Review

Synthesis of neoproteoglycans using the transglycosylation reaction as a reverse reaction of endo-glycosidases

By Masahiko ENDO[*1,†] and Ikuko KAKIZAKI[*2]

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Abstract: A method for the synthesis of carbohydrate chains (glycosaminoglycans) and their coupling to peptides was investigated using proteoglycans. Glycosidases generally catalyze a hydrolytic reaction, but can also mediate the reverse reaction, which in this case is a transglycosylation. In the transglycosylation reaction of bovine testicular hyaluronidase, which is an endoglycosidase, glycosaminoglycans (hyaluronan and chondroitin sulfates) release disaccharide (uronic acid-*N*-acetylhexosamine) moieties from non-reducing terminal sites, and then the liberated disaccharides are transferred immediately to the non-reducing termini of other glycosaminoglycan chains. Using such continuous reactions, it is possible to synthesize glycosaminoglycan chains according to a specific design. It then becomes possible to transfer glycosaminoglycan chains synthesized on a peptide to other peptides using the transglycosylation reaction of endo-*β*-xylosidase acting on the linkage region between a peptide and glycosaminoglycan chains of proteoglycans. We believe this approach will open a new field for the synthesis of homogeneous proteoglycans or their corresponding analogues.

Keywords: neoproteoglycan, glycosaminoglycan, hyaluronidase, endo-*β*-xylosidase, transglycosylation, sugar chain reconstruction

Introduction

The proteoglycans, which are members of the glycoconjugate family of molecules, are found in the extracellular matrix and on the surface of many cell types.[1)] The proteoglycans are composed of a core protein (with a molecular weight of 10 k to 400 k) and one or more covalently bound glycosaminoglycans (GAGs). GAGs are linear polysaccharides made up of hundreds of repeating disaccharide units, which are composed of an amino sugar (*N*-acetylglucosamine, GlcNAc, *N*-acetylgalactosamine, GalNAc or glucosamine) and uronic acid (glucuronic acid, GlcUA or L-iduronic acid, IdoUA) or galactose (Gal, only keratan sulfate). The GAG chains are generally covalently linked *via* an *O*-glycosidic bond to a serine alcohol of the core peptide of proteoglycan through a linkage region, GlcUA*β*1-3Gal*β*1-3Gal*β*1-4Xyl*β*1-Ser (GlcUA-Gal-Gal-Xyl-Ser) (Fig. 1).

The fine structure of GAG chains can have considerable diversity due to various patterns of modification, such as epimerization[2)] and sulfation.[2)] GAG chains display many biological functions, which can be ascribed to the domain structures of only a few oligosaccharides, such as inhibition activity against blood coagulation.[1)]

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Abbreviations: GAG: glycosaminoglycan; HA: hyaluronan; Ch: chondroitin; ChS: chondroitin sulfate; Ch4S: chondroitin 4-sulfate; Ch6S: chondroitin 6-sulfate; ChSE: chondroitin sulfate E; DS: dermatan sulfate; Xyl: D-xylose; Gal: D-galactose; GlcUA: D-glucuronic acid; IdoUA: L-iduronic acid; GlcNAc: *N*-acetyl-D-glucosamine; GalNAc: *N*-acetyl-D-galactosamine; GlcUA-GlcNAc: -4GlcUA*β*1-3GlcNAc*β*1-; GlcUA-GalNAc: -4GlcUA*β*1-3GalNAc*β*1-; GlcUA-GalNAc4S: -4GlcUA*β*1-3GalNAc(-4-sulfate)*β*1-; GlcUA-GalNAc6S: -4GlcUA*β*1-3GalNAc(-6-sulfate)*β*1-; GlcUA-GalNAc4S,6S: -4GlcUA*β*1-3GalNAc(-4,6-disulfates)*β*1-; IdoUA-GalNAc4S: -4IdoUA*α*1-3GalNAc(-4-sulfate)*β*1-; GlcUA-Gal-Gal-Xyl-Ser: GlcUA*β*1-3Gal*β*1-3Gal*β*1-4Xyl*β*1-Ser. PA, 2-aminopyridine; MU: 4-methylumbelliferone; Boc: *t*-butyloxycarbon; MCA: 4-methylcoumaryl-7-amide; HPLC: high performance liquid chromatography.

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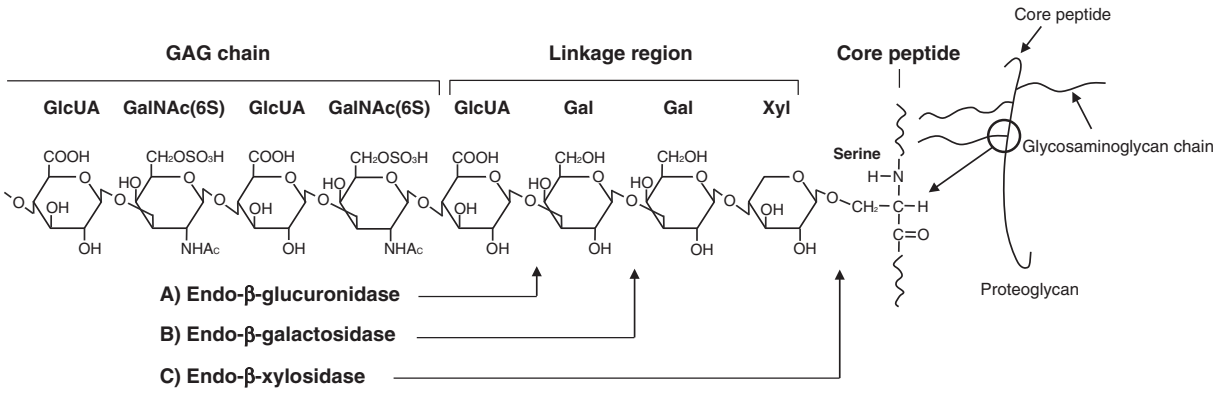

Fig. 1. Chemical structure of the linkage region between a core peptide and a GAG chain of a proteoglycan and the action of endoglycosidases on the linkage region.
GlcUA, glucuronic acid; GalNAc, *N*-acetylgalactosamine; Gal, galactose; Xyl, xylose. A) Takagaki, K. *et al.* (1988) J. Biol. Chem., **263**, 7000; B) Takagaki, K. *et al.* (1990) J. Biol. Chem., **265**, 854; C) Takagaki, K. *et al.* (1992) J. Biol. Chem., **267**, 18558.

Recombinant proteins produced by genetic engineering sometimes have reduced biological activity *in vivo* caused by incomplete or missing carbohydrate chains.[3),4)] This is because the engineered DNA includes no direct information concerning the biosynthesis of the carbohydrate chains. Therefore, it is very difficult to regulate the linkage and elongation of the carbohydrate chains of recombinant proteins encoded by gene technology. Consequently, glycotechnology, which is concerned with the synthesis of carbohydrate chains having a physiological function, and coupling of the synthesized carbohydrate chain to the recombinant protein, is required to resolve the problem.

The synthesis of GAG chains of proteoglycans using standard organic chemistry is problematic because the severe conditions necessary for the synthetic route tend to denature the peptide. The selective chemical synthesis of an α,β-configuration with the desired glycosidic position is technically very difficult to achieve. Moreover, chemical routes to GAG chains are currently time consuming and prohibitively expensive. However, enzymatic synthesis of carbohydrate chains by means of glycosyltransferases is also difficult because of a lack of suitable enzymes.

It is known that glycosidases, which are hydrolytic enzymes, can mediate the reverse reaction (*i.e.*, transglycosylation) under suitable conditions.[5),6)] We reasoned that by exploiting the transglycosylation activity of glycosidases it may be feasible to both synthesize a new GAG chain as well as couple it to a suitable peptide. Therefore, we have employed glycotechnology for the synthesis of neoproteoglycans with long GAG chains by using glycosidases, especially endo-glycosidases.

Testicular hyaluronidase (hyaluronidase), which acts on hyaluronan (HA) and chondroitin sulfates (ChSs), is one such endo-glycosidase. The hydrolysis of GAGs releases oligosaccharides, which are then transferred to the non-reducing termini of other GAG chains.[7)–10)]

Using the transglycosylation activity of hyaluronidase, it has been confirmed that both natural and unnatural GAG chains can be synthesized. Examples include hybrid chains of HA and ChSs that have not been reported previously. It is also known that a GAG chain can be coupled directly to a peptide using an endo-β-xylosidase that acts on the linkage region between the core peptide and the GAG chain of a proteoglycan (Fig. 1). It is likely that transglycosylation mediated by endo-glycosidases together with their subsequent coupling to a desired peptide core will open up a new field of glycotechnology for engineering GAGs.[11)] Using neoproteoglycans, some physiological functions of proteoglycans will be clarified. Here, we review the synthesis of novel proteoglycans using the transglycosylation reaction as a reverse reaction hydrolysis of endo-glycosidases.

