



HOKKAIDO UNIVERSITY

Title	Identification of human hyaluronidase-4 as a novel chondroitin sulfate hydrolase that preferentially cleaves the galactosaminidic linkage in the trisulfated tetrasaccharide sequence
Author(s)	Kaneiwa, Tomoyuki; Mizumoto, Shuji; Sugahara, Kazuyuki et al.
Citation	Glycobiology, 20(3), 300-309 https://doi.org/10.1093/glycob/cwp174
Issue Date	2010-03
Doc URL	https://hdl.handle.net/2115/44894
Rights	This is a pre-copy-editing, author-produced PDF of an article accepted for publication in Glycobiology following peer review. The definitive publisher-authenticated version Glycobiology 2010 20(3):300-309 is available online at: http://glycob.oxfordjournals.org/cgi/content/abstract/20/3/300
Type	journal article
File Information	Gly20-3_300-309.pdf



**Identification of human hyaluronidase-4 as a novel chondroitin sulfate hydrolase
that preferentially cleaves the galactosaminidic linkage in the trisulfated
tetrasaccharide sequence**

Key words: chondroitin sulfate/glycosaminoglycan/hyaluronan/hyaluronidase/sulfated oligosaccharide

Tomoyuki Kaneiwa, Shuji Mizumoto, Kazuyuki Sugahara, and Shuhei Yamada[†]

Laboratory of Proteoglycan Signaling and Therapeutics, Hokkaido University

Graduate School of Life Science, Sapporo 001-0021

[†]To whom correspondence may be addressed: Laboratory of Proteoglycan Signaling and Therapeutics, Graduate School of Life Science, Hokkaido University, Nishi 11-choume, Kita 21-jo, Kita-ku, Sapporo, Hokkaido 001-0021, Japan. Tel.: 81-(11)-706-9055; Fax: 81-(11)-706-9055; E-mail: tjohej@sci.hokudai.ac.jp

Running title: Novel Human Chondroitin Sulfate Hydrolase

Supplementary data: Supplementary data for this article is included and listed as follows; Supplementary Fig. 1. Expression of CSHY in COS-7 cells. Supplementary Fig. 2. Substrate specificity of bacterial CSase ABC and AC-II.

Abstract

Human hyaluronidases have been considered to be the enzymes acting at the initial step in the catabolism of chondroitin sulfate (CS) *in vivo*. However, human hyaluronidase-1 digests CS more slowly than hyaluronan (HA), and its preferred substrate is HA rather than CS. We have identified a chondroitin hydrolase in *Caenorhabditis elegans*, which effectively degrades chondroitin but depolymerizes HA to a much lesser extent (Kaneiwa T, Yamada S, Mizumoto S, Montañó AM, Mitani S, Sugahara K. 2008. J Biol Chem. 283:14971-14979), suggesting the existence of CS-specific endoglycosidases in mammalian systems. In this study, human hyaluronidase-4 was demonstrated to be a CS-specific endo- β -*N*-acetylgalactosaminidase. This is the first demonstration of a CS hydrolase in higher organisms. The specificity of a purified recombinant form of the enzyme was investigated in detail through the characterization of degradation products. The best substrate of the CS hydrolase was the galactosaminidic linkage in the sequence of a trisulfated tetrasaccharide GlcUA(2-*O*-sulfate)-GalNAc(6-*O*-sulfate)-GlcUA-GalNAc(4-*O*- or 6-*O*-sulfate), where GlcUA and GalNAc represent D-glucuronic acid and *N*-acetyl-D-galactosamine, respectively. The disaccharide unit on the nonreducing side, GlcUA(2-*O*-sulfate)-GalNAc(6-*O*-sulfate) (D unit), is rich in shark fin cartilage CS-D

among various CS isoforms. CS hydrolase will be a useful tool for investigating CS-specific functions in tissues and cells. In addition, it may well be applicable to the treatment of acute spinal cord injuries as in the case of, or instead of, the bacterial CS lyase which has been used for recent clinical trials.

Introduction

Chondroitin sulfate (CS) chains are linear polymers composed of the repeating disaccharide unit $\text{—4GlcUA}\beta\text{1-3GalNAc}\beta\text{1—}$, where GlcUA and GalNAc represent D-glucuronic acid and *N*-acetyl-D-galactosamine, respectively, which are sulfated at different positions in various combinations (Rodén 1980; Sugahara and Yamada 2000; Sugahara et al. 2003). CS chains are covalently linked to a wide range of core proteins, forming proteoglycans (PGs), which are ubiquitous components of the extracellular matrix of connective tissues and are also found at the surface of a variety of cell types (Rodén 1980; Iozzo 1998). They are involved in the regulation of various biological processes such as cell proliferation, differentiation, and migration, cell-cell recognition, extracellular matrix deposition, and tissue morphogenesis (Esko and Selleck 2002; Sugahara et al. 2003; Rauch and Kappler 2006; Uyama et al. 2007). The biological functions involved in such events have attracted much attention to the mechanism of CS biosynthesis (Sugahara and Kitagawa 2000; Pavão et al. 2006; Uyama et al. 2007). However, not only biosynthesis but also catabolism is important for the regulation of the biological functions of CS. Despite this, the mechanism underlying the catabolism of CS is not well understood.

The cellular degradation of CS occurs predominantly in lysosomes (Prabhakar

and Sasisekharan 2006). Following the fragmentation of polysaccharides by an endo-type hydrolase, the oligosaccharide products are degraded sequentially from the nonreducing end by exo-type glycosidases and sulfatases to liberate monosaccharide moieties. Although no endoglycosidases specific to CS have been reported, hyaluronan (HA)-degrading enzymes, hyaluronidases, are considered to be the enzymes acting at the initial stage of the degradation process, because HA is similar in structure to nonsulfated CS, chondroitin (Chn). The sugar stereoconfiguration, the substitution pattern of the backbone hydroxy groups, and the glycosidic linkages are identical in Chn and HA. The only difference in structure is the configuration at the C-4 position of the hexosamine residues.

Although human hyaluronidase-1 (HYAL1) and testicular hyaluronidase (SPAM1) can degrade not only HA but also CS, they digest CS to a limited extent (Sugahara et al. 1996; Csoka et al. 2001; Jedrzejewski and Stern 2005) and therefore their preferred substrate is HA rather than CS. These hyaluronidases do not cleave the galactosaminidic linkages in the -GalNAc-IdoUA- and -GalNAc-GlcUA(2-*O*-sulfate)-sequences, which are often found in dermatan sulfate (DS), an isomer of CS, and in a highly sulfated CS isomer, CS-D, respectively. Therefore, a putative glycosaminoglycan (GAG) hydrolase, which cleaves CS/DS rather than HA, is

expected to be involved in the catabolism of CS/DS.

Previously we identified a homolog of human hyaluronidases in *Caenorhabditis elegans* as a Chn-specific hydrolase (Kaneiwa et al. 2008). Thus, uncharacterized members of the human hyaluronidase family may have the activity of a CS-specific hydrolase. Although there are 6 genes encoding a hyaluronidase or a hyaluronidase-like enzyme in the human genome, HYAL1, HYAL2, HYAL3, HYAL4, SPAM1, and HYALP1 (Csoka et al. 2001), enzymatic activity toward HA has been demonstrated only for HYAL1, HYAL2, and SPAM1. *HYALP1* is a pseudogene, which is translated as a truncated and inactive form (Csóka et al. 1999). The enzymatic properties including catalytic activities of HYAL3 and HYAL4 have hardly been characterized. Only one citation is available for hyaluronidase activity of HYAL3 (Lokeshwar et al. 2002), although it was not detected by a purified enzyme but a tissue culture situation. Csoka *et al.* (2001) described HYAL4 as a CS-specific enzyme based on preliminary, unpublished observations. The actual data or conditions for detection of the activity, however, were not presented. Previously, we were unable to detect CS-degrading activity of HYAL4 by agarose gel electrophoresis (Benchetrit et al. 1977) using Chn, CS-A, or CS-C as substrates (Kaneiwa et al. 2008). In the present study, the unique enzymatic activity of HYAL4 toward CS was clearly demonstrated using highly

sulfated CS-D chains as a substrate, and the specificity of HYAL4 was characterized.

Results

Demonstration of the CS-degrading Activity.

To facilitate the functional analysis of HYAL4, a soluble form of the protein was generated as a fusion protein with a FLAG tag by replacing the putative signal sequence with a cleavable leader sequence of preprotrypsin and by deleting the putative glycosylphosphatidylinositol (GPI)-anchored domain as described under "Materials and methods." The soluble protein was expressed in COS-7 cells at 37 °C as a recombinant protein fused with the FLAG tag. The fusion protein secreted in the medium was adsorbed onto an anti-FLAG[®] M2 affinity gel for eliminating endogenous glycosidases, and then the protein-bound resin was used as an enzyme source. The purity of the enzyme was examined by silver staining as well as western blotting (Supplementary Fig. 1). In contrast to the sample from mock-transfected cells, the band was discernible at a molecular mass of 80 kDa. Since the expected molecular mass of this polypeptide is 53 kDa, the recombinant protein detected by western blotting seems to be posttranslationally modified, presumably by glycosylation.

The bound fusion protein was assayed for CS-degrading activity at 37 °C for 12

h using fluorescein 5(6)-isothiocyanate (FITC)-GAG isoforms as substrates, and each digest was analyzed by gel filtration HPLC on a Superdex 200 column (Fig. 1). However, it was not clear whether FITC-HA and/or FITC-CS-A were depolymerized or not by the enzyme (Figs. 1A-1D). Interestingly, however, when FITC-CS-C was used as a substrate, the peak became broader upon digestion with HYAL4 (Figs. 1E and 1F), suggesting weak activity toward CS-C. Although both CS-A and CS-C contain the “A” disaccharide unit [GlcUA β 1-3GalNAc(4-*O*-sulfate)] (76 and 13%, respectively) and “C” disaccharide unit [GlcUA β 1-3GalNAc(6-*O*-sulfate)] (24 and 79%, respectively), the “D” disaccharide unit [GlcUA(2-*O*-sulfate) β 1-3GalNAc(6-*O*-sulfate)] is present only in CS-C (8%) (Li et al. 2008). Therefore, FITC-labeled CS-D, which is rich in D units (23%), was examined. The chromatogram revealed that HYAL4 had strong activity to degrade FITC-CS-D (Figs. 1G and 1H). No detectable catalytic activity was observed in the control sample prepared from a conditioned medium of mock-transfected cells (data not shown). Thus, CS-degrading activity was demonstrated for HYAL4. FITC-Chn, -DS, and -CS-E were also tested. Although FITC-CS-E was degraded to some extent, no significant depolymerization of FITC-Chn or -DS was observed (data not shown). Since HYAL4 did not act on HA but acted specifically on CS as shown here, the enzyme was designated CS hydrolase (CSHY).

