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Generation and characterization of a series of monoclonal antibodies that specifically recognize [HexA(±2S)-GlcNAc]n epitopes in heparan sulfate

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Abstract Five monoclonal antibodies AS17, 22, 25, 38 and 48, a single monoclonal antibody ACH55, and three monoclonal antibodies NAH33, 43, 46, that recognize acharan sulfate (IdoA2S-GlcNAc)n, acharan (IdoA-GlcNAc)n and *N*-acetyl-heparosan (GlcA-GlcNAc)n, respectively, were generated by immunization of mice with keyhole limpet hemocyanin-conjugated polysaccharides. Specificity tests were performed using a panel of biotinylated GAGs that included chemically modified heparins. Each antibody bound avidly to the immunized polysaccharide, but did not bind to chondroitin sulfates, keratan sulfate, chondroitin nor hyaluronic acid. AS antibodies did not bind to heparan sulfate or heparin, but bound to 6-*O*-desulfated, *N*-desulfated and re-*N*-acetylated heparin to varying degrees. ACH55 bound to tri-desulfated and re-*N*-acetylated heparin but hardly bound to other modified heparins. NAH antibodies did not bind to heparin and modified heparins but bound to heparan sulfate to varying degrees. NAH43 and NAH46 also bound to partially *N*-de-acetylated *N*-acetyl-heparosan. Immunohistochemical analysis in rat cerebella was performed with the antibodies. While NAH46 stained endothelia, where heparan sulfate is typically present, neither ACH55 nor

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AS25 stained endothelia. On the contrary ACH55 and AS25 stained the molecular layer of the rat cerebella. Furthermore, ACH55 specifically stained Purkinje cells. These results suggest that there is unordinary expression of IdoA2S-GlcNAc and IdoA-GlcNAc in specific parts of the nervous system.

Keywords Monoclonal antibody · Heparan sulfate · Acharan sulfate · Acharan · *N*-acetyl-heparosan

Abbreviations

NAc-HSs	acharan sulfate, acharan and *N*-acetyl-heparosan
HS	heparan sulfate
GlcA	D-glucuronic acid
IdoA	L-iduronic acid
HexA	unspecified hexuronic acid
ΔHexA	4,5-unsaturated hexuronic acid
GlcNAc	*N*-acetyl-D-glucosamine
$GlcNH_2$	*N*-unsubstituted D-glucosamine
2S	2-*O*-sulfated
6S-	6-*O*-sulfated
NS	*N*-sulfated
GAGs	glycosaminoglycans
NDS/NAc-Hep	*N*-desulfated/*N*-acetylated heparin
6DS-Hep	6-*O*-desulfated heparin
6DS/NAc-Hep	6-*O*-desulfated/*N*-acetylated heparin
6DS/NDS/NAc-Hep	6-*O*-desulfated/*N*-desulfated/*N*-acetylated heparin
2DS/NDS/NAc-Hep	2-*O*-desulfated/*N*-desulfated/*N*-acetylated heparin
6DS/2DS-Hep	6-O-desulfated/2-*O*-desulfated heparin
6DS/2DS/NAc-Hep	6-O-desulfated/2-*O*-desulfated/*N*-acetylated heparin
TriDS/NAc-Hep	6-*O*-desulfated/2-*O*-desulfated/*N*-desulfated/*N*-acetylated heparin
PDeNAcNAH	partially *N*-de-acetylated *N*-acetyl-heparosan
HPLC	high-performance liquid chromatography
PDP	2-pyridyldisulfide-propionated
KLH	keyhole limpet hemocyanin
ELISA	enzyme-linked immunosorbent assay

Introduction

Monoclonal antibodies are rapid and sensitive tools to profile specific sequences of sugar chains. However, a small number of antibodies towards heparan sulfate (HS) were generated, and the epitopes were sometimes unclear, when compared to those recognizing sugar sequences of glycoproteins and glycolipids [1]. This is mainly because of the extreme heterogeneity in the structures, which usually contain three elements: (i) hexuronic acids (HexAs, either GlcA or IdoA), (ii) sulfations, such as *N*-, 2-*O*-, 6-*O*-, and 3-*O*- sulfations and (iii) carbohydrate chains of different lengths [2]. Since the phage display was introduced [3], the number of antibodies towards glycosaminoglycans (GAGs) has been growing. However, these antibodies have not always been characterized in terms of specificity and antibodies that recognize several important sequences have not yet been reported.

Although HS and heparin containing IdoA-GlcNAc and IdoA2S-GlcNAc have been reported [2, 4], the biosynthetic pathways are still uncertain. Disaccharide analysis using bacterial HS/heparin digestive enzymes is convenient [5] and widely used. However, it can not discriminate IdoA and GlcA because the enzymes are eliminases and thereby convert both HexAs to the common 4,5-unsaturated hexuronic (ΔHexA) [4]. Disaccharide analysis based on the technique of deaminative cleavage by sequential treatment with hydrazine and nitrous acid, is also a useful approach, but this does not simultaneously discriminate GlcNAc, $GlcNH_2$ and GlcNS [6, 7]. In fact, GlcNAc is resistant to deamination cleavage by nitrous acid, while GlcNH2 and GlcNS are selectively cleaved by nitrous acid at pH 4 and 1.5, respectively [8]. Therefore, simple but specific tools to overcome these difficulties are required. We prepared acharan sulfate (IdoA2S-GlcNAc)n and the desulfated form of acharan (IdoA-GlcNAc)n from the giant African snail, *Achantina fulica* [9]. We also prepared *N*-acetyl-heparosan (GlcA-GlcNAc)n from *Escherichia coli* K5 [10]. After conjugating the polysaccharides of NAc-HSs (hereinafter these three polysaccharides are, referred to as NAc-HSs) to keyhole limpet hemocyanin, respectively, we immunized mice and generated three types of monoclonal antibodies to specifically recognize these structures. Using these antibodies, we demonstrated the unordinary occurrence of IdoA-GlcNAc and IdoA2S-GlcNAc in rat cerebella.