I Reconstruction of a GAG chain using hyaluronidase

I-1. Enzymatic reaction of hyaluronidase. Bovine testicular hyaluronidase (EC3.2.1.35, hyaluronidase) is an endo-β-*N*-acetyl-D-hexosaminidase that hydrolyzes the internal β-*N*-acetyl-D-hexosami-

nide bond contained in HA and ChSs. The hyaluronidase used in these studies was from a commercial source (bovine testicular hyaluronidase, type I), and was further purified.[12] HA ($M_r = 800{,}000$ and 80,000) derived from human umbilical cord was purified by gel filtration chromatography.[13] When HA is digested with hyaluronidase, the final reaction products are found to contain mainly tetrasaccharides and hexasaccharides, which have *N*-acetylglucosamine at the reducing termini and glucuronic acid at the non-reducing termini.

Hyaluronidase can catalyze both the hydrolytic reaction and a transglycosylation as a reverse reaction.[7)–10)] Hexasaccharide, which is one of the hydrolytic reaction products, is the smallest substrate susceptible to this enzyme. Using the transglycosylation reaction, it is possible to synthesize and reconstruct GAG chains. Using the transglycosylation reaction, it is possible to synthesize and reconstruct GAG chains.

1) Hydrolysis and transglycosylation reaction of hyaluronidase. Hydrolysis reactions of HA and ChSs with hyaluronidase were carried out mainly in 0.1 M sodium acetate buffer (pH 5.0) containing 0.15 M NaCl.[14)–16)] However, a typical transglycosylation reaction with hyaluronidase was carried out in 0.1 M Tris-HCl buffer (pH 7.0) (NaCl free), using PA-hexasaccharides as acceptors and polysaccharides as donors by using combinations of GAGs (HA, chondroitin (Ch), chondroitin 4-sulfate (Ch4S) and chondroitin 6-sulfate (Ch6S), and desulfated dermatan sulfate (DS)). The reaction mixtures were then assayed by HPLC and mass spectrometry.

Structural analyses of the newly synthesized GAGs and their oligosaccharides were performed by ion-spray mass spectrometry[17] and HPLC. These techniques were used directly or after digestion with structural specific enzymes. Labeling the reducing termini of GAG chains with a fluorogenic reagent, 2-aminopyridine (PA)[18] makes it possible to distinguish the reducing terminus from the non-reducing terminus of GAG chains by HPLC analysis. This is because the transglycosylation reaction can proceed at the non-reducing terminus but not at the reducing terminus, which is blocked by PA.

I-2. Principle of the transglycosylation reaction of hyaluronidase. *1) Substrate specificity of the transglycosylation reaction.* To study the substrate specificity of the transglycosylation reaction of hyaluronidase,[14)–16)] HA-oligosaccharides (from di- to dodecasaccharide), which were either unmodified or PA-labeled at the reducing termini,

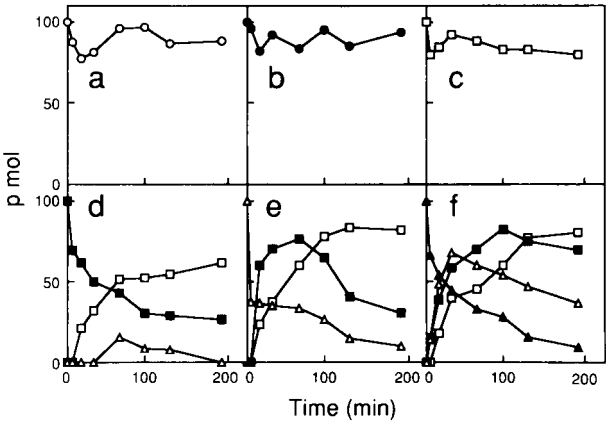

Fig. 2. Time course of hyaluronidase digestion of PA-oligosaccharides.
PA-di- (a), PA-tetra- (b), PA-hexa- (c), PA-octa- (d), PA-deca- (e) and PA-dodecasaccharide of HA (f) were incubated with hyaluronidase, and then aliquots were analyzed by mass spectrometry. Only PA-containing oligosaccharides were selectively detected: (○) PA-di-, (●) PA-tetra-, (□) PA-hexa-, (■) PA-octa, (△) PA-deca- and (▲) PA-dodecasaccharide. [Reprinted with permission from Takagaki, K. *et al.* (1994) Biochemistry **33**, 6503.]

were incubated with hyaluronidase under optimal hydrolytic conditions. The reaction was then continuously monitored by mass spectrometry. The unmodified hexasaccharides were hydrolyzed to disaccharides and tetrasaccharides. A hexasaccharide was the minimum GAG chain length that was susceptible to hydrolysis by hyaluronidase. PA-di- and tetrasaccharides were not subject to hydrolysis or elongation. Unlike the unmodified hexasaccharides, PA-hexasaccharides were not hydrolyzed, because their reducing termini were blocked with PA (Fig. 2, c), but were susceptible to elongation. PA-hexasaccharide is the minimum GAG chain length for the transglycosylation reaction mediated by hyaluronidase. Consequently, PA-hexasaccharides were used as acceptors for the transglycosylation reaction of hyaluronidase in all further experiments.

Upon incubation of PA-octasaccharide with hyaluronidase, PA-hexasaccharides were produced initially along with PA-decasaccharides (Fig. 2, d). These results suggested that hyaluronidase hydrolyzed the sequential release of disaccharide (GlcUAβ1-3GlcNAc, (GlcUA-GlcNAc)) units from the non-reducing terminus of a donor polysaccharide. However, the amount of disaccharide in the reaction mixture was less than anticipated from the simple hydrolysis reaction. Rather, the liberated disaccha-

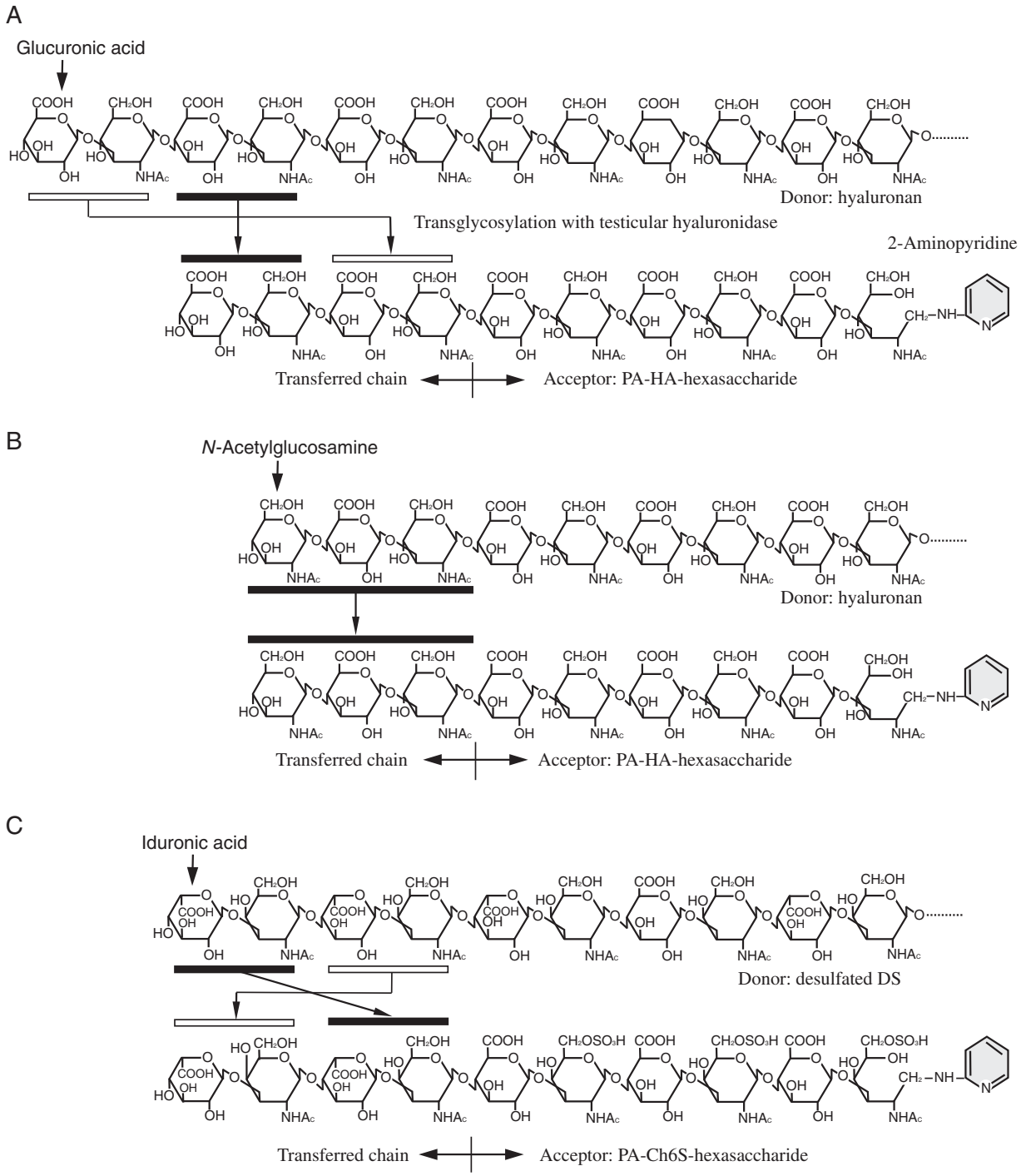

Fig. 3. Schematic diagrams showing the mechanisms of hyaluronidase transglycosylation.
Incubations were performed using PA-GAG-oligosaccharides as acceptors and GAGs as donors. A: PA-HA-hexasacccharide as an acceptor and HA as a donor with a glucuronic acid residue at the non-reducing terminus. B: PA-HA-hexasaccharide as an acceptor and HA as a donor with an *N*-acetylglucosamine residue at the non-reducing terminus. C: PA-Ch6S-hexasaccharide as an acceptor and desulfated DS as a donor with an L-iduronic acid residue at the non-reducing terminus.