Substrate Specificity of CSHY.

To further characterize the specificity of CSHY, non-labeled Chn, CS-A, DS, CS-C, CS-D, CS-E, heparan sulfate, and HA from *S. pyogenes* were used as substrates. The incubation mixtures were analyzed by gel filtration HPLC on a Superdex peptide column after labeling with 2-aminobenzamide (2AB) to detect the newly formed reducing ends (Fig. 2). The chromatograms showed that CSHY exhibited activity to degrade CS-D (Fig. 2A), CS-C (Fig. 2B), CS-A (Fig. 2C), and CS-E (data not shown) into oligosaccharides, whereas HA, Chn, DS, 2,6-di-*O*-sulfated DS, and heparan sulfate were not depolymerized (data not shown). Although the same amount of each substrate was used under the same conditions, the amounts of the products generated by the digestion were different. The relative rates of the degradation of these polysaccharides suggested that the substrates ranked as follows in order of preference by CSHY: CS-D>CS-C>CS-A>CS-E.

The Optimum Temperature and pH for the Catalytic Activity of CSHY.

The effects of temperature on the CSHY activity were examined by incubating at 20, 25, 30 or 37 °C. Taking the activity detected at 37 °C as 1.0, the relative activity at 20, 25

and 30 °C was 0.31, 0.56 and 0.82, respectively. Hence, subsequent experiments were conducted at 37 °C.

The effects of pH on the CSHY activity were examined by incubating CSHY with CS-D over a range of pH values from 3.5 to 6.5 (Fig. 3). The results indicated the optimum pH of CSHY to be 4.5-5.0.

Kinetic Analysis of CSHY.

The initial reaction rates and substrate concentrations (as disaccharide) were used for a kinetic analysis with Lineweaver-Burk plots (Fig. 4). The apparent Michaelis-Menten constants as well as $V_{\max}$ for CS-D, CS-A, CS-C, and CS-E were determined and are shown in Table I. The apparent K_m value towards CS-D was approximately 2, 3, and 20-times smaller than that towards CS-C, CS-A, and CS-E, respectively, indicating that CS-D is the best substrate among the CS preparations used. This preference for CS-D was consistent with the results obtained by quantification of the newly formed reducing ends by incubating various GAGs with CSHY under the same conditions as shown in Fig. 2. It should be noted, however, that the degree to which the various CS isoforms susceptible to CSHY were degraded differed.

Characterization of the Saccharide Sequence Recognized by CSHY.

To investigate the activity of CSHY in more detail, the CSHY digest of CS-D was labeled with 2AB and subjected to gel filtration HPLC on a Superdex peptide column (Fig. 2A). Three major peaks, D-1, D-2, and D-3, which were eluted at the positions of tetra-, hexa-, and octasaccharides, were collected separately (Fig. 2A).

When D-1 was analyzed by anion-exchange HPLC, a single predominant peak was detected at the elution position of trisulfated tetrasaccharide-2AB. The major compound in this peak was resistant to chondroitinase (CSase) AC-II (Fig. 5A), suggesting that the internal GlcUA residue is most likely 2-*O*-sulfated (Supplementary Fig. 2) (Deepa et al. 2007). To determine the saccharide sequence of the major compound in D-1, the CSHY digest of CS-D was subjected to gel filtration HPLC without labeling with 2AB by monitoring UV-absorption at 215 nm, and the peak corresponding to a tetrasaccharide (D-1') was collected (data not shown). The unlabeled tetrasaccharide fraction was digested with CSase ABC and then the digest was labeled with 2AB and analyzed by anion-exchange HPLC. Two major peaks were detected at the elution positions of GlcUA-GalNAc(4S)-2AB and Δ HexUA(2S)-GalNAc(6S)-2AB (Fig. 5D and Table II), suggesting that the saccharide sequence of the predominant tetrasaccharide in the CSHY digest of CS-D was

GlcUA-GalNAc(4S)-GlcUA(2S)-GalNAc(6S) (Table III).

On digestion with CSase AC-II, D-2 yielded a single peak eluted at the position of trisulfated tetrasaccharide-2AB upon HPLC (Fig. 5B). The digest of D-3 obtained with CSase AC-II also gave a single peak eluted at the same position as that of the major compound in the digest of D-2 (Fig. 5C). When these digests were co-chromatographed individually with 2AB-labeled authentic unsaturated tetrasaccharides, they each co-eluted with Δ HexUA-GalNAc(4S)-GlcUA(2S)-GalNAc(6S)-2AB (data not shown). Thus, it was concluded that CSHY recognized the tetrasaccharide sequence, GlcUA-GalNAc(4S)-GlcUA(2S)-GalNAc(6S), as the structure on the nonreducing side of the cleavage sites in the CS-D chains.

To investigate the structure on the reducing side of the cleavage site in CS-D, the disaccharide units at the nonreducing termini of the compounds in the CSHY-produced D-2 and D-3 fractions were investigated. The hexasaccharide fraction D-2 was digested with CSase ABC, and the digest was labeled with 2AB. The 2AB-labeled digest was analyzed by anion-exchange HPLC, which showed three peaks at the elution positions of GlcUA-GalNAc(4S)-2AB, GlcUA-GalNAc(6S)-2AB, and Δ HexUA-GalNAc(4S)-GlcUA(2S)-GalNAc(6S)-2AB, respectively, in a molar ratio of

0.34 : 0.38 : 1.0 (Fig. 5E and Table II), suggesting that fraction D-2 contained the following two major components in a molar ratio of 47:53 (Table III):

GlcUA-GalNAc(4S)-GlcUA-GalNAc(4S)-GlcUA(2S)-GalNAc(6S) and
GlcUA-GalNAc(6S)-GlcUA-GalNAc(4S)-GlcUA(2S)-GalNAc(6S).

Upon digestion of the octasaccharide fraction D-3 with CSase ABC followed by 2AB-derivatization, the CSase digest yielded 2AB-derivatives of two saturated disaccharides, three unsaturated disaccharides and Δ HexUA-GalNAc(4S)-GlcUA(2S)-GalNAc(6S) derived from the reducing termini of the compounds in fraction D-3 (Fig. 5F). Their proportions are shown in Table II. Saturated and unsaturated disaccharides are derived from the nonreducing and internal positions of the octasaccharides in fraction D-3, respectively. The possible saccharide sequences of the major octasaccharides in fraction D-3 are listed in Table III.

Since CS-A and CS-C were also depolymerized by CSHY to some extent (Figs. 1 and 2), the reducing terminal structure of the oligosaccharide products in the CSHY digests of CS-A and CS-C was analyzed to investigate the specificity of CSHY. The 2AB-derivative of the respective digest was treated with CSase AC-II and analyzed by anion-exchange HPLC (Fig. 6). The only product from the reducing end of the CSHY digest of CS-A was detected at the elution position of Δ HexUA-GalNAc(6S)-2AB,

suggesting that GlcUA-GalNAc(6S) is the predominant disaccharide unit occupying the reducing terminus of the major component in the CSHY digest of CS-A. Since the major disaccharide unit in CS-A is GlcUA-GalNAc(4S) (76%), not GlcUA-GalNAc(6S) (24%), it can be concluded that 6-*O*-sulfation on the GalNAc residue at the cleavage site is essential for the recognition by CSHY.

When the 2AB-derivatized CSHY digest of CS-C was analyzed by anion-exchange HPLC after further digestion with CSase AC-II, Δ HexUA-GalNAc(6S)-2AB and Δ HexUA-GalNAc(4S)-GlcUA(2S)-GalNAc(6S)-2AB were detected as major oligosaccharides in a molar ratio of 1.2 : 1.0 (Fig. 6B), suggesting that GlcUA-GalNAc(6S) and GlcUA(2S)-GalNAc(6S) are reducing terminal disaccharides in the major resultant oligosaccharides in the CSHY digest of CS-C (Table III). The molar ratio of GlcUA-GalNAc(6S) to GlcUA(2S)-GalNAc(6S) in the parent CS-C chains is 10.7 : 1.0 (Li et al. 2008). Although the proportion of GlcUA-GalNAc(6S) in the parent CS-C is much higher than that of GlcUA(2S)-GalNAc(6S), the two units were similarly detected as the major reducing terminal disaccharides in the CSHY digest of CS-C, suggesting that CSHY prefers the GlcUA(2S)-GalNAc(6S) structure to GlcUA-GalNAc(6S) as the cleavage site in CS-C chains. Thus, 2-*O*-sulfation of the GlcUA residue on the immediate nonreducing side of

the target GalNAc(6S) residue appears to be preferred by CSHY as indicated by an asterisk in Fig. 7A.

Discussion

In the present study, we demonstrated the catalytic activity of HYAL4 toward CS and characterized its specificity. Although HYAL4 was identified as a homolog of hyaluronidases, it turned out to be a CSHY: the enzyme hardly acted on HA and clearly preferred CS. HYAL1 and SPAM1 can also degrade CS, although the catabolism occurs more slowly than that of HA (Csoka et al. 2001; Jedrzejewski and Stern 2005), and therefore their preferred substrate is HA rather than CS. Thus, these human hyaluronidases and the chondroitin hydrolase of nematodes cannot be termed CS-degrading enzymes. In this study, we demonstrated the CS-degrading activity of HYAL4, characterized its substrate specificity in detail, and identified the sugar sequences of the cleavage site in CS chains recognized by this enzyme.

