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Novel proteoglycan glycotechnology: chemoenzymatic synthesis of chondroitin sulfate-containing molecules and its application

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Abstract Proteoglycans consist of a protein core, with one or more glycosaminoglycan chains (*i.e.,* chondroitin sulfate, dermatan sulfate and heparin sulfate) bound covalently to it. The glycosaminoglycan chains account for many of the functions and properties of proteoglycans. The development of proteoglycan glycotechnology to exploit the functionality of glycosaminoglycan chains is an extremely important aspect of glycobiology. Here we describe an efficient and widely applicable method for chemoenzymatic synthesis of conjugate compounds comprising intact long chondroitin sulfate (ChS) chains. An alkyne containing ChS was prepared by an enzymatic transfer reaction and linked with a chemically synthesized core compound containing an azido group using click chemistry. This method enabled highly efficient introduction of ChS into target materials. Furthermore, the ChS-introduced compounds had marked stability against proteolysis, and the chemically linked ChS chain contributed to the stability of these core compounds. We believe the present method will contribute to the development of proteoglycan glycobiology and technology.

Keywords Chondroitin sulfate · Click chemistry · Glycosaminoglycan · Neo-proteoglycan · Proteoglycan

Abbreviations

AMC	7-amino-4-methylcoumarin
Boc	*t*-butoxycarbonyl
BSA	bovine serum albumin
ChS	chondroitin sulfate
DCC	dicyclohexylcarbodiimide
DMT-MM	4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium chloride
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
ESI-MS-MS	electrospray ionization tandem mass spectrometry
GAG	glycosaminoglycan
Gal	galactose
GalNAc	*N*-acetylgalactosamine
GlcA	glucuronic acid
HPLC	high-performance liquid chromatography
NMM	*N*-methylmorpholine
RP	reverse phase
SEC	size exclusion chromatography
TEA	triethylamine
Xyl	xylose

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Introduction

Proteoglycans are present in the tissues of many mammalian species, both at the cell surface and in the extracellular matrix. These compounds consist of a protein core, which has one or more glycosaminoglycan side chains bound covalently to it. The highly sulfated glycosaminoglycan chondroitin sulfate (ChS) is a composite element of proteoglycan in cartilage [1]. ChS has various important biological roles in cell migration, recognition, and morphogenesis [2]. ChS has many types of structural domains that are known to participate in specific physiological functions. Recently, greater attention has been directed towards the functions of ChS in the brain, optic nerves and chondrocytes [3, 4].

Recent advances in gene engineering have now made it possible to mass-produce extremely useful proteins, but the technology involved is not ideal for the creation of glycoproteins. This is because DNA incorporated by gene recombination has no direct information about biosynthesis of the carbohydrate chains. Many reports have described that those proteins have few biological activities because of the incompletion of carbohydrate chains [5, 6].

The saccharide moieties of glycoproteins contribute to their solubility and thermal stability, and to protection against proteolysis [7]. Therefore, to study the functionality and applications of ChS in detail, there is a need to develop a method for probing ChS and attaching it to peptides and proteins. Unfortunately there are a number of obstacles to achieving this. Although a method for effective chemical synthesis of the linkage region tetrasaccharide [*i.e.,* GlcAβ(1-3)Galβ(1-3)Galβ(1-4)Xyl] [8] and ChS oligosaccharides has been reported [9, 10], it is difficult to supply an intact ChS, including the linkage region tetrasaccharide, employing organic synthesis. In addition, it is not possible to synthesize an artificial proteoglycan consisting of an intact ChS and core protein by organic synthesis alone.

Several strategies for glycopeptides and glycoproteins have been reported [11–19], but these methods are difficult to use for proteoglycans. Because the molecular weight of ChS ranges from 6.5 to 3.5×10^4, and furthermore its hydroxyl groups are highly and randomly sulfated [2, 20], it is difficult to artificially reproduce the ChS in homogeneity that is found in the natural compounds. Therefore, to address these problems, we have developed original approaches for proteoglycan glycotechnology [21–24], and have recently applied it for effective synthesis of neoproteoglycans [25].