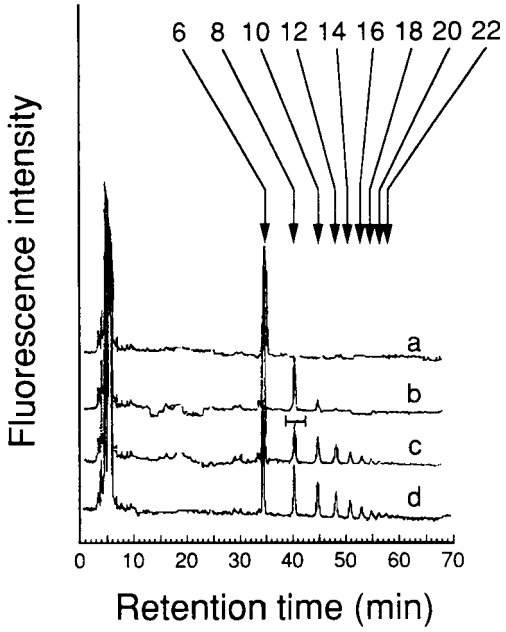

Fig. 4. Time course of PA-HA-oligosaccharide production using the transglycosylation reaction of hyaluronidase.
PA-HA-hexasaccharide as an acceptor and HA as a donor were incubated with hyaluronidase at 37°C for 0 (a), 15 (b), 60 (c) and 90 min (d) in Tris-HCl buffer, pH 7.0, and then subjected to HPLC (PALPAK Type S). Arrows indicate the elution positions of PA-HA-oligosaccharide standards. [Reprinted with permission from Saitoh, H. *et al.* (1995) J. Biol. Chem. **270**, 3741.]

rides appeared to be simultaneously transferred to the non-reducing termini of other oligosaccharides (Fig. 3, A). Under optimal transglycosylation conditions (0.1 M Tris-HCl buffer, pH 7.0 (NaCl free), at 37°C for 1 h), the disaccharide units were sequentially transferred from the GAG chains to the non-reducing terminus of the acceptor, PA-hexasaccharide and subsequently elongated up to docosasaccharide within 1 h (Fig. 4). For the transglycosylation reaction, it was more efficient to use longer-chain oligosaccharides as donors and shorter-chain oligosaccharides as acceptors.

The transferred HA-disaccharide structures were investigated by mass spectrometry and enzymatic digestion using chondroitinase AC-II, which acts on the structure of GlcNAcβ1-4GlcUA. These analyses revealed that the new glycosyl bond between the *N*-acetylglucosamine transferred from the donor and glucuronic acid at the reducing terminus of the acceptor was the same as the original bond *i.e.*, GlcNAcβ1-4GlcUA. The transglycosylation reaction of hyaluronidase thus restored the original form of bonding.

2) Efficiencies of transfer of GAG combinations. The efficiencies of various combinations of HA (having a GlcUA-GlcNAc unit at the non-reducing terminus), Ch (-4GlcUAβ1-3GalNAcβ1-, (GlcUA-GalNAc)), Ch4S (-4GlcUAβ1-3GalNAc(-4-sulfate)β1-, (GlcUA-GalNAc4S)), Ch6S (-4GlcUAβ1-3GalNAc(-6-sulfate)β1-, (GlcUA-GalNAc6S)), DS (-4IdoUAα1-3GalNAc(-4-sulfate)β1-, (IdoUA-GalNAc4S)), as donors and acceptors were investigated.[15] Donor efficiency increased in the order of HA > Ch > Ch4S > Ch6S, whereas acceptor efficiency increased in the order of HA > Ch > Ch4S > Ch6S. However, DS could not act as either a donor or acceptor in the reaction. These findings showed that combinations of HA (glucosaminoglycan) and Ch, Ch4S and Ch6S (galactosaminoglycans) led to the formation of novel hybrid GAG chains.

3) Transfer involving various carbohydrate structures at the non-reducing terminus of the acceptor. PA-HA-oligosaccharides (penta- and heptasaccharides) having an *N*-acetylglucosamine at the non-reducing terminus after digestion with β-glucuronidase, were investigated as potential donors for transglycosylation catalyzed by hyaluronidase.[14] Pentasaccharides remained unchanged, while heptasaccharides were hydrolyzed to the tri- and tetrasaccharides, GlcNAcβ1-4GlcUAβ1-3GlcNAc and (GlcUAβ1-3GlcNAc-)$_2$, respectively. Trisaccharides were released from the non-reducing terminus, and then transferred to the non-reducing terminus of other oligosaccharides (Fig. 3, B). However, no additional transfer of disaccharides occurred as the non-reducing terminus bearing *N*-acetylglucosamine.

DS-oligosaccharide, IdoUA-GalNAc4S, bearing an L-iduronic acid at the non-reducing terminus was investigated as a potential donor for hyaluronidase catalyzing transglycosylation. However, no disaccharides from DS were transferred.[19] DS-oligosaccharides, IdoUA-GalNAc, desulfated by methanolysis were transferred to other oligosaccharides as donors, and the resulting oligosaccharides bearing IdoUA-GalNAc at the non-reducing terminus were also useful as acceptors (Fig. 3, C).

Oligosaccharides bearing a disulfated disaccharide unit, -4GlcUAβ1-3GalNAc(-4,6-disulfates) β1-, (GlcUA-GalNAc4S,6S) at the non-reducing terminus, obtained from disulfated ChSE by enzyme digestion, were not transferred to the non-reducing termini of other oligosaccharides. However, the oligosaccharides were effective as acceptors for the transglycosylation reaction[20] (Fig. 5(B) and (C)).

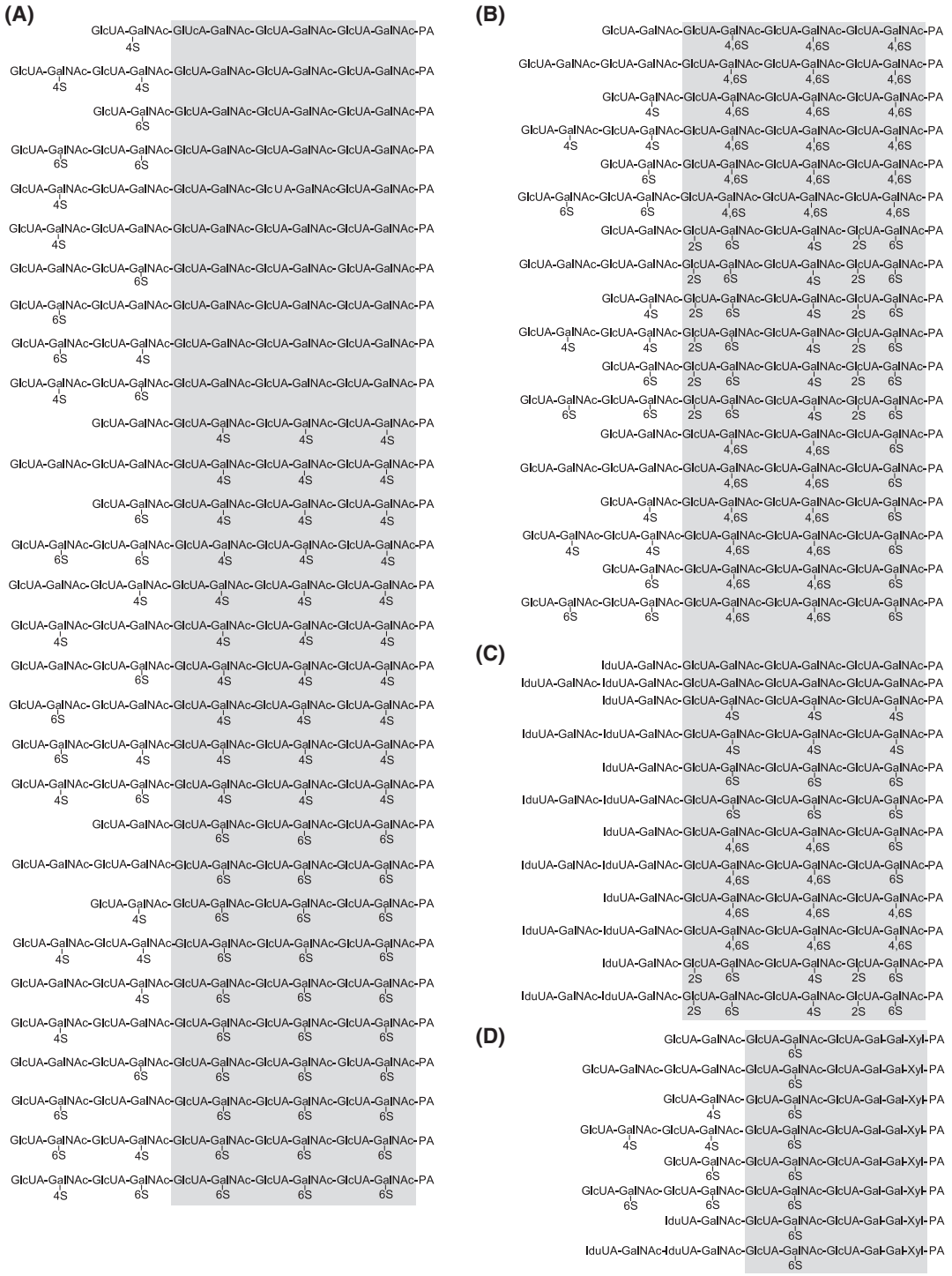

Fig. 5. Library of GAG-oligosaccharides synthesized using PA-GAG-hexasaccharides as acceptors with the transglycosylation of hyaluronidase.