The CSHY protein possesses a putative GPI-anchored domain in the C-terminal region and appears to be a GPI-anchored molecule. However, the optimal pH of this enzyme was determined to be 5.0 (Fig. 3), suggesting it to be active mostly in lysosomes. Although HYAL2 is also a GPI-anchored molecule and has been assigned to

the plasma membrane closely associated with CD44 (Rai et al. 2001; Duterme et al. 2009), it was shown to have an optimal pH of 3.8 (Lepperdinger et al. 1998). Recently, the HA-degrading activity of HYAL2 was detected in the membrane fraction of cells co-expressing HYAL2 and CD44 at pH 6.0-7.0 (Harada and Takahashi 2007). Therefore, we hypothesize that HYAL4 might also function on the cell surface. Investigation of the cellular distribution of this enzyme is in progress.

CSHY can degrade CS but the Chn and HA preparations tested here were hardly depolymerized at all, suggesting that sulfate groups are required for the enzymatic activity in contrast to the Chn hydrolase in *C. elegans*, which prefers Chn to CS (Kaneiwa et al. 2008). Since DS was not digested by CSHY, the steric configuration of the C-5 position of uronic acid residues is distinct. In addition, the structure of CS oligosaccharides generated by the hydrolytic action of CSHY was determined. All the reducing GalNAc residues of the tested oligosaccharides in the CSHY digests of CS-A, -C and -D were 6-*O*-sulfated (Table III), suggesting that the GalNAc(6S) structure is essential for the hydrolyzing activity of CSHY. All the uronate residues at the nonreducing ends of the oligosaccharides in the CSHY digests of CS-A, -C and -D were nonsulfated GlcUA (Table III), suggesting that the 2-*O*-sulfated GlcUA structure of the disaccharide unit on the reducing side of the cleavage sites is not recognized by CSHY.

Based on these results, the minimal sequence recognized by CSHY was concluded to be -GalNAc(6S)-GlcUA-.

To further investigate the patterns of sulfation on the GalNAc/GlcUA residues adjacent to the target glycosidic linkage, the disaccharide units located on both sides of the site of cleavage by CSHY were characterized. The reducing terminal disaccharide units of the oligosaccharides in the CSHY digests of CS-A, -C and -D were identified as C [GlcUA-GalNAc(6S)] or D [GlcUA(2S)-GalNAc(6S)] units (Table III). Although the proportion of D units in CS-C and CS-D (7% and 23%, respectively) is much lower than that of C units (79% and 45%, respectively) (Li et al. 2008), the D disaccharide unit was observed as a major unit even in the CSHY digest of CS-C (Fig. 5, Fig. 6B, and Table III), suggesting that the structure of the D unit is preferred by CSHY. Thus, 2-*O*-sulfation of the GlcUA residue on the nonreducing side of the target GalNAc residue seems to have a promoting effect on the action of CSHY. On the other hand, the nonreducing terminal disaccharides of the oligosaccharides in the CSHY digests were identified as A [GlcUA-GalNAc(4S)] or C units (Tables II and III). Although the CS-D preparation used contained a significant proportion (10%) of O [GlcUA-GalNAc] units, only sulfated disaccharide units were detected, suggesting that sulfation of the GalNAc residue at the cleavage site is required for the recognition by CSHY. The position of

sulfation on the GalNAc residue seems to be tolerated. It appears that the 6-*O*-sulfation on the immediate reducing side of the target GlcUA residue can be replaced by 4-*O*-sulfation. The findings demonstrated the importance of the sulfated structure for the degradation of CS chains by CSHY. Notably, the positions of sulfation preferred by CSHY were revealed; the enzyme cleaves in principle the galactosaminidic linkage in the tetrasaccharide sequence GlcUA(2S)-GalNAc(6S)-GlcUA-GalNAc(4S or 6S) in CS polysaccharides (Fig. 7A).

A schematic representation of the action of CSHY on CS-D is shown in Fig. 7B. Based on the structural analysis of oligosaccharides isolated from CS-D, some characteristic sulfation patterns in CS-D have been revealed (Nadanaka and Sugahara 1997; Sugahara and Yamada 2000). “A” unit could be located on the reducing side of “C” unit, which results in a C-A tetrasaccharide sequence. Oligosaccharides containing the reverse arrangement, A-C, have seldom been isolated from CS-D. “D” unit has been found on the reducing side of “A” unit as well as the nonreducing side of “A” and “C” unit, which forms the A-D, D-A, and D-C tetrasaccharide sequences. These observations may reflect the substrate specificity of CS sulfotransferases. Since the D-D and C-D tetrasaccharide sequences have never been reported, it remains to be investigated whether the glycosidic bonds in these sequences can be hydrolyzed by

CSHY.

The protein sequences of the full-length human hyaluronidase genes have been used in studies of sequence homology, providing information regarding the primary, secondary, and three-dimensional structural properties of these proteins (Jedrzejewski and Stern 2005; Stern and Jedrzejewski 2006; Zhang et al. 2009). Three-dimensional models demonstrated that hyaluronidases are globular proteins encompassing a large cleft, which is where HA (or CS) is thought to bind and be broken down. The surface of the cleft is lined with conserved amino acid residues, most of them positively charged or hydrophobic. The amino acid sequence of CSHY (HYAL4) is displayed with those of HYAL1 and *C. elegans* Chn hydrolase in Fig. 8. Based on the three-dimensional modeling for human hyaluronidases, Stern and Jedrzejewski (2006) claimed that a group of residues including Asp129, Tyr202, Tyr247, and Trp321 (numbering for HYAL1, indicated by asterisks in Fig. 8), which are conserved strictly among the human hyaluronidases, are responsible for their hydrolytic activity, and that Cys263 in HYAL4 replaced by Tyr247 in HYAL1 may cause the distinct substrate specificity of HYAL4. Chn is the preferred substrate for Chn hydrolase from *C. elegans*, while the sulfated sequences in CS are preferred by CSHY. To evaluate the contribution of the amino acid residues to the specificity of CSHY, future studies using site-directed mutagenesis as

well as the preparation and characterization of chimeric proteins consisting of HYAL1 or Chn hydrolase with CSHY are required.

CSHY is not the only candidate for the enzyme involved in the catabolism of CS/DS because its expression is not ubiquitous but restricted to the placenta and skeletal muscle (Csóka et al. 1999). Since HYAL1 is ubiquitously expressed and can degrade CS chains to some extent (Kresse and Glössl 1987; Csoka et al. 2001; Jedrzejak and Stern 2005), it may also be involved in the catabolism of CS under certain conditions. However, no endoglycosidase which can act on DS has been identified. How are DS chains degraded under physiological conditions? It has been reported that free DS chains specifically accumulate in the amyloidotic spleen during amyloid fibrillogenesis (Stenstad et al. 1994), implying the existence of a DS-specific endoglycosidase and its involvement in the formation of amyloid fibrils. The possibility cannot be excluded that another enzyme having no homology with the human hyaluronidases, hydrolyzes CS/DS *in vivo*. Odd-numbered oligosaccharides have been isolated from a porcine skin DS preparation after digestion with a bacterial CS lyase (Sugahara et al. 1994b). Their reducing terminal saccharide residues were identified as GlcUA not GalNAc by a ¹H NMR analysis, indicating that the intact DS chains are liberated from DS-PGs by an unidentified endo- β -glucuronidase, not by a

hyaluronidase-like endo- β -hexosaminidase. Although heparanase is an endo- β -glucuronidase and involved in the catabolism of heparan sulfate/heparin by the endolytic cleavage of long polysaccharide chains at the initial stage of the degradation process (Hopwood 1989; Okada et al. 2002; van den Hoven et al. 2007), no endo-hexuronidase specific to CS/DS has been reported.

Chn/CS, but no HA, has been demonstrated in nematodes (Nematoda) (Yamada et al. 1999) and hydrozoan (Cnidaria) (Yamada et al. 2007). Isolation of HA has been reported only in the mollusc bivalve *Mytilus galloprovincialis* (Volpi and Maccari 2003) and vertebrates in metazoan to our knowledge. Chn/CS is most likely occurred in evolution prior to HA. Previously we demonstrated the human hyaluronidase homolog in *C. elegans* to be a Chn hydrolase. This implies that HYAL4 (CSHY) might be the primordial gene for the hyaluronidase family. However, data base searches suggested that the amino acid sequence of *C. elegans* Chn hydrolase displayed similar identity with the human hyuronidase family members (27-28%) (Kaneiwa et al. 2008), and that the homology with CSHY was not the highest. Rather *C. elegans* Chn hydrolase was similar to a hyaluronidase from bee venom based on the phylogenetic tree produced using the ClustalW algorithm (data not shown). Detailed studies on comparative genomics of hyaluronidase/CSHY are required to clarify this important issue.

Recently, approaches using a bacterial CS lyase against glial scar CS/DS PGs have received much attention in the treatment of acute spinal cord injuries (Bradbury et al. 2002). At sites of damage to the central nervous system, a glial scar containing CS/DS develops and is inhibitory to axon growth. Removal of CS chains in glial scars by treatment with the bacterial CS lyase attenuates the inhibitory activity (Bradbury et al. 2002). However, the use of the bacterial CS lyase may not be most suitable for the treatment of an acute spinal cord injury. The enzyme is not a hydrolase but an eliminase, and therefore the degradation products contain an artificial unsaturated hexuronic acid at the nonreducing end, contributing to a strong antigenicity. Further, this bacterial enzyme depolymerizes not only CS/DS but also HA. In contrast, CSHY is a CS-specific hydrolase. Hence, this enzyme may be a better tool for the treatment of acute spinal cord injury than the bacterial lyase.