Materials and methods

Materials

HS from Engelbreth–Holm–Swarm tumors was prepared as previously described [11]. Other GAGs, monoclonal antibodies, and enzymes were obtained from Seikagaku Biobusiness Corp. (Tokyo, Japan). The GAGs were HS (from bovine kidney), hyaluronic acid (pig skin), chondroitin sulfate-A (whale cartilage), chondroitin sulfate-B (pig skin), chondroitin sulfate-C (shark cartilage), chondroitin sulfate-D (shark cartilage), chondroitin sulfate-E (squid cartilage),

chondroitin (desulfated chondroitin sulfate-C), and keratan sulfate (bovine cornea). The antibodies used were 10E4 and 3G10 [12]. The enzymes used were heparitinase I (heparin lyase III, *Flavobacterium heparinum*), heparitinase II (heparin lyase II, *Flavobacterium heparinum*) and heparinase (heparin lyase I, *Flavobacterium heparinum*). Female BALB/C mice (6 weeks of age) and male Sprague-Dawley rats (8 weeks of age) were obtained from Charles River Laboratories Japan, Inc. (Atsugi, Japan).

Preparation of NAc-HSs (acharan sulfate, acharan and *N*-acetyl-heparosan) and modified heparins

Acharan sulfate was prepared from *Achatina fulica* as previously described [9]. *N*-acetyl-heparosan was purified from *Escherichia coli* K5 [10]. It was further converted into partially *N*-de-acetylated *N*-acetyl-heparosan (PDeNAcNAH), by hydrazinolysis according to the method of Kariya *et al*. [7] with a slight modification. Briefly, after being dissolved in 70% of hydrazine solution containing 1% of hydrazine sulfate, it was divided to three parts and heated at 96°C for 30, 60 and 120 min, respectively. After being dialyzed against water and lyophilized, each part was dissolved in ice-cold 0.25 N HIO_3, to convert the hydrazide generated at GlcA residues into GlcA. I_2 formed was removed by liquid partitioning between diethyl ether and water. After being dialyzed and lyophilized, finally, PDeNAcNAH-1, PDeNAcNAH-2, and PDeNAcNAH-3 were obtained, respectively. Heparin from pig intestines was purchased from Scientific Protein Laboratories (Waunakee, WI). The heparin was regio-selectively, *N*-desulfated, 6-*O*-desulfated and 2-*O*-desulfated as previously described [13–16]. *N*-Acetylation was performed according to the methods of Danishefsky [17]. In total eight types of de-sulfated and/or *N*-acetylated heparins were prepared; these were *N*-desulfated/*N*-acetylated heparin (NDS/NAc-Hep), 6-*O*-desulfated heparin (6DS-Hep), 6-*O*-desulfated/*N*-acetylated heparin (6DS/NAc-Hep), 6-*O*-desulfated/*N*-desulfated/*N*-acetylated heparin (6DS/NDS/NAc-Hep), 2-*O*-desulfated/*N*-desulfated/*N*-acetylated heparin (2DS/NDS/NAc-Hep), 6-*O*-desulfated/2-*O*-desulfated heparin (6DS/2DS-Hep), 6-*O*-desulfated/2-*O*-desulfated/ *N*-acetylated heparin (6DS/2DS/NAc-Hep), and 6-*O*-desulfated/2-*O*-desulfated/*N*-desulfated/*N*-acetylated heparin (TriDS/NAc-Hep). Acharan was prepared from acharan sulfate by desulfation and re-*N*-acetylation [17].

Preparation of KLH-conjugated NAc-HSs and BSA-conjugated NAc-HSs

NAc-HSs were reductively aminated in ammonium chloride with sodium cyanoborohydride. Next, 2-pyridyl disulfhide was introduced into the reductively aminated NAc-HSs and keyhole limpet hemocyanin, respectively, and consequently products of 2-pyridyldisulfide-propionated NAc-HSs (PDP-NAc-HSs) and PDP-KLH were generated [18]. PDP-NAc-HSs were further converted into thiol containing NAc-HSs (SH-NAc-HSs) in the presence of dithiothreitol, SH-NAc-HSs and PDP-KLH were mixed in 0.1 M NaCl/0.1 M phosphate buffer (pH 7.5), and finally KLH-conjugated-NAc-HSs were thus generated. NAc-HSs and bovine serum albumin (BSA) were conjugated in 0.1 M 2-morpholinoethanesulfonic acid buffer (pH 5.5) in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide.

Generations of monoclonal antibodies

One milligram of KLH-conjugated NAc-HSs were dissolved in a small volume of water and mixed with 2 ml of TiterMAX Gold (TiterMax USA, Inc. Norcross, GA). One hundred microliter of the mixture was subcutaneously injected into BALB/C mice, every 2 weeks. Sera from these mice were periodically examined by enzyme-linked immunosorbent assay (ELISA) using microtiter plates coated with BSA-conjugated-NAc-HSs. Bound antibody was detected using horseradish peroxidase-labeled anti-mouse immunoglobulin antibodies (DAKO, Glostrup, Denmark). When the titer of antibodies was sufficiently elevated, a final round of immunizations was carried out. 3 days later, the mice were ethically sacrificed and their spleen cells were fused to mouse myeloma cells. Hybridoma cells were injected to BALB/C mice and ascites containing crude antibodies was collected.

Specificity test of monoclonal antibodies using a panel of biotinylated GAGs

Antibodies were purified from ascites with ammonium sulfate precipitation. IgM antibodies were further purified by gel filtration using Sepharose CL-4B (GE Healthcare, Buckinghamshire, UK), and IgG antibodies were purified by HiTrap Protein G HP (GE Healthcare). Purity of each antibody confirmed by SDS-polyacrylamide gel electrophoresis was more than 80%. Various GAGs were biotinylated through their carboxyl groups using biotin LC hydrazine (Pierce, Rockford IL) according to the instructions of the manufacturer. Streptavidin coated plates were prepared using 96 well Maxisorp plates (eBioscience, Inc. San Diego, CA). One hundred microliter of biotinylated GAGs (1-1000 ng/ml) in phosphate buffered saline containing 0.05% Tween 20, 0.1% Proclin 950 (Sigma-Aldrich Corp. St. Louis, MO) and 5% ApplieDuo (Seikagaku Biobusiness Corp.) was added to each well. After 0.5 h at room temperature, the wells were washed and 100 μl of appropriately diluted monoclonal antibodies were added to the wells. After 1 h, the wells were washed and 100 μl of 2000-fold diluted horseradish

peroxidase-labeled anti-mouse immunoglobulin antibody was added to the wells. After 1 h, 100 μl of 3,3′, 5,5′-tetramethylbenzidine was added and absorbances at 450 nm (reference wave length at 630 nm) were measured with a plate-reader (Wellreader SK603, Seikagaku Biobusiness Corp.)