(A), Nonsulfated and monosulfated disaccharide units; (B), polysulfated disaccharide units; (C), L-iduronic acid containing disaccharide units; (D), linkage region containing disaccharide units. The transglycosylation reaction with hyaluronidase was carried out in 0.1 M Tris-HCl buffer (pH 7.0) (NaCl free) using PA-hexasaccharide as an acceptor and each polysaccharides as donor. The shaded area shows PA-hexasaccharides used as acceptors. Lists of other synthesized oligosaccharides are shown in Tables 1–4. [Reprinted with permission from Takagaki, K. *et al.* (2000) Trends Glycosci. Glycotechnol. **12**, 303.]

Table 1. Sensitivity of oligosaccharides to hydrolysis by hyaluronidase SD

Number	Structures	Digestibility with hyaluronidase SD
1	GlcUA-GalNAc-PA	-
2	GlcUA-GalNAc-GlcUA-GalNAc-PA	-
3	GlcUA-GalNAc-GlcUA-GalNAc-GlcUA-GalNAc-PA	+
4	GlcUA-GalNAc-GlcUA-GalNAc-GlcUA-GalNAc-GlcUA-GalNAc-PA	+
5	GlcUA-GalNAc(4S)-GlcUA-GalNAc(4S)-GlcUA-GalNAc(4S)-PA	-
6	GlcUA-GalNAc(6S)-GlcUA-GalNAc(6S)-GlcUA-GalNAc(6S)-PA	-
7	GlcUA-GalNAc-GlcUA-GalNAc(4S)-GlcUA-GalNAc(4S)-GlcUA-GalNAc(4S)-PA	+
8	GlcUA-GalNAc-GlcUA-GalNAc-GlcUA-GalNAc(4S)-GlcUA-GalNAc(4S)-GlcUA-GalNAc(4S)-PA	+
9	GlcUA-GalNAc-GlcUA-GalNAc(6S)-GlcUA-GalNAc(6S)-GlcUA-GalNAc(6S)-PA	+
10	GlcUA-GalNAc-GlcUA-GalNAc-GlcUA-GalNAc(6S)-GlcUA-GalNAc(6S)-GlcUA-GalNAc(6S)-PA	+
11	GlcUA-GalNAc(4S)-GlcUA-GalNAc(4S)-GlcUA-GalNAc-GlcUA-GalNAc-GlcUA-GalNAc-PA	+
12	GlcUA-GalNAc(6S)-GlcUA-GalNAc(6S)-GlcUA-GalNAc-GlcUA-GalNAc-GlcUA-GalNAc-PA	+
13	GlcUA-GalNAc(4S)-GlcUA-GalNAc-GlcUA-GalNAc(6S)-GlcUA-GalNAc(6S)-GlcUA-GalNAc(6S)-PA	+

The arrows indicate the glycosidic linkages cleaved by hyaluronidase SD.
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4) Library of GAG oligosaccharides synthesized using the transglycosylation reaction of hyaluronidase. Galactosaminoglycan oligosaccharides from Ch, Ch4S, Ch6S, desulfated DS and ChS E were prepared as donors.[21),22)] PA-hexasaccharides and PA-linkage region hexasaccharides, GlcUA-GalNAc6S-GlcUA-Gal-Gal-Xyl-PA, for the linkage region between a core peptide and a ChS chain of proteoglycans were prepared as acceptors. A library, including many types of GAG-oligosaccharides synthesized by the transglycosylation reaction of hyaluronidase, was finally obtained using various combinations of these donors and acceptors (Fig. 5). It was also possible to remove PA chemically from all of the PA-oligosaccharides and to restore their original structures before pyridylamination.[23)]

I-3. Applications of GAG-oligosaccharides synthesized using hyaluronidase. *1) Examination of the substrate specificity of hyaluronidase SD.* Hyaluronidase SD (from *Streptococcus dysgalactiae*) is an eliminase, which catalyzes the eliminating cleavage of the β1-3 glycosidic bond between *N*-acetylglucosamine and glucuronic acid residues of HA.[24)] However, the precise details of the cleavage of ChS are lacking because of the complicated structures. Hybrid GAG-oligosaccharides with various combinations of disaccharide units (*i.e.*, GlcUA-GalNAc from Ch, GlcUA-GalNAc4S from Ch4S and GlcUA-GalNAc6S from Ch6S) were synthesized using the transglycosylation reaction of hyaluronidase, as model substrates (Tables 1 and 2).

Seven substrate oligosaccharides (Nos. 1–6 and 16) were prepared from native GAG, and then labeled with PA. Other oligosaccharides were reconstructed using the transglycosylation reaction of hyaluronidase. These oligosaccharides were incubated with hyaluronidase SD, and the chemical structures of the reaction products were determined by mass spectrometry.

Table 2 shows the sensitivities of the hybrid oligosaccharides to hyaluronidase SD. The cleavage specificity using hyaluronidase SD was as follows: (i) at least one unsulfated disaccharide unit (GlcUA-GalNAc) is necessary for the enzymatic action; (ii)

Table 2. Hydrolysis of oligosaccharides by hyaluronidase SD

Number	Structures[b]	Hydrolysis (%)[a]
14	GlcUA-GalNAc-GlcUA-GalNAc(6S)-GlcUA-GalNAc(6S)-GlcUA-GalNAc(6S)-PA	100
15	GlcUA-GalNAc(4S)-GlcUA-GalNAc(6S)-GlcUA-GalNAc(6S)-GlcUA-GalNAc(6S)-PA	0
16	GlcUA-GalNAc(6S)-GlcUA-GalNAc(6S)-GlcUA-GalNAc(6S)-GlcUA-GalNAc(6S)-PA	0
17	GalNAc-GlcUA-GalNAc(6S)-GlcUA-GalNAc(6S)-GlcUA-GalNAc(6S)-PA	0
18	GlcUA-GlcNAc-GlcUA-GalNAc(6S)-GlcUA-GalNAc(6S)-GlcUA-GalNAc(6S)-PA	100
19	IdoUA-GalNAc-GlcUA-GalNAc(6S)-GlcUA-GalNAc(6S)-GlcUA-GalNAc(6S)-PA	62
20	GlcUA-GalNAc-GlcUA-GalNAc(4S)-GlcUA-GalNAc(4S)-GlcUA-GalNAc(4S)-PA	100
21	GlcUA-GalNAc-GlcUA-GalNAc(4, 6S)-GlcUA-GalNAc(4, 6S)-GlcUA-GalNAc(6S)-PA	100

[a]Hydrolysis (%) was measured by HPLC of PA-labeled products. [b]The vertical broken line indicates the sites of cleavage by hyaluronidase SD.
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cleavage is inhibited by sulfation of *N*-acetylgalactosamine; (iii) the enzyme reaction releases GlcUA-GalNAc and IdoUA-GalNAc units as well as GlcUA-GlcNAc of HA. Thus, hyaluronidase SD can specifically and endolytically cleave the internal unsulfated regions of ChS chains.

2) Determination of the domain structure of chondroitin sulfate E octasaccharides binding to type V collagen. Chondroitin sulfate E (ChSE) is known to interact with type V collagen.[25] Therefore the domain structures of ChSE responsible for this interaction were investigated using oligosaccharides synthesized by the transglycosylation reaction of hyaluronidase.[20]

Eleven ChSE-oligosaccharides were isolated by HPLC from commercially available ChSE (squid cartilage) following digestion with hyaluronidase (Table 3, nos. 1–11). Ch, Ch4S, Ch6S and desulfated DS were used as donors and incubated with PA-ChSE-hexasaccharide (no. 6) as an acceptor under conditions suitable for the transglycosylation reaction. In all, five new oligosaccharides (PA-octasaccharides nos. 12–16) having different disaccharide units at the non-reducing termini were prepared. Oligosaccharide no. 17 was prepared from no. 3 by fluorescence labeling with PA, and no. 18 was obtained by β-glucuronidase digestion of no. 17. Interaction of oligosaccharides with type V collagen was analyzed by surface plasmon resonance using a type V collagen-immobilized sensor chip.