Materials and methods

Materials

The following sugars and enzymes were purchased from Seikagaku Corp. (Tokyo, Japan): CS-A from whale cartilage, DS from pig skin, CS-C from shark cartilage, Chn, a chemically desulfated derivative of CS-A, CS-D from shark cartilage, CS-E from

squid cartilage, heparan sulfate from bovine kidney, seven unsaturated standard disaccharides derived from CS, CSase ABC from *Proteus vulgaris* (EC 4.2.2.4), and CSase AC-II from *Arthrobacter aureescens* (EC 4.2.2.5.). 2,6-Di-*O*-sulfated DS from *Ascidia nigra*, which mainly consists of IdoUA(2-*O*-sulfate)-GalNAc(6-*O*-sulfate) disaccharide unit, was from Iduron Ltd. (Manchester, UK). HA of human umbilical cord was obtained from Sigma. An HA preparation, which has an average molecular mass of 35 kDa, from *Streptococcus pyogenes* was purchased from R&D Systems, Inc. (Minneapolis, MN). COS-7 cells were obtained from Japan Health Sciences Foundation (Tokyo, Japan). SuperdexTM Peptide and 200 10/300 GL columns, and prepacked disposable PD-10 columns containing Sephadex G-25 (medium) were obtained from GE Healthcare. FITC-labeled GAG isoforms, Chn, CS-A, DS, CS-C, CS-D, CS-E, and HA from human umbilical cord, were prepared as described (Kaneiwa et al. 2008). All the FITC-labeled GAGs were sensitive to at least CSase ABC and can be degraded into small oligosaccharides (data not shown). 2AB-Derivatives of unsaturated trisulfated tetrasaccharides, $\Delta\text{HexUA-GalNAc(4S)-GlcUA(2S)-GalNAc(6S)}$, $\Delta\text{HexUA-GalNAc(6S)-GlcUA(2S)-GalNAc(6S)}$, $\Delta\text{HexUA(2S)-GalNAc(6S)-GlcUA-GalNAc(4S)}$, and $\Delta\text{HexUA(2S)-GalNAc(6S)-GlcUA-GalNAc(6S)}$, were prepared as described (Sugahara

et al. 1994a, Deepa et al. 2007).

Cloning of Human HYAL4 cDNA

The putative full-length open reading frame encoding HYAL4 was amplified from the human placenta PCR Ready First Strand cDNA library (BioChain Institute Inc., Hayward, CA) by two-rounds of PCR using specific primers corresponding to the sequences in the 5'- and 3'-noncoding regions. The first PCR was performed with the primers, 5'-GGT TTG GAG CCA TTG CTG GAC ATC C-3' and 5'-TTT CCT AGC CAG ACT GGA GGC-3'. The second PCR was performed with the nested primers, 5'-GCA CCA AGG TGA CTA AAG GAC CA-3' and 5'-CAT CCT TCT TTA AAT GAC TAG GC-3'. Each PCR was carried out with the KOD-Plus DNA polymerase (Toyobo, Tokyo) in the presence of 5% (v/v) dimethyl sulfoxide for 30 cycles at 94 °C for 30 s, 55 °C for 45 s, and 68 °C for 2 min. The amplified cDNA fragment of expected size (~1.4 kbp) was subcloned into a pGEM®-T Easy vector (Promega, Tokyo) and sequenced¹ in a CEQ 8000 DNA sequencer (Beckman Coulter, Fullerton, CA) at the OPEN FACILITY in Sousei Hall, Hokkaido University.

Construction of an Expression Vector Containing a cDNA Fragment Encoding a

Soluble Form of HYAL4

The DNA fragment which encodes the putative HYAL4 protein lacking both the first *N*-terminal 33 amino acids (a hydrophobic region) and the last *C*-terminal 19 amino acids (the putative GPI-anchored region), was amplified by PCR with the pGEM®-T Easy vector containing the full-length form of HYAL4 as a template, using a 5' primer containing an in-frame *EcoRV* site (5' -GCG ATA TCG TGT CTA AAA CCT GCT C-3') and a 3' primer containing a *BamHI* site (5'-GCG GAT CCT CAA GGA GAA GGG GAA A-3'). PCR was carried out with the KOD-Plus DNA polymerase for 30 cycles at 95 °C for 30 s, 55 °C for 45 s, and 68 °C for 2 min. The amplified fragment was subcloned into the *EcoRV* and *BamHI* sites of the expression vector p3XFLAG-CMV-8 (Sigma), resulting in the fusion of HYAL4 to the preprotrypsin leader sequence and the FLAG tag sequence present in the vector.

Expression of a Soluble Form of HYAL4

The expression plasmid (6.7 µg) was introduced into COS-7 cells (2.2×10^6 cells) using FuGENE™6 (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. After 3 days of culture at 37 °C, 1 ml of the culture medium was collected and incubated with 10 µl of ANTI-FLAG® M2 affinity gel (Sigma) overnight at 4 °C.

The resin was washed with 25 mM Tris-buffered saline containing 0.1% Tween-20, subjected to SDS-PAGE, and analyzed by silver staining as well as western blotting as described (Kaneiwa et al. 2008).

Measurement of the Enzymatic Activity

The cells transfected with *HYAL4* were cultured for 3 days and 1 ml of the medium was purified with 10 μ l of ANTI-FLAG[®] M2 affinity resin overnight at 4 °C. The resin was washed with 25 mM Tris-buffered saline containing 0.1% Tween-20 and subsequently with 50 mM phosphate buffer, pH 6.0, containing 150 mM NaCl, and then resuspended individually in 4 μ l of the same buffer containing ~10 μ g of FITC-labeled GAG isoforms. The mixture was incubated at 37 °C for 12 h. The resin was removed by filtration using an Ultrafree-MC filter (Millipore), and the sample solution was subjected to gel filtration chromatography on a Superdex 200 column equilibrated with 0.2 M NH_4HCO_3 . Eluates were monitored by measuring fluorescence with excitation and emission wavelengths of 490 and 520 nm, respectively.

The purified enzyme-bound resin (5 μ l) was also incubated with FITC-CS-D (~20 μ g) in 50 mM phosphate buffer, pH 5.0, at 20, 25, 30, or 37 °C for 1 h to determine its optimal temperature. The CS-degrading activity of *HYAL4* was assessed based on

the proportion (in percentage terms) of low molecular weight fragments of FITC-CS-D in the digests as described previously (Kaneiwa et al. 2008).

To determine the optimal pH, the purified enzyme-bound resin (5 μ l) was incubated with non-labeled CS-D (~ 5 μ g) in 50 mM phosphate, acetate, or citrate buffers, pH 3.5 – 6.5, at 37 °C for 1 h. The resin was then removed by filtration using an Ultrafree-MC filter, each sample was labeled with 2AB (Kinoshita and Sugahara 1999), and excess 2AB-derivatizing reagents were removed by extraction with chloroform (Kawashima et al. 2002). The 2AB-derivatives were digested by CSase AC-II (5 mIU) in 50 mM sodium acetate buffer, pH 6.0, and the digests were analyzed by anion-exchange HPLC on an amine-bound silica PA03 column (4.6 X 250 mm, YMC Co., Kyoto, Japan) using a linear gradient of NaH_2PO_4 from 16 to 800 mM over 60 min at a flow rate of 1 ml/min. Eluates were monitored by measuring fluorescence at excitation and emission wavelengths of 330 and 420 nm, respectively. The CS-degrading activity of HYAL4 was assessed based on the proportion (in percentage terms) of 2AB-labeled di- or tetrasaccharides formed, which was determined by measuring the peak areas. Taking the total amount of disaccharide as 100%, the proportion of each 2AB-labeled di- or tetrasaccharide was calculated.

Non-labeled Chn, CS-A, DS, CS-C, CS-D CS-E, heparan sulfate,

2,6-di-*O*-sulfated DS, and HA from *S. pyogenes* (10 µg each) were incubated individually with the CSHY-bound resin at 37 °C. The resin was then removed by filtration using an Ultrafree-MC filter, and each sample was labeled with 2AB and analyzed by gel filtration HPLC on a Superdex peptide column equilibrated with 0.2 M NH₄HCO₃.

Various concentrations of non-labeled CS-A, CS-C, CS-D, and CS-E were individually incubated with the CSHY-bound resin at 37 °C. The resin was removed by filtration using an Ultrafree-MC filter, and each sample was labeled with 2AB. The 2AB-derivatives of the degradation products were digested with CSase AC-II and then analyzed by anion-exchange HPLC on an amine-bound silica PA03 column as described above. The reaction rate was measured as moles of the products formed/min, and apparent Michaelis-Menten constants were determined by fitting the data to the Michaelis-Menten equation ($V=V_{\max}[\text{CS disaccharide}]/K_m + [\text{CS disaccharide}]$).

Determination of the Saccharide Sequence Required for Digestion by CSHY

The 2AB-labeled oligosaccharides generated by the digestion of CS-D with CSHY were fractionated by gel filtration HPLC on a Superdex peptide column and individually digested with CSase AC-II. An aliquot of each digest was analyzed by anion-exchange

HPLC on an amine-bound silica PA03 column and another aliquot was labeled with 2AB again, purified by paper chromatography, and analyzed by anion-exchange HPLC. The non-labeled digest of CS-D was also fractionated by gel filtration HPLC on a Superdex peptide column with absorption monitored at 215 nm. The tetrasaccharide fraction was digested with CSase ABC, labeled with 2AB, and analyzed by anion-exchange HPLC.

Funding

The work was supported in part by Grants-in-aid for Scientific Research C-19590052 (to S. Y.) and Scientific Research (B) 18390030 (to K. S.) from MEXT, the Human Frontier Science Program RGP0018/2005 (to K. S.), and the Core Research for Evolutional Science and Technology (CREST) of the Japan Science and Technology (JST) agency (to K. S.).

Footnotes

¹The nucleotide sequence reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number AB470346.

Abbreviations

2AB, 2-aminobenzamide; Chn, chondroitin; CS, chondroitin sulfate; CSase,

chondroitinase; CSHY, CS hydrolase; DS, dermatan sulfate; FITC, fluorescein
5(6)-isothiocyanate; GalNAc, *N*-acetyl-D-galactosamine; GlcUA, D-glucuronic acid;
GPI, glycosylphosphatidylinositol; HA, hyarulonon; Δ HexUA,
4-deoxy- α -L-*threo*-hex-4-enepyranosyluronic acid; IdoUA, L-iduronic acid; PG,
proteoglycan; 2S, 2-*O*-sulfate; 4S, 4-*O*-sulfate; 6S, 6-*O*-sulfate.