Analysis of disaccharides produced by bacterial heparitinases/heparinase

Disaccharides from NAc-HSs, HS, heparin and modified heparins after treatment with heparitinases/heparinase were analyzed as previously described [5]. Briefly these GAGs (50 μg) were treated with a mixture of heparitinase I (50 mU), heparitinase II (50 mU) or heparinase (50 mU) in 200 μl of 20 mM sodium acetate and 2 mM calcium acetate (pH 7.0) at 37°C for 2 h, respectively. The disaccharides generated were monitored at 230 nm by strong anion-exchange HPLC using a Dionex CarboPac PA-1 column.

Immunohistochemical staining of neural tissues from rats

Frozen sections (4 μm) of rat cerebella were cut with a cryostat and acetone-fixed. After eliminating endogenous peroxidase, avidin and biotin, non-specific binding sites were blocked by incubating the sections in PBS containing 0.1% casein for 1 h. After being washed, sections were incubated with the primary antibodies (0.5–5 μg/ml) with or without the given GAGs in PBS containing 0.1% casein and 0.1% Triton X 100, at 4°C, overnight. After being washed, sections were incubated with the secondary antibody, biotinylated F(ab')2 fragment goat anti-mouse immunoglobulin (Jackson ImmunoResearch, Laboratories, West Grove, PA) in PBS containing 10% rat serum, 0.1% casein and 0.1% Triton X 100 at 4°C for 1 h. Sections were washed, treated with horseradish peroxidase-streptavidin and visualized with 3,3′-diaminobenzidine, tetrahydrochloride solution. After nuclei were stained with hematoxylin, the sections were examined by microscopy. Some sections were pretreated with heparitinase I and/or II dissolved in a solution containing 20 mM sodium acetate containing 1 μM calcium acetate (pH7.0) before staining with antibodies. For 3G10, 5 mU/slide and for other antibodies, 20mU/slide of the enzymes were used.

Results and discussion

Comparisons of antibodies recognizing acharan sulfate in terms of specificity and affinity

We obtained five antibodies, AS17 (IgG2a), AS22 (IgM), AS25 (IgG1), AS38 (IgG1) and AS48 (IgM), and their specificities towards various GAGs were summarized (Fig. 1A). These antibodies strongly bound to acharan sulfate but did not bind to non-HS/heparin GAGs such as chondroitin sulfate-A, -B, -C, -D, -E, keratan sulfate, chondroitin and hyaluronic acid. Immunoreactivity towards HS, HS from Engelbreth–Holm–Swarm tumors, native heparin and chemically modified heparins were compared. Figure 2 showed an elution profile of the disaccharides from 6DS/NAc-Hep as a representative chromatogram of the modified heparins and Table 1 showed the disaccharides compositions of the heparins determined by strong anion exchange HPLC.

In addition to acharan sulfate, the IgGs AS17, AS25, and AS38 bound to 6DS/NDS/NAc-Hep, although AS38 had relatively weak affinity to 6DS/NDS/NAc-Hep. Since major HexA of heparin is IdoA and the major disaccharide of 6DS/NDS/NAc-Hep is ΔHexA2S-GlcNAc (77.3%), it is reasonable that these antibodies bound well to 6DS/NDS/NAc-Hep. Interestingly, AS17 had a higher affinity to 6DS/NDS/NAc-Hep than to the original immunogen, acharan sulfate, though the reason for this is unclear. Although the IgMs AS22 and AS48 exhibited similar affinity to acharan sulfate and 6DS/NDS/NAc-Hep, they also bound to 6DS/NAc-Hep, which contains 18.4% of ΔHexA2S-GlcNAc and to NDS/NAc-Hep which contains 10.6% of ΔHexA2S-GlcNAc, with similar affinity. The IgM type of AS antibodies may recognize the short sequence IdoA2S-GlcNAc. It should be noted that all AS antibodies, regardless of whether they were IgGs or IgM, did not recognize IdoA2S-GlcNS or IdoA2S-GlcNS6S because no antibody bound to 6DS-Hep (62.7% ΔHexA2S-GlcNS) or native heparin (60% ΔHexA2S-GlcNS6S), indicating that GlcNAc, but not GlcNS/GlcNS6S is necessary for epitope recognition. AS48 exhibited some affinity to TriDS/NAc-Hep (90% ΔHexA-GlcNAc) and this is reasonable because AS48 cross-reacts with acharan. The antibody MW3G3, which recognizes (IdoA2S-GlcNAc)n was generated by phage display previously and was found to have some affinity to heparin from pig intestine [19]. Since the AS antibodies we generated hardly bound to heparin from pig intestines, they are more specific to acharan sulfate than antibody MW3G3. The affinity of the antibodies towards acharan sulfate was compared by ELISA (Fig. 1B). AS38 and AS 25 exhibited strong, AS17 moderate and, AS22 and AS48 weak, affinities, respectively.

Comparisons of antibodies recognizing acharan and *N*-acetyl-heparosan in terms of specificity towards various GAGs

Antibody ACH55 (IgM) bound specifically to acharan, but did not bind to other naturally occurring GAGs including acharan sulfate and *N*-acetyl-heparosan (Fig. 3A). These results indicate that the antibody can discriminate not only