Here we report a highly efficient chemoenzymatic method for synthesizing several types of novel ChS-containing compounds. Furthermore, with a view to potential application of the synthesized ChS-containing compounds, we also describe their proteolytic stabilities.

Results

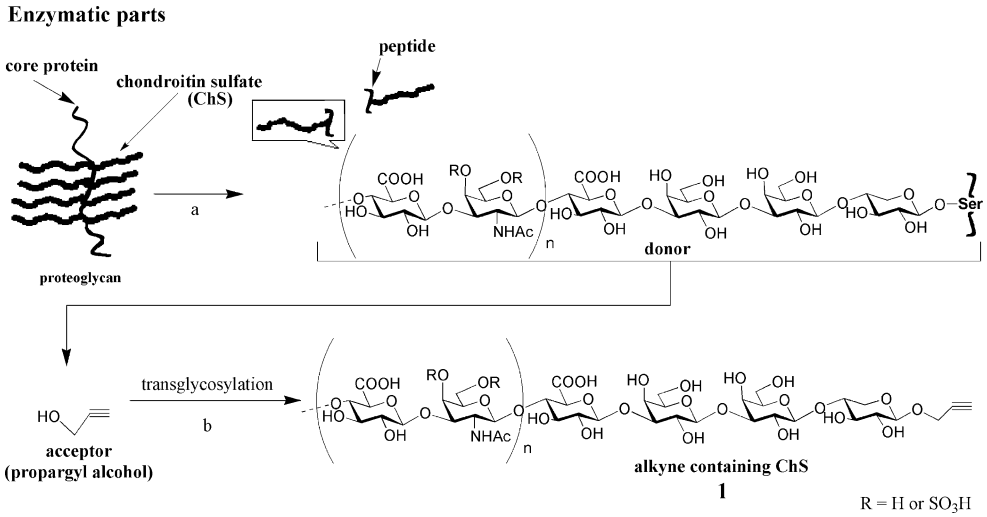
ChS probe design and synthesis strategy

Our strategy in the present study consisted of three steps. The first step was the preparation of alkyne-containing ChS **1** using an enzymatic method. In order to do this, we employed peptide chondroitin sulfate (Peptide-ChS) as a donor, which was prepared from proteoglycan derived from salmon nose cartilage [26]. Use of proteoglycan derived from a non-mammalian source reduces the risk of contamination with pathogenic agents. The peptide-ChS was treated with endo-β-xylosidase [22] in the presence of propargyl alcohol. This enzyme specifically cleaved the β-xyloside linkage between the reducing end xylose and the hydroxyl group of the Ser side chain, and at the same time introduced the alkyne into the xylose in accordance with its transglycosylation activity [25]. There are several advantages of using propargyl alcohol as an acceptor, as the transglycosylation yield is better than that of a peptide or protein. In our previous study, we had tried direct transglycosylation of ChS to the target peptide and protein of the Ser residue, but the yield of the target compounds was very low; when peptide and protein were used as acceptors, the yields were >5% and 0%, respectively [25]. Furthermore, it is not necessary to consider degeneration of the acceptor material and the reaction is easily quenched by boiling. Also, any residual excess propargyl alcohol can easily be removed by lyophilization (Scheme 1). The second step was the synthesis of a core compound containing an azido group in order to introduce the alkyne-containing ChS. For synthesis of the compound, we employed 4-azido benzoic acid (Tokyo Kasei Kogyo Co., Japan) as the azidolation reagent. The reagent was introduced into the target compound by forming an ester and an amide using a carbodiimide reagent (DCC, EDC) and a non-carbodiimide reagent (DMT-MM; 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium chloride), as appropriate [25, 27] (Scheme 2). The last step was the ligation reaction for ChS **1** and the core compound **2**, utilizing click chemistry to afford the target ChS-linked compound **3**. The conditions of click chemistry afford superior regioselectivity, high tolerance of other functionalities, and almost quantitative transformation under mild conditions [25, 28–31] (Scheme 2).