References

- Benchetrit LC, Pahuja SL, Gray ED, Edstrom RD. 1977. A sensitive method for the assay of hyaluronidase activity. *Anal Biochem.* 79:431-437.
- Bradbury EJ, Moon LD, Popat RJ, King VR, Bennett GS, Patel PN, Fawcett JW, McMahon SB. 2002. Chondroitinase ABC promotes functional recovery after spinal cord injury. *Nature.* 416:636-640.
- Csoka AB, Frost GI, Stern R. 2001. The six hyaluronidase-like genes in the human and mouse genomes. *Matrix Biol.* 20:499-508.
- Csóka AB, Scherer SW, Stern R. 1999. Expression analysis of six paralogous human hyaluronidase genes clustered on chromosomes 3p21 and 7q31. *Genomics.* 60:356-361.
- Deepa SS, Yamada S, Fukui S, Sugahara K. 2007. Structural determination of novel sulfated octasaccharides isolated from chondroitin sulfate of shark cartilage and their application for characterizing monoclonal antibody epitopes. *Glycobiology.* 17:631-645.
- Duterme C, Mertens-Strijthagen J, Tammi MI, Flamion B. 2009. Two novel functions of hyaluronidase-2 (Hyal2): Formation of the glycocalyx and control of CD44-ERM interactions. *J Biol Chem.* doi:10.1074/jbc.M109.044362.

- Esko JD, Selleck SB. 2002. Order out of chaos: assembly of ligand binding sites in heparan sulfate. *Annu Rev Biochem.* 71:435-471.
- Harada H, Takahashi M. 2007. CD44-dependent intracellular and extracellular catabolism of hyaluronic acid by hyaluronidase-1 and -2. *J Biol Chem.* 282:5597-5607.
- Hopwood JJ. 1989. Enzymes that degrade heparin and heparan sulphate. In: Lane DA, Lindahl U, editors. *Heparin*. London: Edward Arnold. p. 191-227.
- Iozzo RV. 1998. Matrix proteoglycans: from molecular design to cellular function. *Annu Rev Biochem.* 67:609-652.
- Jedrzejewski MJ, Stern R. 2005. Structures of vertebrate hyaluronidases and their unique enzymatic mechanism of hydrolysis. *Proteins.* 61:227-238.
- Kanehisa T, Yamada S, Mizumoto S, Montañó AM, Mitani S, Sugahara K. 2008. Identification of a novel chondroitin hydrolase in *Caenorhabditis elegans*. *J Biol Chem.* 283:14971-14979.
- Kawashima H, Atarashi K, Hirose M, Hirose J, Yamada S, Sugahara K, Miyasaka M. 2002. Oversulfated chondroitin/dermatan sulfates containing GlcA β 1/IdoA α 1-3GalNAc(4,6-*O*-disulfate) interact with L- and P-selectin and chemokines. *J Biol Chem.* 277:12921-12930.

- Kinoshita A, Sugahara K. 1999. Microanalysis of glycosaminoglycan-derived oligosaccharides labeled with the fluorophore 2-aminobenzamide by high-performance liquid chromatography: application to disaccharide composition analysis and exosequencing of oligosaccharides. *Anal Biochem.* 269:367-378.
- Kresse H, Glössl J. 1987. Glycosaminoglycan degradation. *Adv Enzymol Relat Areas Mol Biol.* 60:217-311.
- Lepperdinger G, Strobl B, Kreil G. 1998. *HYAL2*, a human gene expressed in many cells, encodes a lysosomal hyaluronidase with a novel type of specificity. *J Biol Chem.* 273:22466-22470.
- Li F, Yamada S, Basappa, Shetty AK, Sugiura M, Sugahara K. 2008. Determination of iduronic acid and glucuronic acid in sulfated chondroitin/dermatan hybrid chains by ¹H-nuclear magnetic resonance spectroscopy. *Glycoconj J.* 25:603-610.
- Lokeshwar VB, Schroeder GL, Carey RI, Soloway MS, Iida N. 2002. Regulation of hyaluronidase activity by alternative mRNA splicing. *J Biol Chem.* 277:33654-33663.
- Nadanaka S, Sugahara K. 1997. The unusual tetrasaccharide sequence GlcA beta 1-3GalNAc(4-sulfate)beta 1-4GlcA(2-sulfate)beta 1-3GalNAc(6-sulfate) found in the hexasaccharides prepared by testicular hyaluronidase digestion of shark cartilage

chondroitin sulfate D. *Glycobiology*. 7:253-263.

Okada Y, Yamada S, Toyoshima M, Dong J, Nakajima M, Sugahara K. 2002. Structural recognition by recombinant human heparanase that plays critical roles in tumor metastasis: hierarchical sulfate groups with differential effects and the essential target disulfated trisaccharide sequence. *J Biol Chem*. 277:42488-42495.

Prabhakar V, Sasisekharan R. 2006. The biosynthesis and catabolism of galactosaminoglycans. *Adv Pharmacol*. 53:69-115.

Pavão MS, Vilela-Silva AC, Mourão PA. 2006. Biosynthesis of chondroitin sulfate: from the early, precursor discoveries to nowadays, genetics approaches. *Adv Pharmacol*. 53:117-140.

Rai SK, Duh FM, Vigdorovich V, Danilkovitch-Miagkova A, Lerman MI, Miller AD. 2001. Candidate tumor suppressor HYAL2 is a glycosylphosphatidylinositol (GPI)-anchored cell-surface receptor for jaagsiekte sheep retrovirus, the envelope protein of which mediates oncogenic transformation. *Proc Natl Acad Sci U S A*. 98:4443-4448.

Rauch U, Kappler L. 2006. Chondroitin/dermatan sulfates in the central nervous system: their structures and functions in health and disease. *Adv Pharmacol*. 53:337-356.

- Rodén L. 1980. Structure and metabolism of connective tissue proteoglycans. In: Lennarz, WJ, editor. *The Biochemistry of Glycoproteins and Poteoglycans*. New York: Plenum Publishing Corp. p. 267-371.
- Stenstad T, Magnus JH, Husby G. 1994. Characterization of proteoglycans associated with mouse splenic AA amyloidosis. *Biochem J.* 303:663-670.
- Stern R, Jedrzejewski MJ. 2006. Hyaluronidases: their genomics, structures, and mechanisms of action. *Chem Rev.* 106:818-839.
- Sugahara K, Kitagawa H. 2000. Recent advances in the study of the biosynthesis and functions of sulfated glycosaminoglycans. *Curr Opin Struct Biol.* 10:518-527.
- Sugahara K, Mikami T, Uyama T, Mizuguchi S, Nomura K, Kitagawa H. 2003. Recent advances in the structural biology of chondroitin sulfate and dermatan sulfate. *Curr Opin Struct Biol.* 13:612-620.
- Sugahara K, Shigeno K, Masuda M, Fujii N, Kurosaka A, Takeda K. 1994a. Structural studies on the chondroitinase ABC-resistant sulfated tetrasaccharides isolated from various chondroitin sulfate isomers. *Carbohydr Res.* 255:145-163.
- Sugahara K, Takemura Y, Sugiura M, Kohno Y, Yoshida K, Takeda K, Khoo K-H, Morris HR, Dell A. 1994b. Chondroitinase ABC-resistant sulfated trisaccharides isolated from digests of chondroitin/dermatan sulfate chains. *Carbohydr Res.*

255:165-182.

Sugahara K, Tanaka Y, Yamada S, Seno N, Kitagawa H, Haslam SM, Morris HR, Dell

A. 1996. Novel sulfated oligosaccharides containing 3-*O*-sulfated glucuronic acid from king crab cartilage chondroitin sulfate K: unexpected degradation by chondroitinase ABC. *J Biol Chem.* 271:26745-26754.

Sugahara K, Yamada S. 2000. Structure and function of oversulfated chondroitin sulfate variants: unique sulfation patterns and neuroregulatory activities. *Trends Glycosci Glycotechnol.* 12:321-349.

Uyama T, Kitagawa H, Sugahara K. 2007. Biosynthesis of glycosaminoglycans and proteoglycans. In: Kamerling, JP, editor. *Comprehensive Glycoscience* Volume 3. Amsterdam: Elsevier. p. 79-104.

van den Hoven MJ, Rops AL, Vlodavsky I, Levidiotis V, Berden JH, van der Vlag J. 2007. Heparanase in glomerular diseases. *Kidney Int.* 72:543-548.

Volpi N, Maccari F. 2003. Purification and characterization of hyaluronic acid from the mollusc bivalve *Mytilus galloprovincialis*. *Biochimie.* 85:619-625.

Yamada S, Van Die I, Van den Eijnden DH, Yokota A, Kitagawa H, Sugahara K. 1999. Demonstration of glycosaminoglycans in *Caenorhabditis elegans*. *FEBS Lett.* 459:327-331.

Yamada S, Morimoto H, Fujisawa T, Sugahara K. 2007. Glycosaminoglycans in *Hydra magnipapillata* (Hydrozoa, Cnidaria): demonstration of chondroitin in the developing nematocyst, the sting organelle, and structural characterization of glycosaminoglycans. *Glycobiology*. 17:886-894.

Zhang L, Bharadwaj AG, Casper A, Barkley J, Barycki JJ, Simpson MA. 2009. Hyaluronidase activity of human Hyal1 requires active site acidic and tyrosine residues. *J Biol Chem*. 284:9433-9442.

Figure legends

Fig. 1. Gel filtration HPLC analysis of the FITC-labeled GAG isoforms digested with HYAL4.

FITC-labeled HA (**A** and **B**), CS-A (**C** and **D**), CS-C (**E** and **F**), and CS-D (**G** and **H**) were analyzed by gel filtration HPLC on a column of Superdex 200 before (**A**, **C**, **E**, and **G**) and after (**B**, **D**, **F**, and **H**) incubation with the purified HYAL4 protein, and monitored by measuring the fluorescent intensity of FITC with excitation and emission wavelengths of 490 and 520 nm, respectively. V_0 , void volume; V_t , total volume.

Fig. 2. Comparison of the digestibility of various GAGs to CSHY and fractionation of the reaction products.