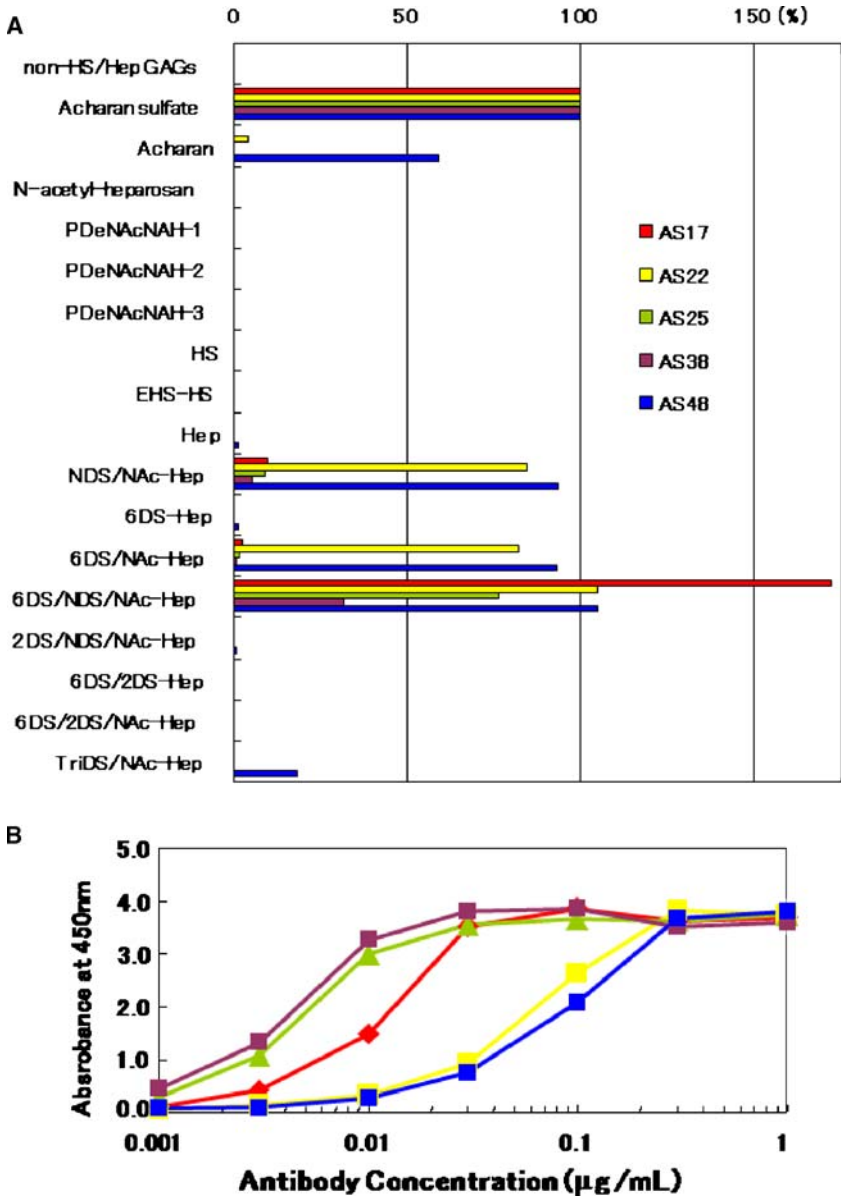

Fig. 1 Comparisons of antibodies recognizing acharan sulfate in terms of specificity and affinity. A, Comparison of specificity. non-HS/Hep GAGs include chondroitin sulfate-A, -B, -C, -D, -E, keratan sulfate, chondroitin and hyaluronic acid. EHS-HS indicates HS from Engelbreth-Holm-Swarm tumors. The specificity was examined by ELISA using microtiter plates precoated biotinylated GAGs. B, Comparison of affinity to acharan sulfate by ELISA. Microtiter plates were precoated with 1 μg/ml of biotinylated acharan sulfate and ELISA was performed. AS25 and 38 exhibited the strongest affinity, AS17 exhibited moderate affinity, and AS 22 and 48 the weakest affinity of the antibodies tested

between IdoA and IdoA2S but also between IdoA and GlcA. ACH55 also bound strongly to TriDS/NAc-Hep (90% ΔHexA-GlcNAc, Table 1) and weakly to 6DS/NDS/NAc-Hep (22.7% ΔHexA-GlcNAc), 2DS/NDS/NAc-Hep (16.7% ΔHexA-GlcNAc) and 6DS/2DS/NAc-Hep (28.8% ΔHexA-GlcNAc). It is reasonable that ACH55 bound to these modified heparins, because the major HexA of heparin is IdoA. Interestingly, ACH55 hardly bound to 6DS/NAc-Hep (11.4% ΔHexA-GlcNAc) and 6DS/2DS-Hep (11.2% ΔHexA-GlcNAc). Probably, ACH may require some repetitive unit of (-IdoA-GlcNAc-)n, and/or the units may be too little for the antibody recognition.

We obtained three IgM monoclonal antibodies NAH33, NAH43 and NAH46 towards *N*-acetyl-heparosan. The specificities of these antibodies against various GAGs were shown (Fig. 3B). While these antibodies bound strongly to *N*-acetyl-heparosan, they did not bind to sulfated and non-sulfated GAGs including chondroitin or hyaluronic acid, suggesting that the antibodies discriminate not only between -GlcA-GalNAc- (chondroitin) and -GlcA-GlcNAc- (*N*-acetyl-heparosan and HA), but also between -4GlcAβ1-4GlcNAcα1- (*N*-acetyl-heparosan) and -4Glcβ1-3GlcNAcβ1- (hyaluronic acid). NAH33, 43 and 46 bound moderately to HS and this is expected, because more than 50% of the disaccharide composition of HS is ΔHexA-GlcNAc (Table 1), which corresponds to GlcA-GlcNAc. On the other hand these antibodies hardly bound to heparin, suggesting that the disaccharide unit of ΔHexA-GlcNAc in heparin may be too little (4.3%) for these antibodies to be recognized as an epitope. NAH33

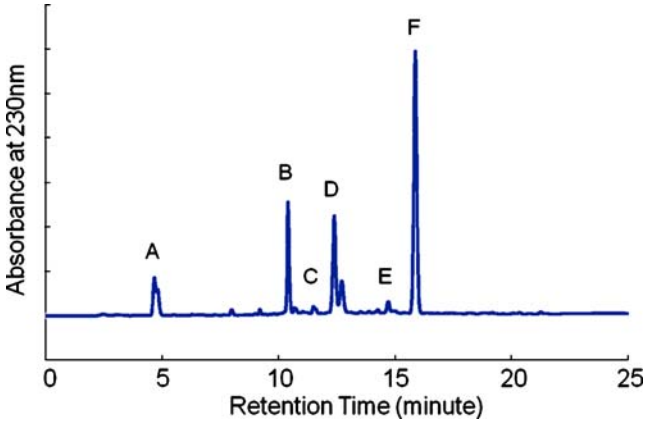

Fig. 2 Elution profile of the disaccharides from 6DS/NAc-Hep as a representative chromatogram of the modified heparins determined by strong anion exchange HPLC. Peaks, *A*, *B*, *C*, *D*, *E* and *F* indicate, ΔHexA-GlcNAc, ΔHexA-GlcNS, ΔHexA-GlcNAc6S, ΔHexA2S-GlcNAc, ΔHexA-GlcNS6S and ΔHexA2S-GlcNS, respectively

