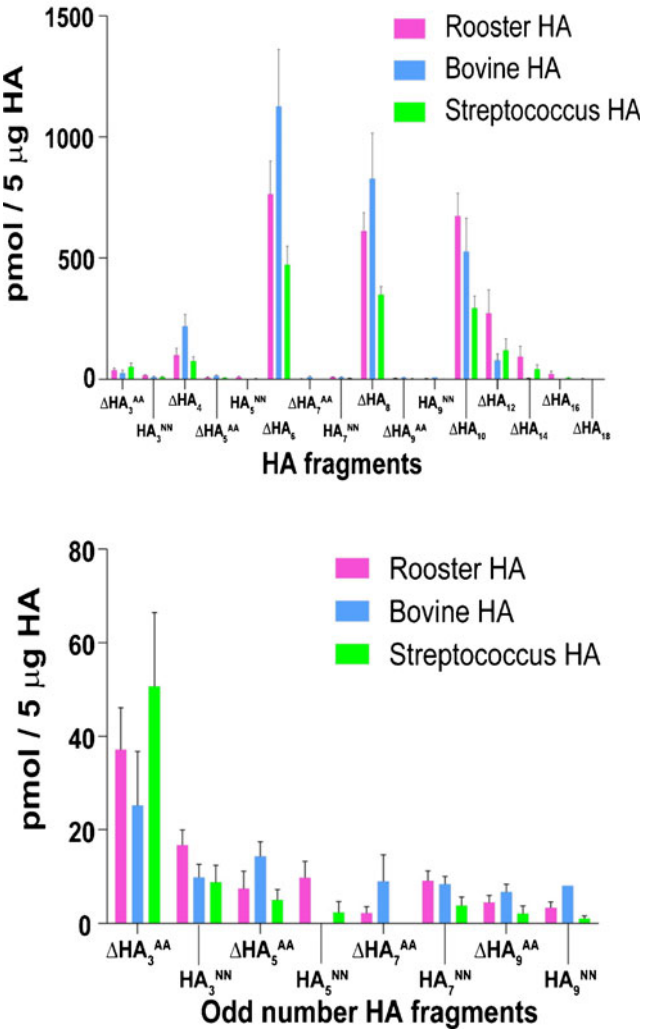


Fig. 6 Profile of HA degradation products from three HAs by treating with *Streptomyces* HAase (0.2 U). Top; whole fragments, bottom; odd number HA fragments. Error bar means the average of the raw MALDI-TOFMS data (n=6) for all trials

standard spiked, and compositions and estimated structures. It was concluded that *Streptomyces* HAase produces significantly HA fragments composed of odd number sugar residues from ΔHA_3 to ΔHA_9 while the fragments of even number sugar residues from ΔHA_4 to ΔHA_{14} were major products (Fig. 6). Overall profiles of HA degradation products appeared to be dependent strongly on the characteristics of HAases employed rather than macromolecular HA sources while there were slight but detectable differences in the molecular size and distribution between three typical HAs from roosted comb, bovine and *Streptococcus* (Supporting Information, Figure S9 and S10).

Conclusion

We demonstrated the feasibility of glycoblotting method for high throughput and quantitative characterization of HA degradation products during enzymatic processing. Solid-phase esterification of carboxyl groups of GlcA residues allowed for facile MALDI-TOF mass spectrometric analyses at positive ion mode within a wide dynamic range (1~1000 pmole) of HA degradation fragments and high precision structural characterization by MS/MS. It was revealed that HA degradation products involve unusual oligosaccharides such as GlcNAcβ1-4GlcAβ1-3GlcNAc (ΔHA_3^{NN}), GlcAβ1-3GlcNAc β1-4GlcA (ΔHA_3^{AA}), or GlcAβ1-3GlcNAc (HA_2) generated as minor and low abundant substances. Considering the importance of biological functions of HA metabolites during cell differentiation, inflammatory and malignancy, merit of the present protocol is evident because on-bead enrichment and chemical manipulation do not need any chromatographic purification of HA fragments from the reaction mixture. HA oligosaccharides can be produced both by digestion using various HAases [10] and/or by engineered HA synthases (HAS) [9, 31]. In fact, we have communicated that an engineered human HAS2 can synthesize HA oligosaccharides (HA_8~HA_{16}) [31]. It is clear that these HA oligosaccharides would become nice tools to investigate the relationship between HA metabolism and their biological roles though the interaction of small HA fragments with endogenous receptor molecules [32]. However, it should also be emphasized that a standardized method for quantitative and qualitative analyses of HA oligosaccharides is crucial because molecular size and the distribution might affect greatly cellular homeostatic balance controlled by the dynamic mechanism in HA metabolism [33]. Monitoring the generation of HA fragments in some human cell lines are under way and the results will be reported as soon as possible.

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