Ten micrograms each of commercial CS-D (**A**), CS-C (**B**), or CS-A (**C**) was incubated with CSHY under the same conditions, and each digest was derivatized with 2AB. An aliquot (750 ng) of each 2AB-derivatized digest was analyzed by gel filtration HPLC on a column of Superdex peptide. Peaks indicated by *arrowheads* in panel **A** were collected, digested with CSases, and analyzed by anion-exchange HPLC to identify the structure of the major component in each fraction. V_0 , void volume; V_t , total volume.

Fig. 3. Effects of pH on the hydrolytic activity of CSHY.

CS-D was incubated with purified CSHY in 50 mM phosphate (circles), acetate (squares), or citrate (triangles) buffers, pH 3.5-6.5, containing 150 mM NaCl. The digests were labeled with 2AB, digested with CSase AC-II, and then analyzed by anion-exchange HPLC on an amine-bound silica column. Degradation of CS-D was assessed by calculations based on the proportion (in percentage) of 2AB-labeled di- and tetrasaccharides formed as described under "Materials and methods".

Fig. 4. Lineweaver-Burk plots of the initial velocities obtained by enzymatic hydrolysis of varying concentrations of CS isoforms by CSHY.

CSHY was incubated with different concentrations of CS-A (**A**), CS-C (**B**), CS-D (**C**), or CS-E (**D**). After incubation, degradation products were labeled with 2AB, and digested with CSase AC-II. The resultant oligosaccharides were quantified by HPLC as described in "Materials and methods". The plots showed linearity and the reaction velocities were used for a kinetic analysis to determine apparent K_m and V_{max} values for CSHY.

Fig. 5. Identification of the degradation products of CS-D by anion-exchange HPLC.

Fractions D-1 (**A**), D-2 (**B**, **E**), and D-3 (**C**, **F**), isolated from the CSHY digest of CS-D (Fig. 2), were analyzed by anion-exchange HPLC after digestion with CSase AC-II (**A-C**). Fraction D-1' was digested with CSase ABC, and the digest was labeled with 2AB and analyzed by anion-exchange HPLC (**D**). Fractions D-2 and D-3 were also digested with CSase ABC, and the digests were labeled with 2AB and analyzed individually (**E** and **F**). The elution positions of the 2AB-derivatives of the authentic unsaturated tetrasaccharides (Sugahara et al. 1994a; Deepa et al. 2007) are indicated by arrows marked by letters in panel **C**: a, $\Delta\text{HexUA}(2\text{S})\text{-GalNAc}(6\text{S})\text{-GlcUA-GalNAc}(6\text{S})$; b, $\Delta\text{HexUA}(2\text{S})\text{-GalNAc}(6\text{S})\text{-GlcUA-GalNAc}(4\text{S})$; c, $\Delta\text{HexUA-GalNAc}(4\text{S})\text{-GlcUA}(2\text{S})\text{-GalNAc}(6\text{S})$; d, $\Delta\text{HexUA-GalNAc}(6\text{S})\text{-GlcUA}(2\text{S})\text{-GalNAc}(6\text{S})$. The peaks marked by asterisks at around 5 min in panels **D-F** were derived from the 2AB reagents. The numbered arrows and arrowheads in panel **F** indicate the elution positions of the 2AB-derivatives of authentic unsaturated and saturated disaccharides (Kinoshita and Sugahara 1999): 1, $\Delta\text{HexUA-GalNAc}$; 2, $\Delta\text{HexUA-GalNAc}(6\text{S})$; 3, $\Delta\text{HexUA-GalNAc}(4\text{S})$; 4, $\Delta\text{HexUA}(2\text{S})\text{-GalNAc}(6\text{S})$; 5, $\Delta\text{HexUA}(2\text{S})\text{-GalNAc}(4\text{S})$; 6, $\Delta\text{HexUA-GalNAc}(4\text{S},6\text{S})$; 7, $\Delta\text{HexUA}(2\text{S})\text{-GalNAc}(4\text{S},6\text{S})$; 8, $\text{GlcUA-GalNAc}(6\text{S})$; 9, $\text{GlcUA-GalNAc}(4\text{S})$.

Fig. 6. Analysis of the reducing terminal structure in the degradation products of CS-A and CS-C.

CS-A (**A**) and CS-C (**B**) were digested individually with CSHY. Each digest was derivatized with 2AB and analyzed after digestion with CSase AC-II by anion-exchange HPLC using an NaH_2PO_4 gradient (indicated by the dashed lines). The numbered arrows in panels **A** indicate the elution positions of the 2AB-derivatives of authentic disaccharides (see the legend to Fig. 5). The open arrow in panel **A** indicates the elution position of the 2AB-derivative of the unsaturated tetrasaccharide, $\Delta\text{HexUA-GalNAc(4S)-GlcUA(2S)-GalNAc(6S)}$ (Deepa et al. 2007).

Fig. 7. Structure of the site of cleavage by CSHY in CS.

(**A**) The arrow indicates the galactosaminidic linkage cleaved by CSHY. A sulfated structure appears to be critical for the hydrolytic action of the enzyme. The 6-*O*-sulfate group on the GalNAc residue on the nonreducing side of the cleavage site is essential, and 4-*O*- or 6-*O*-sulfation on the GalNAc residue on the reducing side of the cleavage site is important for recognition by the enzyme. The 2-*O*-sulfate group on the GlcUA residue on the nonreducing side indicated by an asterisk appears to have a promoting

effect on the hydrolytic action of CSHY. (B) A representative saccharide sequence in CS-D is shown. Disaccharide units are depicted as circles. A, C, and D in the circles stand for A unit, C unit, and D unit, respectively. The glycosidic bonds on the reducing side of D units are preferentially hydrolyzed by CSHY as indicated by black arrows, giving tetra-, hexa-, and octasaccharides as major degradation products.

Fig. 8. Comparison of CSHY with HYAL1 and *C. elegans* Chn hydrolase.

Multiple sequence alignment of CSHY (HYAL4) with HYAL1 and *C. elegans* Chn hydrolase (Chnase) is shown using the ClustalW multiple sequence alignment program (version 1.83) (<http://align.genome.jp/clustalw/>). Introduced gaps are shown by *hyphens*, and aligned identical residues are *boxed*. (*black* for all sequences and *dark gray* for two sequences). Asterisks indicate the amino acid residues, which are conserved strictly among the human hyaluronidases and supposed to be responsible for their hydrolytic activity (Stern and Jedrzejewski 2006). Note that Cys263 in HYAL4 reported by Csoka *et al.* (1999) (accession number: AF009010) was replaced by Gly263 in the sequence of HYAL4 gene cloned by us (accession number: AB470346) and Mammalian Gene Collection Program Team (accession numbers: BC104788 and BC104790) as well as in the human genomic DNA (accession number: NT_007933).

Table I. Kinetic parameters of the recombinant CSHY

Substrate	Apparent K_m	Apparent V_{max}
	mM as disaccharides	pmol/min
CS-D	0.17	7.0
CS-C	0.30	4.9
CS-A	0.46	5.3
CS-E	2.9	3.2

Table II. Composition of the isolated CS-D oligosaccharide fractions

Fraction	Di- or tetrasaccharide	Proportion (%) ^a
D-1' ^b	GlcUA-GalNAc(4S)	56 ± 0.4
	ΔHexUA(2S)-GalNAc(6S)	44 ± 0.4
D-2 ^c	GlcUA-GalNAc(4S)	20 ± 0.9
	GlcUA-GalNAc(6S)	22 ± 0.8
	ΔHexUA-GalNAc(4S)-GlcUA(2S)-GalNAc(6S)	58 ± 1.5
D-3 ^c	GlcUA-GalNAc(4S)	13 ± 0.5
	GlcUA-GalNAc(6S)	15 ± 0.2
	ΔHexUA-GalNAc(4S)	13 ± 1.4
	ΔHexUA-GalNAc(6S)	14 ± 0.2
	ΔHexUA(2S)-GalNAc(6S)	7 ± 1.1
	ΔHexUA-GalNAc(4S)-GlcUA(2S)-GalNAc(6S)	38 ± 2.0

^aThe values are expressed as the mean ± SD for three independent experiments

^bThe unlabeled tetrasaccharide fraction D-1' was digested with CSase ABC and the digest was labeled with 2AB and analyzed by anion-exchange HPLC.

^cThe 2AB-derivatives of the hexasaccharide fraction D-2 and the octasaccharide fraction D-3 were also digested individually with CSase ABC, and each digest was labeled with 2AB and analyzed by anion-exchange HPLC.

Table III. Structure of the major components in the digests of CS obtained with CSHY

Fraction	Substrate	Structure of the products	
Tetrasaccharide (D-1)	CS-D	GlcUA-GalNAc(4S)-GlcUA(2S)-GalNAc(6S)	
Hexasaccharides (D-2)	CS-D	GlcUA-GalNAc(4S)-GlcUA-GalNAc(4S)-GlcUA(2S)-GalNAc(6S) (47%) GlcUA-GalNAc(6S)-GlcUA-GalNAc(4S)-GlcUA(2S)-GalNAc(6S) (53%)	
Octasaccharides (D-3)	CS-D	GlcUA-GalNAc(4S)- GlcUA-GalNAc(6S)- GlcUA-GalNAc(6S)- { { } GlcUA-GalNAc(4S)- GlcUA-GalNAc(6S)- GlcUA(2S)-GalNAc(6S)- } } } GlcUA-GalNAc(4S)-GlcUA(2S)-GalNAc(6S)	
Reducing terminus ^a	CS-A	-GlcUA-GalNAc(6S) (100%)	
Reducing termini ^a	CS-C	-GlcUA-GalNAc(6S) (55%) -GlcUA-GalNAc(4S)-GlcUA(2S)-GalNAc(6S) (45%)	

^aThe reducing terminal structure of the products of the digestion of CS-A and CS-C by CSHY is shown.