bound well to *N*-acetyl-heparosan, but did not bind to partially *N*-de-acetylated *N*-acetyl-heparosans (PDeNAc-NAHs,) including PDeNAcNAH-1 (77.5% ΔHexA-GlcNAc). On the other hand, NAH33 moderately bound to HS (53% ΔHexA-GlcNAc). Non-sulfated parts of HS are composed of repetitive units of (-GlcA-GlcNAc-)n and there are sulfated domains of HS inserted in the non-sulfated sequences [2]. It is probable that partial de-*N*-acetylation procedure randomly produces (-GlcA-$GlcNH_2$-)n from (-GlcA-GlcNAc-)n in *N*-acetyl-heparosan and that short sequences of (-GlcA-GlcNAc-)n discontinuously inserted in the sequence of (-GlcA-$GlcNH_2$-)n may not be recognized by NAH33. Indeed a continuous stretch of 18-saccharides was reported to be necessary for epitope recognition by a monoclonal antibody 865, which has a similar specificity with NAH antibodies used here [20]. Alternatively the positive charge through the presence of the unsubstituted amino group on $GlcNH_2$ that is disclosed by de-*N*-acetylation may hinder the recognition process. NAH46 bound strongly to *N*-acetyl-heparosan, and PDeNAcNAH-1, and moderately to PDeNAcNAH-2 and HS but hardly to PDeNAcNAH-3. The results suggest that NAH46 may recognize shorter sequences of (GlcA-GlcNAc)n than NAH33. Alternatively, the cationic property of $GlcNH_2$ may hinder epitope recognition by NAH46 more weakly than by NAH33. NAH43 bound to *N*-acetyl-heparosan and PDeNAcNAHs with almost equal affinity, suggesting that it may recognize (GlcA-GlcNAc)n as well as (GlcA-$GlcNH_2$)n. NAH43 may also require a longer stretch of (GlcA-GlcNAc/$GlcNH_2$)n, because NAH43 bound weakly to HS from Engelbreth-Holm-Swarm tumors (33% ΔHexA-GlcNAc). NAH33, 43, and 46 did not bind to TriDS/NAc-Hep, even though this is composed of 80.6% ΔHexA-GlcNAc. It is reasonable that the major HexA of TriDS/NAc-Hep derived from heparin is not GlcA but IdoA, This result is consistent with the finding that NAH antibodies did not bind to acharan, which solely contains IdoA as a HexA.

Immunohistochemistry of rat cerebella

Recently, unordinary structures of chondroitin sulfate have been uncovered in neural tissues using recently generated anti-chondroitin sulfate antibodies [21–24], but such studies of HS are rare. Therefore, we applied our antibodies to immunohistochemical examination of rat cerebella.

Table 1 Compositions of disaccharides from various GAGs produced by heparitinases/heparinse (%)

Disaccharides	ΔHexaA-GlcNH2	ΔHexA-GlcNAc	ΔHexA-GlcNS	ΔHexA-GlcNAc6S	ΔHexA2S-GlcNAc	ΔHexA-GlcNS6S	ΔHexA2S-GlcNS	ΔHexA2S-GlcNAc6S	ΔHexA2S-GlcNS6S
Acharan sulfate					100				
Acharan		100							
N-acetyl-heparosan	0.5	99.5							
PDeNAcNAH-1	22.5	77.5							
PDeNAcNAH-2	35.6	64.4							
PDeNAcNAH-3	55.4	44.6							
HS	Tr	53	16	11	0.9		6.3	7	5.8
EHS-HS	Tr	34.2	60.1	0.3	0.5	1.6	3.2		
Heparin	Tr	4.3	3.3	3.8	2.3	17	7.6	1.5	60.1
NDS/Nac-Hep		5		19	10.6	3.8		61.6	
6DS-Hep		8.7	17.1	1.9	4.3	5.4	62.7		
6DS/NAc-Hep	Tr	11.4	15.3	1.5	18.4	3.2	48.8		
6DS/NDS/NAc-Hep		22.7			77.3				
2DS/NDS/NAc-Hep		16.7		78.8		4.5			
6DS/2DS-Hep		11.2	88.8						
6DS/2DS/NAc-Hep	1.1	28.8	65.1	2.6		2.5			
TriDS/NAc-Hep		90	6.1	4.1					