The following oligosaccharide structures were found to be important for interaction with collagen: (i) a minimum of octasaccharide in size; (ii) three sequential continuous GlcUA-GalNAc4S,6S units at the non-reducing terminus; (iii) a GlcUA-GalNAc4S,6S unit, a GlcUA-GalNAc6S unit or a GlcUA-GalNAc4S unit at the reducing terminus.

3) Structural interactions of Ch4S-mediated adherence of Plasmodium falciparum infected erythrocytes. Pregnancy-associated malaria has severe health consequences for both mother and fetus. Infection with *Plasmodium falciparum* results in adhesion of infected red blood cells (IRBCs) to the placenta, which is mediated by low-sulfated placental Ch4S proteo-

Table 3. Structure of the oligosaccharides and its affinity for collagen V

Oligosaccharide number	Structures	Affinity
1	(GlcUA-GalNAc)-(GlcUA-GalNAc)-(GlcUA-GalNAc)-(GlcUA-GalNAc) 4S,6S 6S 4S,6S 6S	−
2	(GlcUA-GalNAc)-(GlcUA-GalNAc)-(GlcUA-GalNAc)-(GlcUA-GalNAc) 4S,6S 4S,6S 4S,6S	−
3	(GlcUA-GalNAc)-(GlcUA-GalNAc)-(GlcUA-GalNAc)-(GlcUA-GalNAc) 4S,6S 4S,6S 4S,6S 6S	+
4	(GlcUA-GalNAc)-(GlcUA-GalNAc)-(GlcUA-GalNAc)-(GlcUA-GalNAc) 4S,6S 4S,6S 4S,6S 4S	+
5	(GlcUA-GalNAc)-(GlcUA-GalNAc)-(GlcUA-GalNAc)-(GlcUA-GalNAc) 4S,6S 4S,6S 4S,6S 4S,6S	+
6	(GlcUA-GalNAc)-(GlcUA-GalNAc)-(GlcUA-GalNAc) 4S,6S 4S,6S 6S	−
7	(GlcUA-GalNAc)-(GlcUA-GalNAc)-(GlcUA-GalNAc) 4S,6S 4S,6S 4S	−
8	(GlcUA-GalNAc)-(GlcUA-GalNAc)-(GlcUA-GalNAc)-(GlcUA-GalNAc)	−
9	(GlcUA-GalNAc)-(GlcUA-GalNAc)-(GlcUA-GalNAc)-(GlcUA-GalNAc) 4S 4S 4S 4S	−
10	(GlcUA-GalNAc)-(GlcUA-GalNAc)-(GlcUA-GalNAc)-(GlcUA-GalNAc) 6S 6S 6S 6S	−
11	(GlcUA-GalNAc)-(GlcUA-GalNAc)-(GlcUA-GalNAc)-GlcUA 4S,6S 4S,6S 4S,6S	−
12	(GlcUA-GalNAc)-(GlcUA-GalNAc)-(GlcUA-GalNAc)-(GlcUA-GalNAc)−PA 4S,6S 4S,6S 6S	−
13	(GlcUA-GalNAc)-(GlcUA-GalNAc)-(GlcUA-GalNAc)-(GlcUA-GalNAc)−PA 4S 4S,6S 4S,6S 6S	−
14	(GlcUA-GalNAc)-(GlcUA-GalNAc)-(GlcUA-GalNAc)-(GlcUA-GalNAc)−PA 6S 4S,6S 4S,6S 6S	−
15	(IdoUA-GalNAc)-(GlcUA-GalNAc)-(GlcUA-GalNAc)-(GlcUA-GalNAc)−PA 4S,6S 4S,6S 6S	−
16	(GlcUA-GlcNAc)-(GlcUA-GalNAc)-(GlcUA-GalNAc)-(GlcUA-GalNAc)−PA 4S,6S 4S,6S 6S	−
17	(GlcUA-GalNAc)-(GlcUA-GalNAc)-(GlcUA-GalNAc)-(GlcUA-GalNAc)−PA 4S,6S 4S,6S 4S,6S 6S	+
18	GalNAc-(GlcUA-GalNAc)-(GlcUA-GalNAc)-(GlcUA-GalNAc)−PA 4S,6S 4S,6S 4S,6S 6S	−

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glycans. The minimal structural motif involved in IRBCs interaction with placental proteoglycans was determined by means of an *in vitro* inhibition assay using a series of ChS dodecasaccharides with different numbers of 4-sulfate groups (Table 4).[26] It was found that dodecasaccharides with two to four 4-sulfated disaccharide units at the non-reducing terminus were necessary for maximal binding.

Table 4. Ch4S oligosaccharides synthesized by the testicular hyaluronidase transglycosylation reaction

donor	acceptor	products[a]
Ch4S	0S-0S-0S-0S-0S-PA	**4S-0S-0S-0S-0S-0S-PA**
		4S-4S-0S-0S-0S-0S-0S-PA
		4S-4S-4S-0S-0S-0S-0S-0S-PA
		4S-4S-4S-4S-0S-0S-0S-0S-0S-PA
Ch4S	0S-0S-0S-0S-PA	4S-0S-0S-0S-0S-PA
		4S-4S-0S-0S-0S-0S-PA
		4S-4S-4S-0S-0S-0S-0S-PA
		4S-4S-4S-4S-0S-0S-0S-0S-PA
		4S-4S-4S-4S-4S-0S-0S-0S-0S-PA
Ch4S	0S-0S-0S-PA	4S-0S-0S-0S-PA
		4S-4S-0S-0S-0S-PA
		4S-4S-4S-0S-0S-0S-PA
		4S-4S-4S-4S-0S-0S-0S-PA
		4S-4S-4S-4S-4S-0S-0S-0S-PA
Ch4S	4S-0S-0S-PA	4S-4S-0S-0S-PA
		4S-4S-4S-0S-0S-PA
		4S-4S-4S-4S-0S-0S-PA
		4S-4S-4S-4S-4S-0S-0S-PA
Ch4S	0S-4S-4S-PA	4S-0S-4S-4S-PA
		4S-4S-0S-4S-4S-PA
		4S-4S-4S-0S-4S-4S-PA
		4S-4S-4S-4S-0S-4S-4S-PA
		4S-4S-4S-4S-4S-0S-4S-4S-PA
		4S-4S-4S-4S-4S-4S-0S-4S-4S-PA

[a]The oligosaccharides indicated in bold type are used for the inhibition analysis. (0S, GlcUA-GalNAc; 4S, GlcUA-GalNAc4S; 6S, GlcUA-GalNAc6S.)
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II Synthesis of linkage regions between a core peptide and a GAG chain using the transglycosylation reaction of endo-glycosidases

Three types of endo-glycosidase (endo-β-glucuronidase,[27] endo-β-xylosidase[28] and endo-β-galactosidase[29]) act on the linkage region (GlcUA-Gal-Ga1-Xyl-Ser) between the core peptide and the GAG chain of a proteoglycan (Fig. 1).

Endo-β-xylosidase, which acts on the Xylβ1-Ser of proteoglycans, is useful for coupling of the GAG chain to a core peptide by exploiting the transglycosylation reaction (*i.e.*, reverse of the hydrolysis reaction). A carrier substance such as 4-methylumbelliferone (MU) and a peptide, which binds to the reducing terminus of the GAG chain, is needed for the GAG transfer.[30),31]

II-1. Synthesis of linkage regions by endo-β-xylosidase. *1) Discovery of endo-glycosidases.* Urinary ChS was purified from pooled human urine by a combination of selective precipitation followed by column chromatography.[32] The reducing terminal sugars of purified urinary ChS were then identified by gas-liquid chromatography analysis of the hydrolysate derivatives. Thus, it was suggested that endo-type β-xylosidase, β-galactosidase and β-glucuronidase acting on the linkage region between the core peptide and GAG chain of proteoglycans are present in tissues. This led to the isolation of endo-β-glucuronidase from rabbit liver, and endo-β-xylosidase and endo-β-galactosidase from the mid-gut gland of scallop (*Patinopecten yessoensis*).

2) GAG chain carrier bearing the linkage region between a core peptide and a GAG chain. Okayama *et al.* reported that addition of a β-xyloside, such as *p*-nitrophenyl-β-D-xyloside, to the cell culture medium induces elongation of GAG chains, which is initiated by the β-xyloside acting as a primer.[33] Human skin fibroblasts were incubated in the presence of a fluorogenic xyloside, MU-β-D-xyloside (Xyl-MU).[34)–37] From the incubation medium, fluorogenic compounds were isolated, comprising DS and ChS bearing the structure GlcUA-Gal-Gal-Xyl-MU at the reducing terminus (Fig. 6, Step 1). As a substrate of endo-β-xylosidase, these compounds released a fluorogenic product (MU) into the reaction medium, which is useful for estimating enzyme activity.

Therefore, after removal of the non-reducing terminus of the GAG chain bearing the fluorogenic agent using hyaluronidase, the remaining linkage region, GlcUA-Gal-Gal-Xyl-MU, was able to act as an acceptor for elongation of a new GAG chain *via* the transglycosylation reaction (Fig. 6, Step 2). Thus, GlcUA-Gal-Gal-Xyl-MU is also available as a carrier for linkage formation between a GAG chain and a serine residue in the peptide core (Fig. 6, Step 3). The linkage region bearing MU at the reducing terminus is more useful as a carrier than that bearing the peptide, GlcUA-Gal-Gal-Xyl-peptide.