Fig. 1

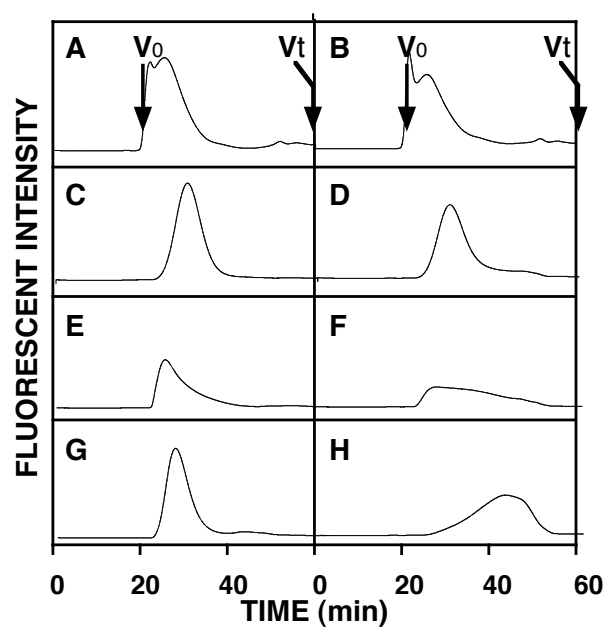


Fig. 2

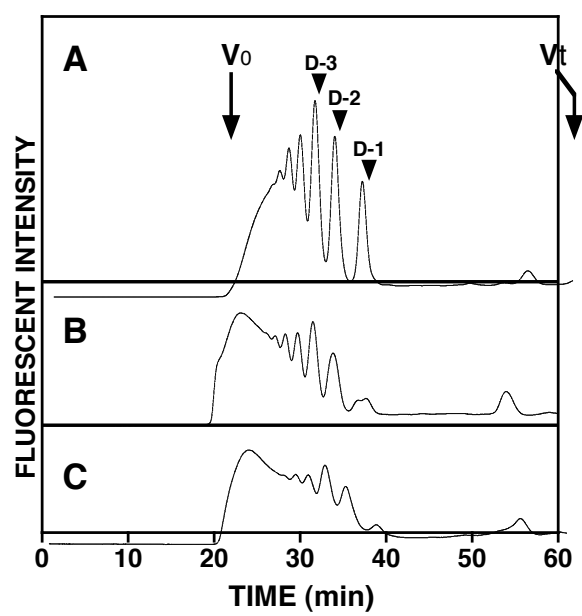
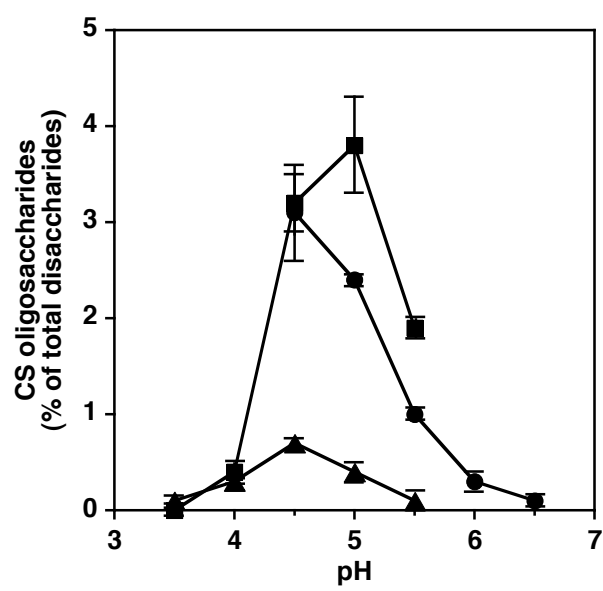


Fig. 3



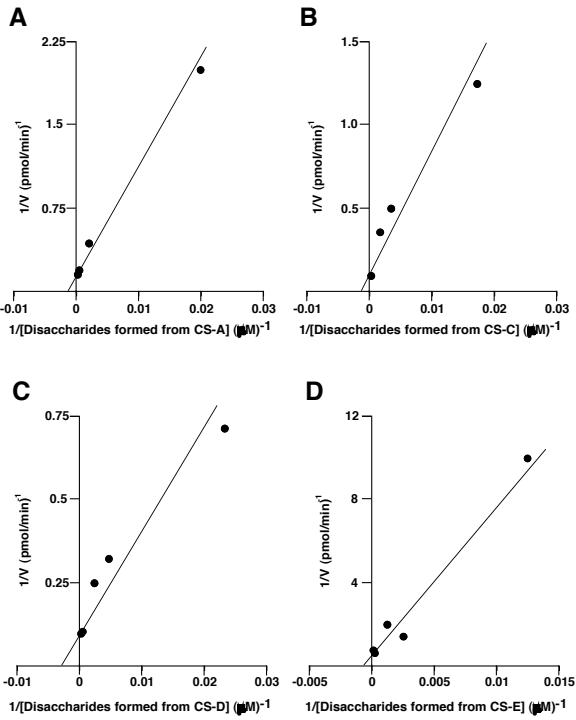


Fig. 4

Fig. 5

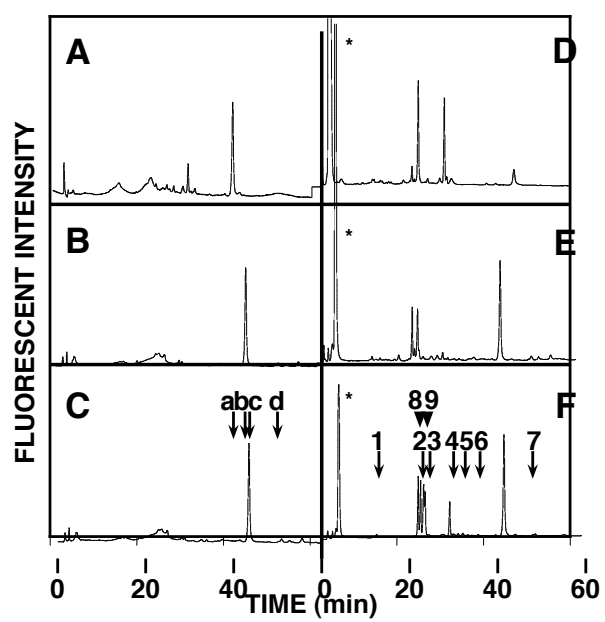
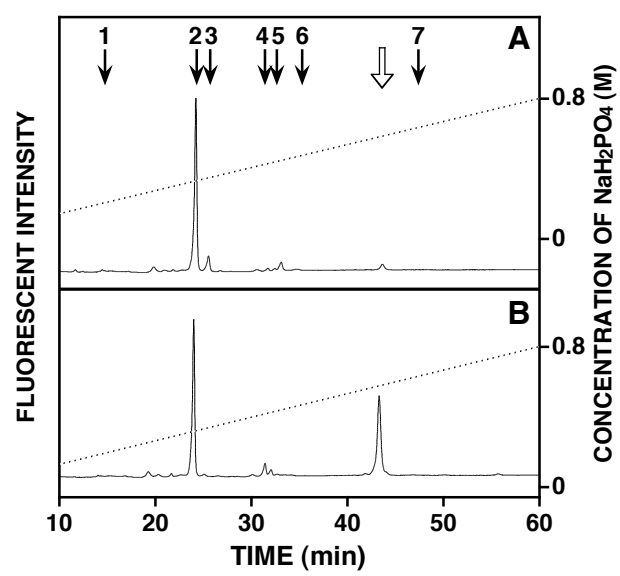
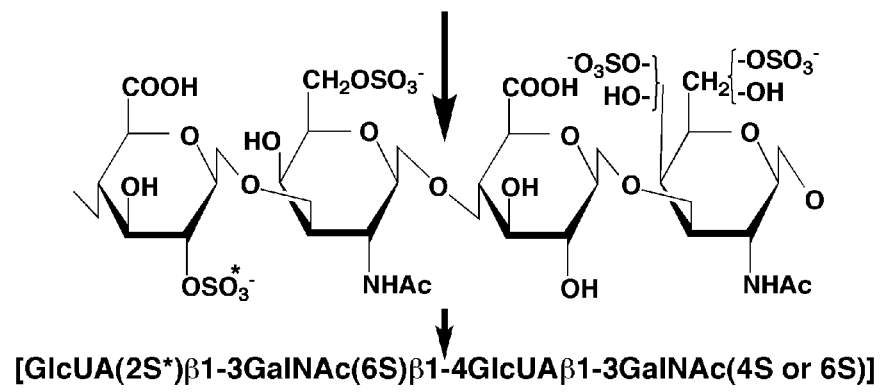
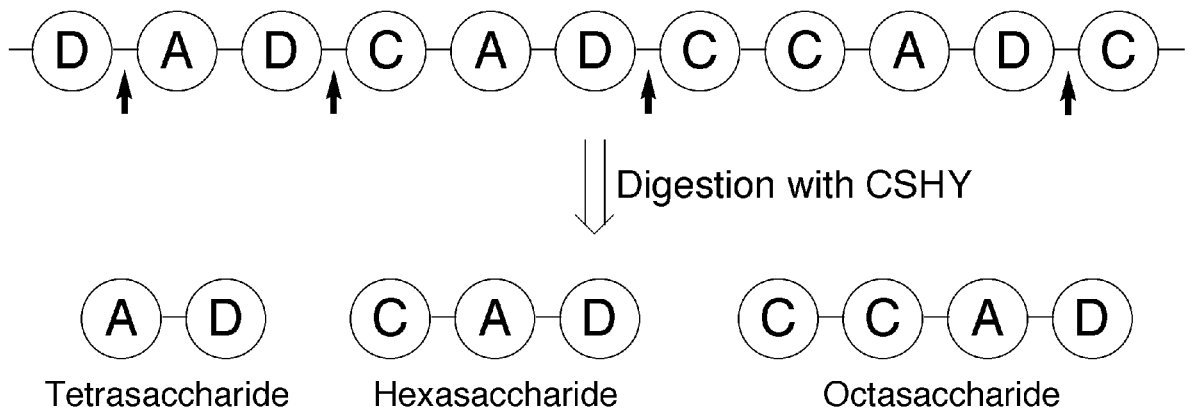