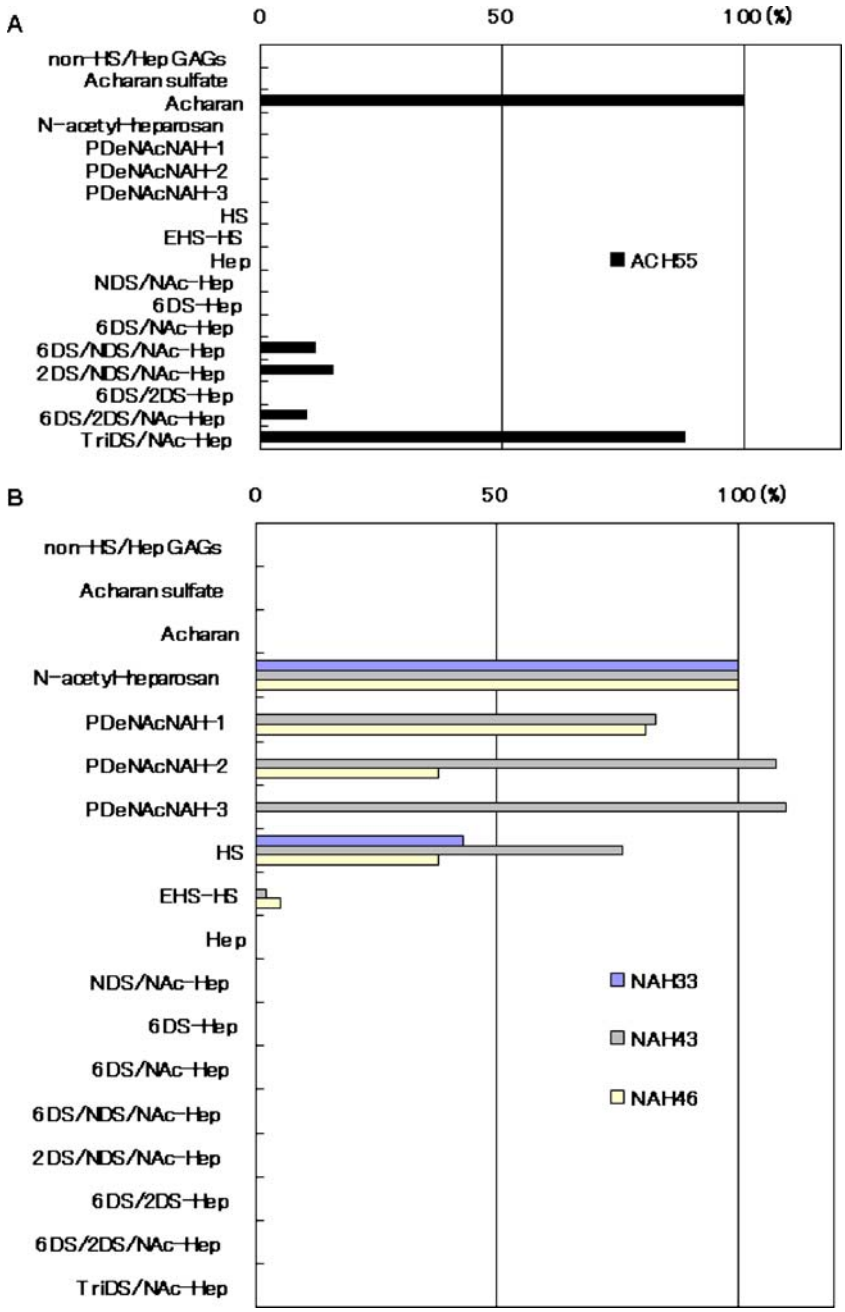

Fig. 3 Antibody specificity towards various GAGs. **A** specificity of ACH55; **B** specificity of NAH33, NAH43 and NAH46

NAH46 similarly stained endothelia as 10E4 [12], but none of AS antibodies or ACH55 stained endothelia (Fig. 4A,B,C-1,D-1). Although the other AS antibodies did not stain the cerebella, AS25 stained molecular layers (Fig. 4C-1), and ACH55 stained molecular layers and Purkinje cells, respectively (Fig. 4D-1 and D-3). When the antibodies were incubated with sections in the presence of the given antigens of acharan sulfate and acharan, both positive signals were decreased (Fig. 4C-2 and D-2), indicating these signals are specific. In order to confirm whether or not the molecular layers and the Purkinje cells contain HS, we pretreated specimens with heparitinase I and II before incubation with antibody 3G10, which recognizes ΔHexA of HS [12]. While pretreatment with heparitinase I disclosed the epitope for 3G10, in endothelia but not in the molecular layer and the Purkinje cells (Fig. 4E-1 and E-2), pretreatment with heparitinase II disclosed the epitope in the molecular layers and the Purkinje cells of the cerebella as well as that in the endothelia (Fig. 3E-3 and E-4). These results suggest that HS in endothelia and HS in the molecular layers and Purkinje cells are very different. It is unclear why the other AS antibodies did not stain the