3) Application of endo-β-xyloside to form a linkage region between a core peptide and a GAG chain. One successful application of endo-β-xylosidase for GAG chain transfer was carried out using an artificial substrate Boc-Leu-Ser-Thr-Arg-MCA of activated protein C, which is a blood coagulation modulator, as an acceptor for the GAG chain (Fig. 7).[31),38] This peptide contains two amino acids (serine and thre-

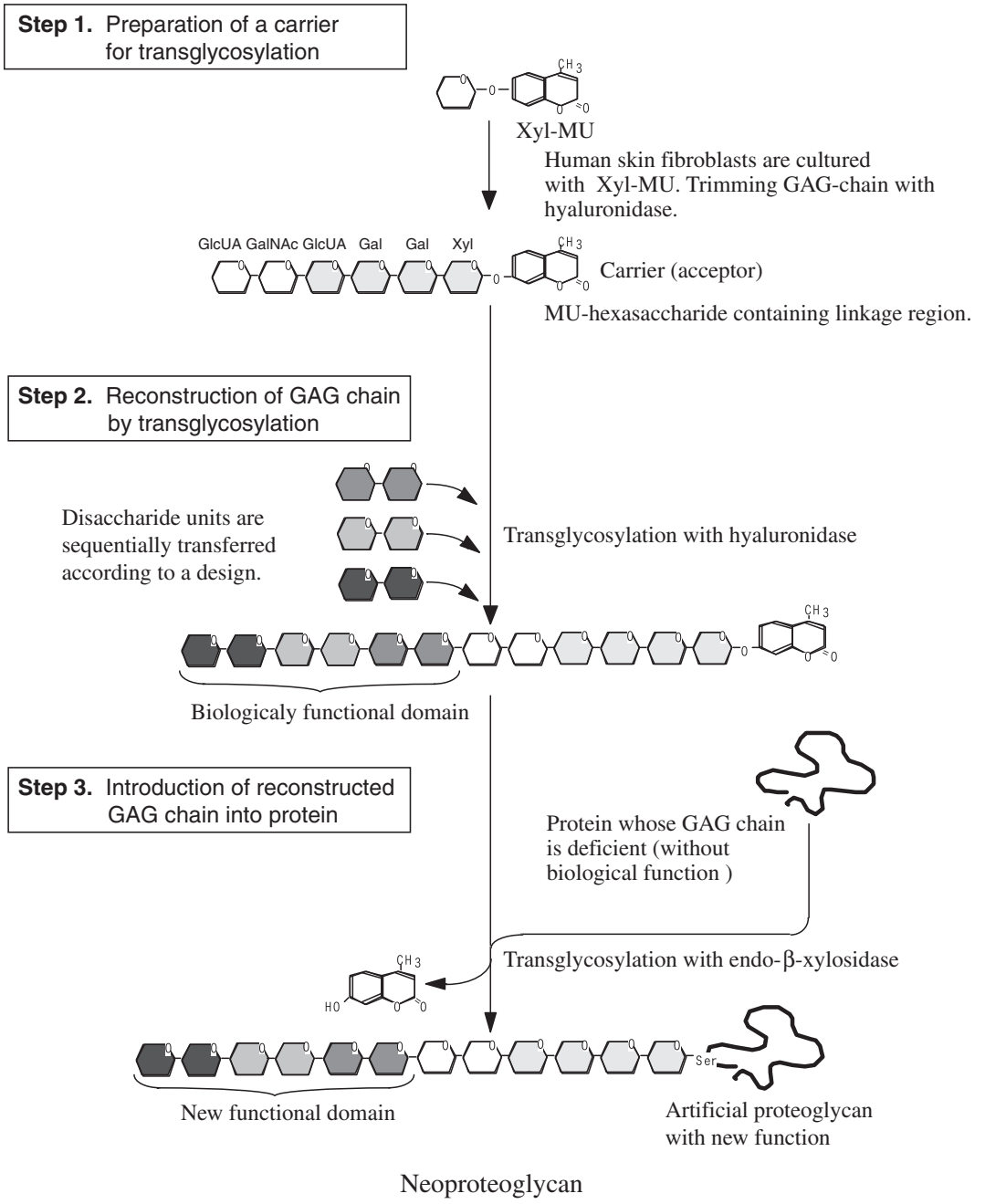

Fig. 6. Strategy for neoproteoglycan synthesis using the transglycosylation reaction of endoglycosidases.

onine) bearing a hydroxyl group that can accept the GAG chain. As such, this peptide is useful for analyzing the transglycosylation reaction mediated by endo-β-xylosidase. The peptide, acting as an acceptor, and peptide-ChS (or a GAG chain bearing MU at the reducing terminus), as a donor, were incubated at 37°C with endo-β-xylosidase in 100 mM sodium acetate buffer (pH 3.0). As shown in Fig. 7, ChS was successfully transferred from the peptidoglycan donor substrate to the alcohol moiety of serine in the peptide.

The essential conditions found for the transglycosylation reaction are as follows:[38] (i) acceptors are peptides bearing a serine residue, and (ii) acceptable donors are any ChS (Ch, Ch4S and Ch6S), DS or heparan sulfate (HS) that possesses

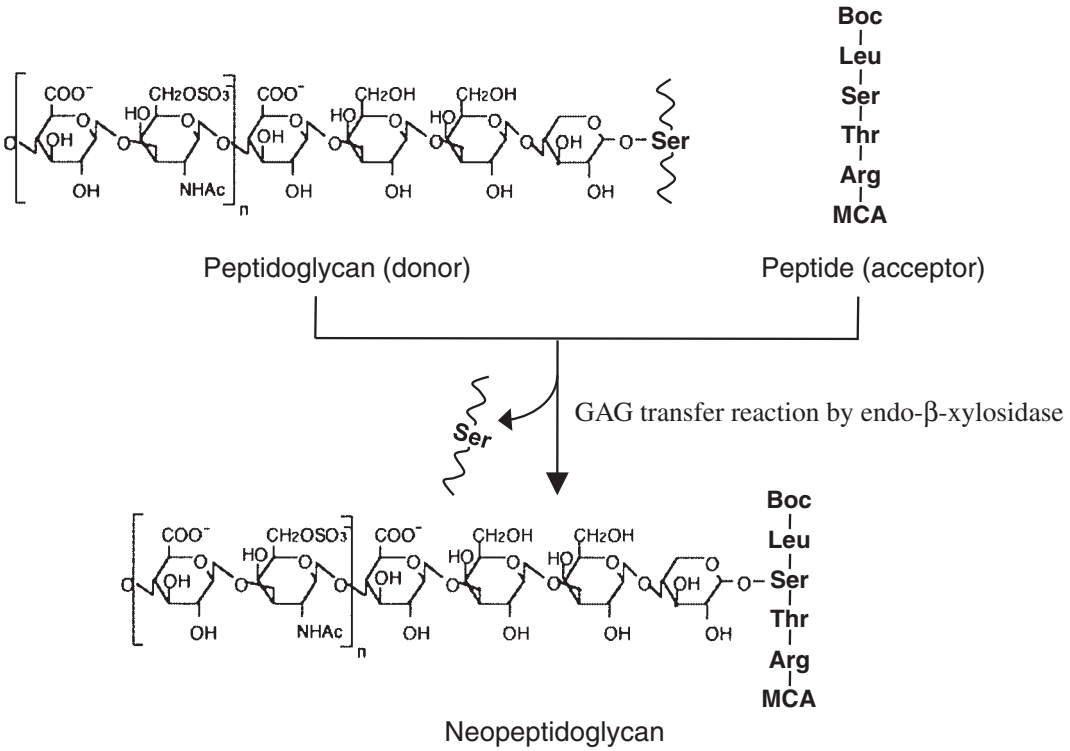

Fig. 7. Scheme for GAG chain coupling to a peptide using the transglycosylation reaction of endo-β-xylosidase.
Boc, *t*-butyloxycarbon; MCA, 4-methylcoumaryl-7-amide.

a linkage region, although the order of efficiency is ChS > DS > HS. The longer the donor GAG chain, the higher the degree of transglycosylation.

II-2. Synthesis of linkage regions between a core peptide and a GAG chain using cellulase. Cellulases, which are widely distributed in plants, microorganisms and fungi, endolytically hydrolyze β-1,4-glucoside bonds in cellulose. Xylose and glucose have the same configurations, except that the C-5 of xylose possesses a hydrogen atom instead of a hydroxymethyl group, which is a typical functional group of hexose derivatives. Therefore, it is likely that cellulase will recognize β-xyloside.