Fig. 6



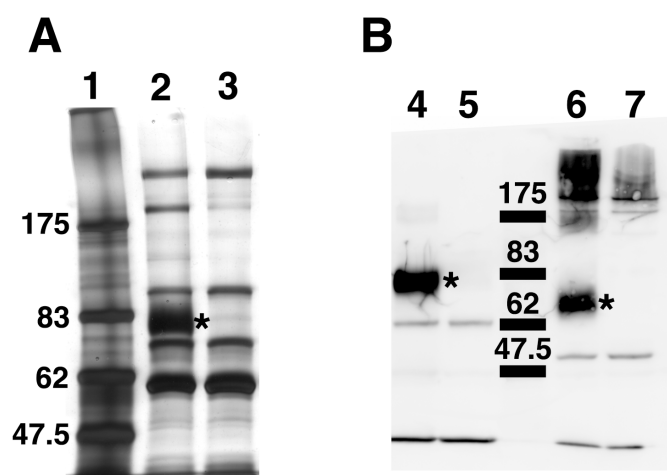
A**B**

HYAL1	1	-----MAAHLLPICALFLTLDDMAQG--FRGPLEPNRPFTTVVNANTQWCLERHGVDDVSVFDVVANPGQTFRC	74
CSHY (HYAL4)	1	MKVLSEGQLKLCVVQPVHLTSWLLIFFILKSISCLKPARLPYQRPFIAAWNAPTQCLIKYNLRNLNLMFPVIGSPLAKARGQNV	90
Chnase	1	-----MVIVVYHQLLLVLLIFIGAAKGAQYIGSGASQPNR-TDVVMVPSWTCCKNEYSIDVEKYGILQNEQD-HFVCGKQFALF	77
HYAL1	75	YSSQLGTYPPYYTPTG--EPVFGGLPQNASTIAHARTFQDILAAIPAPDFSGLAVIDWEAWRPRWAFNWDTKDIYRQSRALVQAQHPDW	162
CSHY (HYAL4)	91	YVNRLGYYPWYTSQG--VPINGGLPQNISLQVHLEKADQDINYYIPAEDFSGLAVIDWEYWRPQWARNWNSKDYYRQKSRKLISDMGKNV	178
Chnase	78	YEHSFKIPYFKAQNESDPKNGGLEPQMGDLEAHLIQAEKDINETIPDENFNCAVILIEEFRPMWELSWGPFVYKTESIRLTRQQHPYW	167
HYAL1	163	PAPQVEAVAQDQFQGAARAWMAGTLQLGRALRPRGLWCFYGFEDCYNDFLSPNYTGCCPSGIRACNDOLGWLWCQSRALYPSIYMPAVL	252
CSHY (HYAL4)	179	SATDIEYLAKVTFEESAKAFMKETIKLGLKSRPKGLWGYLYPDCHNYNVYAPNYSGSCPEDEVLRNNELSWLWNSSAALYPSIGVWKS	268
Chnase	168	STKQIEWQAERDYEKACQKFFIETLRLGKRLRPNAKWGYLFEKCN--GDVGQKSDTDCSTLFQKFNDNLHHLWGESTALFSPSTIYLYPSQ	255
HYAL1	253	E-GTGKSQMYVQHRVAAFRVAVAAG-DPNLPVLPYVQIFY-DTTHFLPLDELEHSLGESAAQCAAGVVLWVSWENTRTKESCQAIKEY	339
CSHY (HYAL4)	269	G-DSENILRFKFRVHESMRISTMTSHDYALPFEVYTRLGYRDEPLFSLSKQDLVSTIGESAALCAAGIVIWGDMNLTASKANCTKVQF	357
Chnase	256	KQNPENFVNSGALITETKRIKRNYPSCIEHVEFKIEYNPYTPDDFYSKQNLASTLDAIKMNAANSVVIWTSQSIG--SRCGSLQTY	343
HYAL1	340	MDTTLGPFILNVTSGALLCSQALCSGHGRCVRRRTSHPKALLLNPAFSFIQLTPGGGPLSLRGALSLEDQAQMAVEFKRCYPGWQAPWC	429
CSHY (HYAL4)	358	VSSDLGSYIANVTRAAEVCSLHLCRNNGRCIRKMWNAPSYLHLNPASYHIEASEDG-EFTVKGKASDSDLAVMADTFSCHCYQGYEGADC	446
Chnase	344	VDNTLGPYLQLTDRNLDKCRMERCEGRCECYLPRPKTNPAIYNFACRCERPFGKSCYGRGRMGVSMPKASQTPQVIPDVTAYFSTSSN	433
HYAL1	430	ERKSMW-----	435
CSHY (HYAL4)	447	REIKTADGCGSVSPSPGSLMTLCLLLASYRSIQL	481
Chnase	434	GTKKYNAPNQFYSTRGGDIKLARKL-----	458

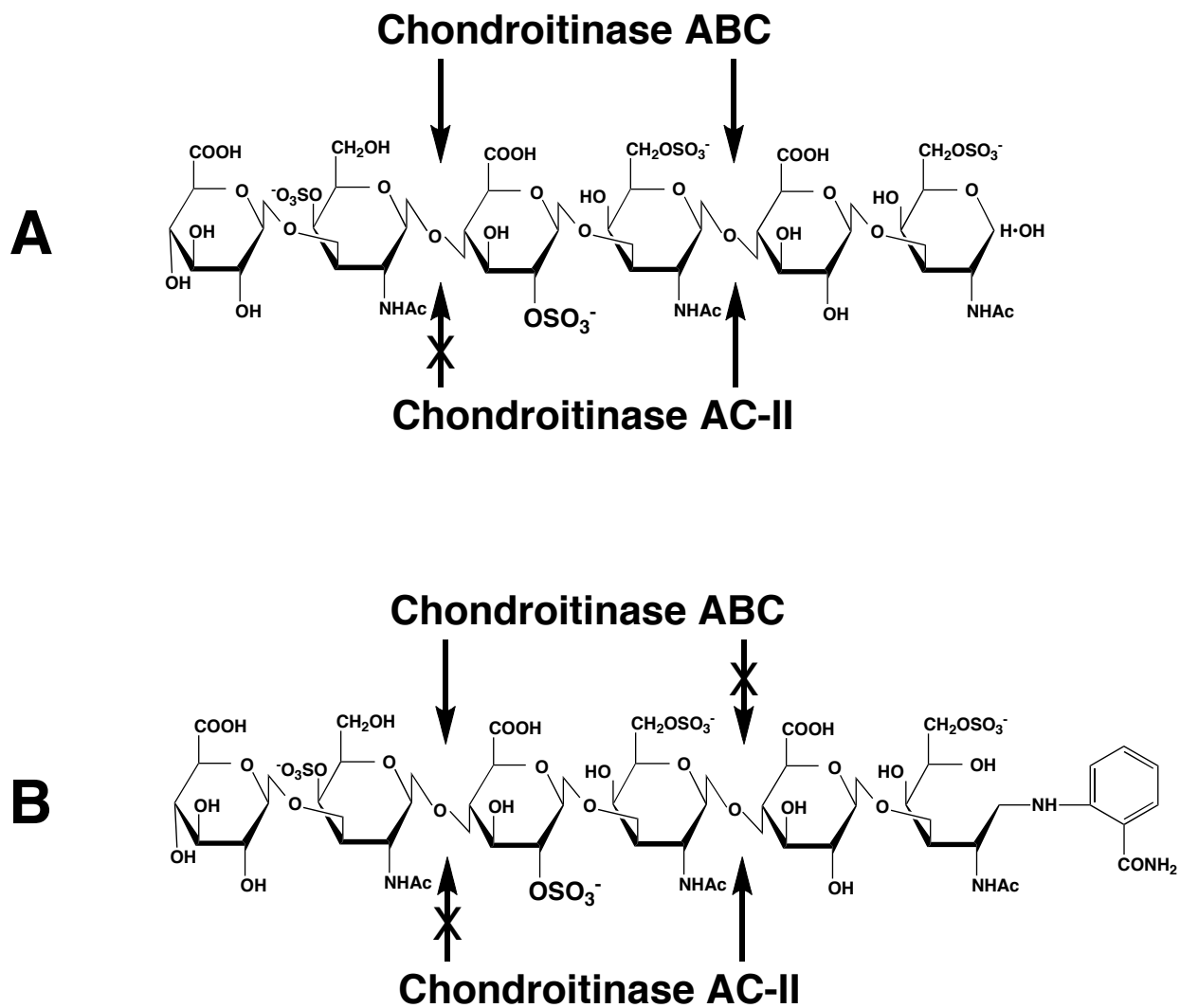
SUPPLEMENTARY DATA

Supplementary Figure 1. Expression of CSHY in COS-7 cells. The culture medium of COS-7 cells transfected with *HYAL4* (lanes 2, 4, and 6) or mock-transfected cells (lanes 3, 5, and 7) was purified with ANTI-FLAG M2 affinity gel. The resin was subjected to SDS-PAGE under reducing (panel **A** and lanes 4 and 5 in panel **B**) and nonreducing (lanes 6 and 7 in panel **B**) conditions and analyzed by silver staining (panel **A**) as well as western blotting (panel **B**) with the anti-FLAG antibody. The apparent molecular weights of the protein standards (lane 1) are indicated. Bands indicated by asterisks were not observed in the mock-transfected samples, suggesting that they are the protein bands of CSHY. Bands observed at around 60 and 30 kDa correspond to those of immunoglobulin heavy and light chains, respectively.

Supplementary Figure 2. Substrate specificity of bacterial CSase ABC and AC-II. CSase ABC cleaves all the *N*-acetylgalactosaminidic linkages in CS chains to yield disaccharides (**A**). However, when the reducing terminal GalNAc residue is derivatized with 2AB, it does not act on the linkage adjacent to the 2AB-labeled disaccharide unit, giving rise to 2AB-tetrasaccharide (**B**). In contrast, CSase AC-II can act on the reducing terminal *N*-acetylgalactosaminidic linkages in CS chains even if they are 2AB-derivatized (**B**). However, 2-*O*-sulfation (shown in larger font size) renders the GalNAc-GlcUA(2S) linkage resistant to the enzyme (**A**, **B**).



Supplementary Figure 1



Supplementary Figure 2