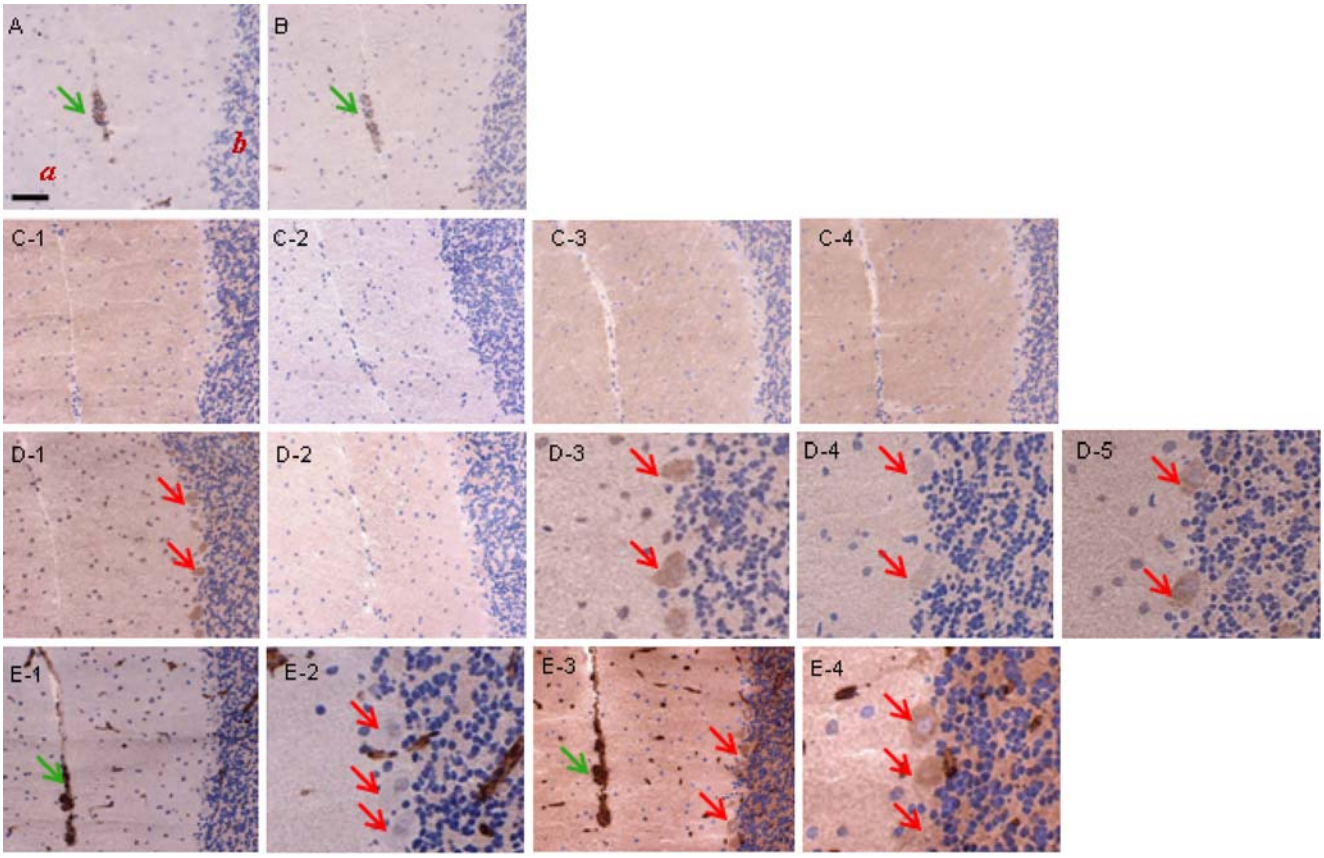

Fig. 4 Immunohistochemical examination of rat cerebella. Cerebella were stained by 10E4 (**A**), NAH46 (**B**), AS25 (**C**–1 to **C**–4), ACH55 (**D**–1 to **D**–5) and 3G10(**E**–1, to **E**–4) antibodies. The bar (**A**) indicates 100 μm. Red *a* and *b* (both in **A**) indicate, the molecular layers and granule cells of the cerebella, respectively. Green and red arrows indicate endothelia and Purkinje cells, respectively. Antibodies 10E4 and NAH46 stained endothelia with similar intensity (**A**, **B**). AS25 stained the molecular layers (**C**–1) and this staining was abolished in the presence of excess antigen, acharan sulfate (**C**–2). Although specimens were pretreated with heparitinase I (**C**–3) or heparitinase II (**C**–4), the intensity of staining was almost similar to that of untreated specimen (**C**–1). ACH55 stained the molecular layers and Purkinje cells (**D**–1 and **D**–3. **D**–3 is high magnification of **D**–1) of the cerebella. These stainings were diminished in the presence of excess antigen, acharan (**D**–2). When specimens were pretreated with heparitinase I (**D**–4) and II (**D**–5), the intensity of staining for ACH55 were diminished and this was especially remarkable in the Purkinje following pretreatment with heparitinase I (**D**–4). 3G10 stained cerebella following pretreatment with heparitinase I (**E**–1 and **E**–2. **E**–2 show a high magnification of **E**–1) and heparitinase II (**E**–3 and **E**–4. **E**–4 show a high magnification of **E**–3). While pretreatment with heparitinase I disclosed epitope for 3G10 at endothelia, pretreatment with heparitinase II disclosed the 3G10 epitope in molecular layers and Purkinje cells

cerebella. However, this could be partly due to relatively high sensitivity of AS25, when compared with the majority of AS antibodies (Fig. 1B). AS38 exhibited similar sensitivity to AS25 in the ELISA (Fig. 1) but it did not stain the cerebella. Probably it might be due to a slight difference in the epitopes for AS 25 and AS38, because AS25 bound strongly to 6DS/NDS/NAc-Hep but AS38 weakly to 6DS/NDS/NAc-Hep (Fig. 1A). After the specimens were pretreated with heparitinases, we stained them with AS25 and ACH55 again. Although the specimens were pretreated with heparitinase I (Fig. 4C-3), heparitinase II (Fig. 4C-4), or the mixture (data not shown), the staining intensity with AS 25 was not abolished, suggesting that HS in the molecular layers that contain IdoA2S-GlcNAc are not sensitive to the enzymes. On the other hand, pretreatment with either heparitinase I or II, abolished the positive signals of ACH55 in the molecular layers and Purkinje cells, markedly (Fig. 4D-4) and slightly (Fig. 4D-5), respectively. Pretreatment with the mixture of both enzymes showed similar result to the case pretreated with heparitinase I (data not shown). Since heparitinase I well digests unsulfated HS and heparitinase II digests sulfated HS [4], the HS in the Purkinje cells may have a similar structure to that of acharan. In fact, acharan was almost digested to disaccharides when it was treated by heparitinase I (data not shown). It will be necessary to further characterize HS sequences containing IdoA-GlcNAc and IdoA2S-GlcNAc in rat neural tissues. The occurrences of IdoA-GlcNAc and IdoA2S-GlcNAc have been described previously, but the biosynthetic pathways that lead to formation of these structures are still unknown. This is largely due to the regulated synthetic pathway that chain modification proceeds

to *N*-deacetylation/*N*-sulfation of GlcNAcs by *N*-deacetylase/*N*-sulfotransferases, then to epimerization of GlcA to IdoA by 5′-uronosyl epimerase, and to 2-*O*-sulfation of IdoA and GlcA by 2-*O*-sulfotransferase [2].

Although current glycomics approaches such as soft ionization mass spectrometry are well established [25, 26], structural determination of sulfated GAGs from small amounts of biological materials is still a developing area [27–29]. In order to determine the detailed structures in the chains, a substantial amount of materials and laborious works are still inevitable. On the other hand, conventional disaccharide analysis of GAGs using bacterial eliminases is very convenient but it cannot discriminate between GlcA and IdoA and furthermore, some sequences in HS appear to be resistant to the enzymes. Another disaccharide analysis using sequential treatments with hydrazine and nitrous acid, is also useful, but on the contrary it cannot discriminate GlcNAc, $GlcNH_2$ and GlcNS in HS [6, 7]. The series of the antibodies directed towards [HexA(±2S)-GlcNAc]n that we present here, will significantly contribute to the analysis of HS, especially for the rapid and simple profiling, combined with current useful analytical methods.