Screening of several cellulases was carried out using GAG-MU with the linkage region of proteoglycans.[39] These studies revealed that cellulase from *Aspergillus niger* cleaves the β-xyloside bond.[39] Moreover, the cellulase purified from *A. niger* displayed the following properties: (i) the enzyme cleaves the linkage region (Xyl-Ser) between the core peptide and the GAG chain; (ii) the shorter the GAG chain the greater is the enzymatic activity; and (iii) the enzyme has broad substrate specificity for the GAG chain (Ch, ChS, DS or heparan sulfate). Therefore, the *A. niger* cellulase could be a useful alternative to endo-β-xylosidase.[39]

III Replacement and reconstruction of the GAG chain

Using the transglycosylation reaction of hyaluronidase and endo-β-xylosidase, a GAG chain and its attachment to a peptide have been successfully reconstructed. However, the selective coupling of a GAG chain to the serine residue at a specific position in a peptide has not yet been accomplished.

Therefore, in order to investigate the physiological functions of proteoglycan GAG chains it is desirable to have the ability to replace one GAG chain with another in a proteoglycan, as well as to be able to reconstruct a GAG chain in accordance with predetermined specifications.

III-1. Trimming and reconstruction of GAG chains using hyaluronidase. Hydrolytic trimming of proteoglycans using hyaluronidase yield peptides bearing hexa-, octa-, or decasaccharides that retain the linkage region. As glucuronic acid residues are present at the non-reducing terminus of these oligosaccharides, elongation of the disaccharide units

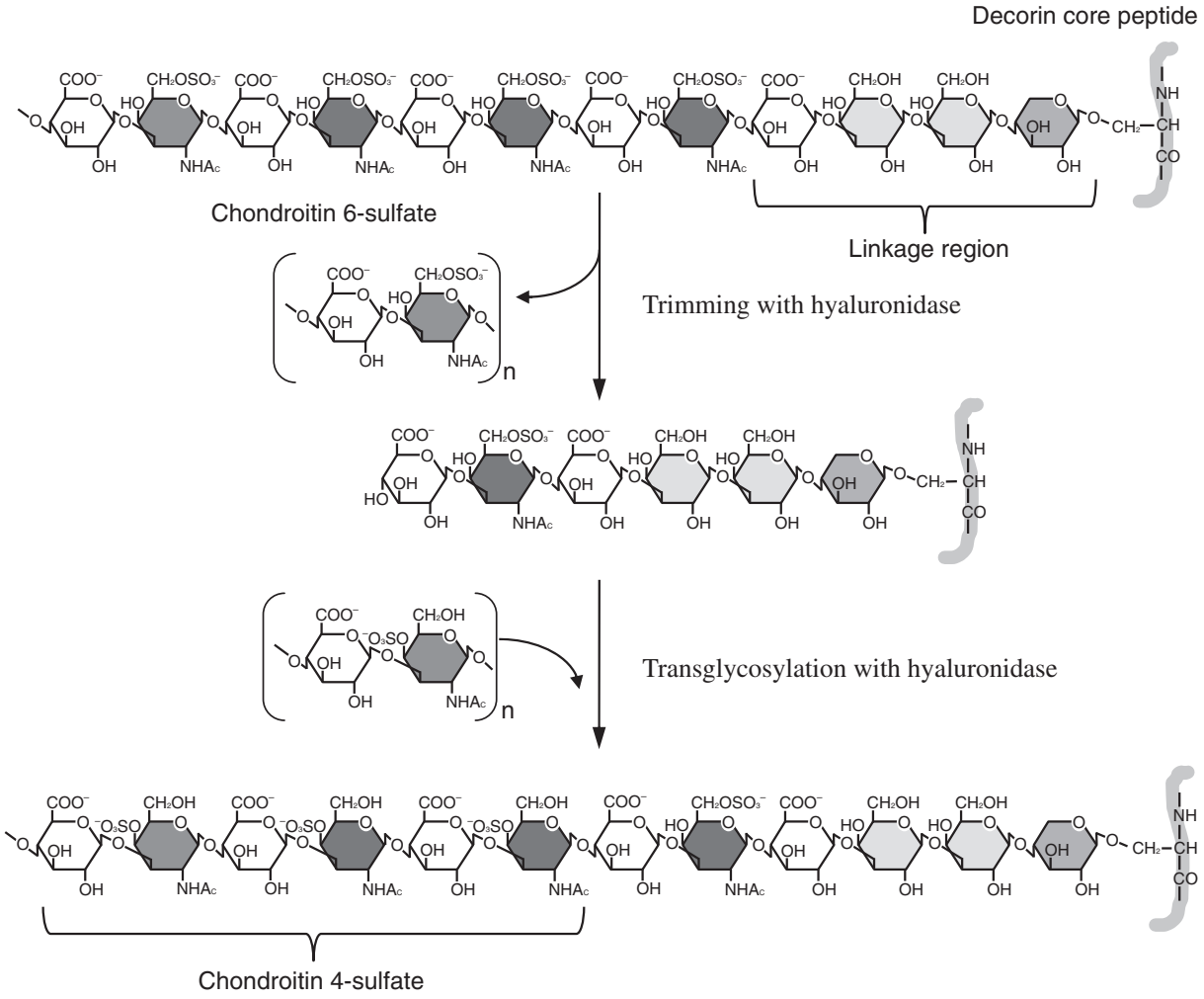

Fig. 8. Replacement of the Ch6S chain by a Ch4S chain in decorin peptide using hyaluronidase.
First, the original Ch6S chain of decorin was trimmed by exhaustive hydrolysis with hyaluronidase. The resulting decorin with only the sugar chain of the linkage region remaining was used as an acceptor in a transglycosylation reaction mediated by hyaluronidase with Ch4S chain as a donor. The final decorin possessed a Ch4S chain.

of GAG is possible using the transglycosylation reaction of hyaluronidase (Fig. 8). Such proteoglycans bearing changed GAG chains may possess novel physiological functions.

1) Reconstruction of GAG chains in decorin. Decorin is a small proteoglycan, which has one or two GAG chains. Decorin having a Ch6S chain from human spinal ligaments was digested using hyaluronidase[40] to yield decorin with hexa-, octa- and decasaccharide coupling to the linkage region (Fig. 8). When the decorin peptide as an acceptor and HA as a donor were incubated with hyaluronidase under optimal conditions for the transglycosylation reaction, the DS chain of decorin increased from hexasaccharide to triacontasaccharide. Similarly, it was possible to reconstruct the DS chain of decorin into Ch, Ch4S and Ch6S chains. Therefore, an artificial family of decorins was synthesized bearing different kinds of GAG chain. An investigation was carried out using plates coated with HA binding protein (HABP). These studies showed the affinity of HABP for decorin was reconstructed with the HA chain, but not for the original decorin. Therefore, the physiological activity of decorin was changed by reconstruction of the GAG chain. The novel physiological activities of decorin are currently being investigated.

III-2. Trimming and replacement of GAG chains using eliminases. *1) Unsaturated uronic acid produced at the non-reducing terminus by chon-*

droitinases. Some eliminases, such as chondroitinases, catalyze an elimination reaction in which *N*-acetylhexosaminide bonds within the GAG chains (Ch, ChSs, DS and HA) are cleaved. In the case of when chondroitin sulfate chains of proteoglycans are incubated with testicular hyaluronidase which is a glycosidase, short oligosaccharides coupling to the linkage region of proteoglycans remain. On the other hand, in the case of when the chondroitin sulfate chains are incubated with bacterial hyaluronidase SD[24] which is an eliminase, only the unsulfated *N*-acetylgalactosaminide bonds of the chains are cleaved, and as a result longer chains remain than with testicular hyaluronidase incubation. Therefore, selective use of eliminases such as hyaluronidase SD, chondroitinase AC-II and ABC,[41] may result in longer chains of GAG containing biological domain structures near the linkage region remaining. The residual GAG oligosaccharides coupled to the proteoglycan peptide have a 4,5-unsaturated uronic acid residue at the non-reducing terminus (Fig. 9, Step 1). Therefore, the hyaluronidase is unable to transfer GAG oligosaccharides from a donor using the transglycosylation reaction because unsaturated uronic acid is not an acceptable residue for GAG chains.

Chondroitinases (chondroitinase AC-II, ABC, etc.) are very useful for the determination of GAG structures that are sulfated at various positions.[41] Therefore, at the non-reducing terminus of a GAG chain, it is necessary to change unsaturated uronic acid to saturated uronic acid.

2) Changing unsaturated uronic acid to saturated uronic acid at the non-reducing terminus of GAG chains. The oxymercuration-demercuration reaction is a useful procedure for synthesizing alcohols from alkenes *via* a two-step method and is useful for converting 4,5-unsaturated uronic acid to the original glucuronic acid.[42] The 4,5-unsaturated uronic acid β1-3-*N*-acetylgalactosamine 4-*O*-sulfate was used as the model compound for the optimization process. The reaction should proceed below 0°C to prevent decomposition of unsaturated uronic acid and the sulfate group, and the most appropriate amounts of mercury (II) acetate ($(CH_3COO)_2Hg$) and sodium borohydride ($NaBH_4$) are 2 and 1.5 molar equivalents, respectively, relative to unsaturated uronic acid.

Addition of excess $(CH_3COO)_2Hg$ led to the conversion of 4,5-unsaturated uronic acid into glucuronic acid (Fig. 9, Step 2). The optimal oxymercuration and demercuration times were 10 min and 60 min, respectively. The converted oligosaccharides are then available as acceptors for the transglycosylation reaction of hyaluronidase (Fig. 9, Step 3).