References

1. Kannagi, R., Hakomori, S.: A guide to monoclonal antibodies directed to glycotopes. Adv. Exp. Med. Biol. **491**, 587–630 (2001)
2. Esko, J.D., Selleck, S.B.: Order out of chaos: assembly of ligand binding sites in heparan sulfate. Annu. Rev. Biochem. **71**, 435–471 (2002)
3. Smits, N.C., Lensen, J.F., Wijnhoven, T.J., Ten Dam, G.B., Jenniskens, G.J., van Kuppevelt, T.H.: Phage display-derived human antibodies against specific glycosaminoglycan epitopes. Methods Enzymol. **416**, 61 – 87 (2006)
4. Yamada, S., Sugahara, K.: Structure of oligosaccharides isolated from heparan sulfate/heparin and substrate specificities of the degrading enzymes of bacterial origin. Trends Glycosci. Glycotech. **10**, 95–123 (1998)
5. Kariya, Y., Yoshida, K., Morikawa, K., Tawada, A., Miyazono, H., Kikuchi, H., Tokuyasu, K.: Preparation of unsaturated disaccharides by eliminative cleavage of heparin and heparan sulfate with heparitinases. Comp. Biochem. Physiol. B. **103**, 473–479 (1992)
6. Guo, Y.C., Conrad, H.E.: The disaccharide composition of heparins and heparan sulfates. Anal. Biochem. **176**, 96–104 (1989)
7. Kariya, Y., Herrmann, J., Suzuki, K., Isomura, T., Ishihara, M.: Disaccharide analysis of heparin and heparan sulfate using deaminative cleavage with nitrous acid and subsequent labeling with paranitrophenyl hydrazine. J. Biochem. (Tokyo) **123**, 240–246 (1998)
8. Shively, J.E., Conrad, H.E.: Formation of anhydrosugars in the chemical depolymerization of heparin. Biochemistry **15**, 3932–3942 (1976)
9. Kim, Y.S., Jo, Y.Y., Chang, I.M., Toida, T., Park, Y., Linhardt, R. J.: A new glycosaminoglycan from the giant African snail *Achatina fulica*. J. Biol. Chem. **271**, 11750–11755 (1996)
10. Vann, W.F., Schmidt, M.A., Jann, B., Jann, K.: The structure of the capsular polysaccharide (K5 antigen) of urinary-tract-infective *Escherichia coli* 010:K5:H4. A polymer similar to desulfo-heparin. Eur. J. Biochem. **116**, 359–364 (1981)
11. Trescony, P.V., Oegema Jr, T.R., Farnam, B.J., Deloria, L.B.: Analysis of heparan sulfate from the Engelbreth–Holm–Swarm (EHS) tumor. Connect. Tissue Res. **19**, 219 – 242 (1989)
12. David, G., Bai, X.M., Van der Schueren, B., Cassiman, J.J., Van den Berghe, H.: Developmental changes in heparan sulfate expression: *in situ* detection with mAbs. J. Cell Biol. **119**, 961–975 (1992)
13. Takano, R., Kanda, T., Hayashi, K., Yoshida, K., Hara, S.: Desulfation of sulfated carbohydrates mediated by silylating reagents. J. Carbohydr. Chem. **14**, 885 – 888 (1995)
14. Takano, R., Ye, Z., Ta, T.-V., Hayashi, K., Kariya, Y., Hara, S.: Specific 6-*O*-desulfation of heparin. Carbohydr. Lett. **3**, 71–77 (1998)
15. Kariya, Y., Kyogashima, M., Suzuki, K., Isomura, T., Sakamoto, T., Horie, K., Ishihara, M., Takano, R., Kamei, K., Hara, S.: Preparation of completely 6-*O*-desulfated heparin and its ability to enhance activity of basic fibroblast growth factor. J Biol. Chem. **275**, 25949–25958 (2000)
16. Ayotte, L., Perlin, A.S.: N.m.r. spectroscopic observations related to the function of sulfate groups in heparin. Calcium binding vs. biological activity. Carbohydr. Res. **145**, 267 – 277 (1986)
17. Danishefsky, I.: Desulfation of heparin. Methods Carbohydr. Chem. **5**, 407–409 (1965)
18. Carlsson, J., Drevin, H., Axen, R.: Protein thiolation and reversible protein-protein conjugation. *N*-Succinimidyl 3-(2-pyridyldithio) propionate, a new heterobifunctional reagent. Biochem. J. **173**, 723 – 737 (1978)
19. ten Dam, G.B., van de Westerlo, E.M., Smetsers, T.F., Willemse, M., van Muijen, G.N., Merry, C.L., Gallagher, J.T., Kim, Y.S., van Kuppevelt, T.H.: Detection of 2-*O*-sulfated iduronate and *N*-acetylglucosamine units in heparan sulfate by an antibody selected against acharan sulfate (IdoA2S-GlcNAc)n. J. Biol. Chem. **279**, 38346 – 38352 (2004)
20. Born, J., Jann, K., Assmann, K.J., Lindahl, U., Berden, J.H.: *N*-Acetylated domains in heparan sulfates revealed by a monoclonal antibody against the *Escherichia coli* K5 capsular polysaccharide. Distribution of the cognate epitope in normal human kidney and transplant kidney with chronic vascular rejection. J. Biol. Chem. **271**, 22802 – 22809 (1996)
21. Maeda, N., He, J., Yajima, Y., Mikami, T., Sugahara, K., Yabe, T.: Heterogeneity of the chondroitin sulfate portion of phosphacan/6B4 proteoglycan regulates its binding affinity for pleiotrophin/heparin binding growth-associated molecule. J. Biol .Chem. **278**, 35805 – 35811 (2003)
22. Bao, X., Pavao, M.S., Dos Santos, J.C., Sugahara, K.: A functional dermatan sulfate epitope containing iduronate (2-*O*-sulfate)alpha1-3GalNAc(6-*O*-sulfate) disaccharide in the mouse brain: demonstration using a novel monoclonal antibody raised against dermatan sulfate of ascidian *Ascidia nigra*. J. Biol. Chem. **280**, 23184 – 23193 (2005)
23. von Holst, A., Sirko, S., Faissner, A.: The unique 473HD-chondroitin sulfate epitope is expressed by radial glia and involved in neural precursor cell proliferation. J. Neurosci. **26**, 4082 – 4094 (2006)
24. Purushothaman, A., Fukuda, J., Mizumoto, S., ten Dam, G.B., van Kuppevelt, T.H., Kitagawa, H., Mikami, T., Sugahara, K.: Functions of chondroitin sulfate/dermatan sulfate chains in brain development. Critical roles of E and iE disaccharide units recognized by a single chain antibody GD3G7. J. Biol. Chem. **282**, 19442–19452 (2007)
25. Jang-Lee, J., North, S.J., Sutton-Smith, M., Goldberg, D., Panico, M., Morris, H., Haslam, S., Dell, A.: Glycomic profiling of cells and tissues by mass spectrometry: fingerprinting and sequencing methodologies. Methods Enzymol. **415**, 59 – 86 (2006)

26. Haslam, S.M., North, S.J., Dell, A.: Mass spectrometric analysis of *N*- and *O*-glycosylation of tissues and cells. Curr. Opin. Struct. Biol. **16**, 584 – 591 (2006)
27. Tissot, B., Gasiunas, N., Powell, A.K., Ahmed, Y., Zhi, Z.L., Haslam, S.M., Morris, H.R., Turnbull, J.E., Gallagher, J.T., Dell, A.: Towards GAG glycomics: analysis of highly sulfated heparins by MALDI-TOF mass spectrometry. Glycobiology **17**, 972 – 982 (2007)
28. Minamisawa, T., Suzuki, K., Hirabayashi, J.: Multistage mass spectrometric sequencing of keratan sulfate-related oligosaccharides. Anal. Chem. **78**, 891 – 900 (2006)
29. Laremore, T.N., Zhang, F., Linhardt, R.J.: Ionic liquid matrix for direct UV-MALDI-TOF-MS analysis of dermatan sulfate and chondroitin sulfate oligosaccharides. Anal. Chem. **79**, 1604 – 1610 (2007)