IV Discussion

Currently, there are three significant problems associated with the glycotechnological manipulation of high-molecular-weight GAG chains: (i) synthesis of extended GAG chains; (ii) introduction of sulfate groups at specific positions on the GAG chains; and (iii) linkage of a GAG chain to a selected serine residue in the peptide of a proteoglycan.

An efficient synthetic chemical route to long-chain GAGs has not been fully achieved. However, the tailored production of long-chain GAG should be feasible by exploiting the transglycosylation reaction as a reverse reaction of glycosidases. Moreover, using combinations of such GAG chains allows the creation of hybrid molecules that have not yet been characterized. Indeed, the authors have succeeded in synthesizing hybrid chains of HA and ChS.[15),24] In the case of GAGs that are not substrates for hyaluronidase, the discovery of novel glycosidases, especially endoglycosidases and eliminases is eagerly anticipated.

The selective introduction of sulfate groups into specific locations in a GAG chain is technically difficult at the present time. Some sulfuryl transferases acting on ChS and DS have been discovered,[43),44] but are currently unavailable. However, the transglycosylation reaction with hyaluronidase is able to transfer GAG-disaccharide units bearing sulfate groups to specific positions. Hence, the selective introduction of sulfate groups is not a major issue. Nonetheless, the sulfation of L-iduronic acid residues in DS chains remains an insoluble problem.

Linkage formation between a core peptide and a GAG chain is now possible using endo-β-xylosidase and cellulase. However, introduction of a GAG chain at a preselected serine residue in a peptide has still not been achieved.

Endo-β-galactosidase[29] acting on the linkage region between a core peptide and a GAG chain will become applicable for linkage formation in the near future. However, cleavage of immature GAG chains using hyaluronidase and chondroitinases, and elongation of new GAG chains at the non-reducing terminus of a residual GAG chain of a proteoglycan can now be done efficiently.

Glycotechnological manipulation using the transglycosylation reaction as a reverse reaction of endoglycosidases has certain characteristics as follows: (i) synthetic methods using this reaction are better than organic chemical methods in that high-

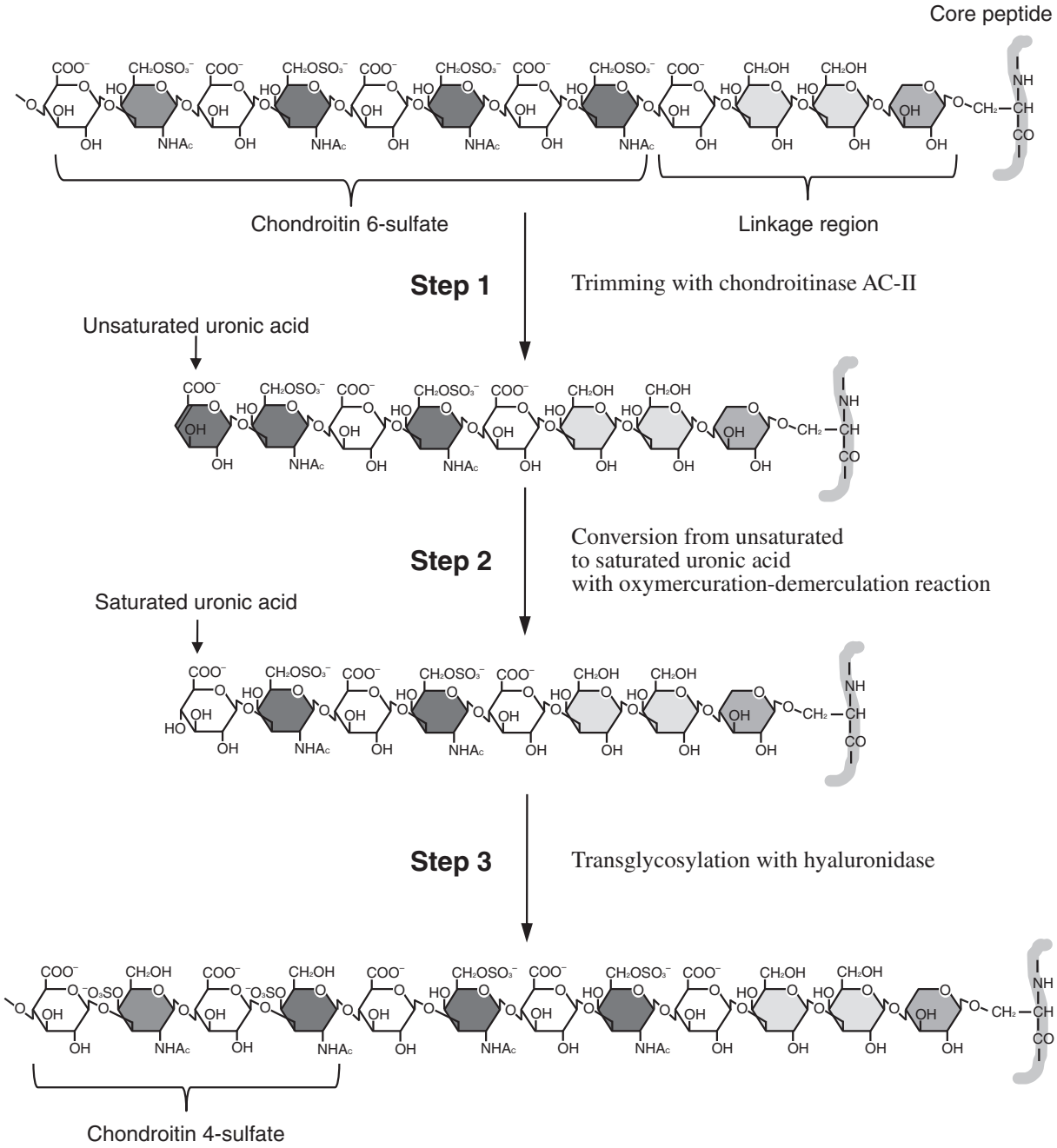

Fig. 9. Replacement of the GAG chain after conversion of unsaturated uronic acid produced by eliminase digestion to saturated uronic acid.

molecular-weight GAG chains can be produced in a short time; (ii) the products synthesized using this reaction have the same chemical structure in terms of the position of the linkage bond and the α,β-anomer, faithfully reflecting the structural specificity of the enzyme substrate; and (iii) use of this reaction makes it possible to generate novel GAG chains.

Therefore, it is highly likely that application of the transglycosylation reaction of glycosidases will facilitate artificial reconstruction of GAG chains that are physiologically functional. A system for reconstruction of GAG chains will undoubtedly open new field of GAG glycotechnology for the development of novel drugs.

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Profile

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After serving as a research associate, a lecturer, and an associate professor, he held the position of Professor in the Department of Biochemistry at Hirosaki University School of Medicine where he worked on research and education in the field of Biochemistry, especially proteoglycan glycotechnology, from 1981 until his retirement in 2002.

From 1996 to 2002, he served as Dean of Hirosaki University School of Medicine, and from 2002 to 2012 as President of Hirosaki University. In 1980 he was conferred with the title of Visiting Associate Professor from Boston University School of Medicine. From 2006 to 2012 while serving as the President, he held an additional post in the Department of Biochemistry, Hirosaki University Graduate School of Medicine. In 2012, he was conferred with the title of Professor Emeritus, and he currently holds the position of Special Contract Professor of the Department of Glycobiochemistry, Hirosaki University Graduate School of Medicine. The prizes he has been awarded include the Kahoku Shinbun Cultural Prize and The Order of the Sacred Treasure, Gold and Silver Star.

His proteoglycan research may be classified into three categories: 1) Metabolism of hyaluronan including the first discovery in the world of "hyaluronuria" which is errors of hyaluronan metabolism, the first inhibitor, 4-Methylumbelliferone of hyaluronan synthesis, etc., 2) Metabolism of proteoglycan including discovery of five new glycosidases on proteoglycan catabolism, 3) Glycotechnology of neoproteoglycan synthesis using transglycosylation reaction of reverse reaction of endoglycosidases.

Profile

Ikuko Kakizaki graduated from the Faculty of Pharmaceutical Sciences, Hokkaido University, Japan in 1992, and received her MS degree from the graduate school of the same university in 1994. She graduated from the Graduate School of Medicine, Hirosaki University, Japan and received her Ph.D in 1998. She started her professional career as a Research Associate in the Department of Biochemistry, Hirosaki University School of Medicine in 1998 under the supervision of Prof. Masahiko Endo, and from 2002, under Prof. Keiichi Takagaki. She studied in the Department of Biochemistry and Molecular Biology, Pennsylvania State University College of Medicine in 2003 under the supervision of Profs. Veer P. Bhavanandan and Channe D. Gowda. Since 2008 she has been the Associate Prof. of the Department of Glycotechnology, Center for Advanced Medical Research, Hirosaki University Graduate School of Medicine. Her research interests are the metabolism and functions of hyaluronan and proteoglycans and she recently focused on the glycotechnology of proteoglycans to synthesize tools for research on the relationships between functions and specific structures of proteoglycan sugar chains.