Synthesis of azido group-containing fluorogenic peptide substrate **5** for trypsin

The title compound **5** was synthesized to investigate the influence of the introduction of a long ChS chain on trypsin digestion. The fluorogenic peptide, peptidyl-4-methylcumaryl-7-amide (MCA), consisted of three amino acid residues: Phe, Ser and Arg. The *N*-terminal amino group was acylated with

Scheme 1 Enzymatic preparation of alkne containing ChS 1. **a** actinase E, 0.1 M Tris-HCl buffer (pH 8.0) containing 10 mM $CaCl_2$, 40°C, 48 h. **b** endo-β-xylosidase, propargyl alcohol, 0.1 M sodium acetate buffer (pH 5.0), 37°C, 24 h

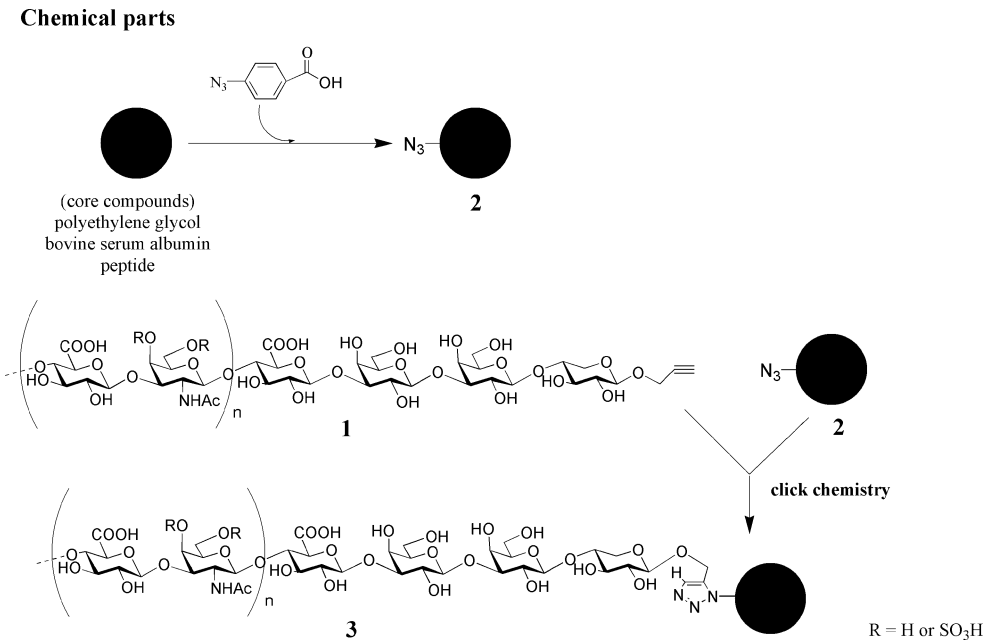


4-azido benzoic acid in order to prepare a site for introduction of the alkyne containing ChS **1** by click chemistry. The synthetic starting material, Boc-Phe-Ser-Arg-MCA **4** [32], was purchased from Peptide Institute Inc., Japan. The Boc group of this peptide was removed by treatment with TFA : CH_2Cl_2=1 : 3, and the resulting *N*-terminal amino group was acylated with 4-azido benzoic acid employing DMT-MM and *N*-methylmorpholine (NMM) in MeOH [27]. After purification, the target peptide was obtained at 65% yield (2 steps; Scheme 3). The characterization of **5** was performed by NMR, amino acid analysis and ESI-MS-MS. The MS-MS data of the precursor ion that matches the molecular mass of compound **5** were extensively analyzed to identify the site of attachment of 4-azido benzoic acid. The fragment ion of *m/z* 293.0 indicated that the 4-azido benzoic acid introduced the *N*-terminal of compound **5** (Fig. 1).

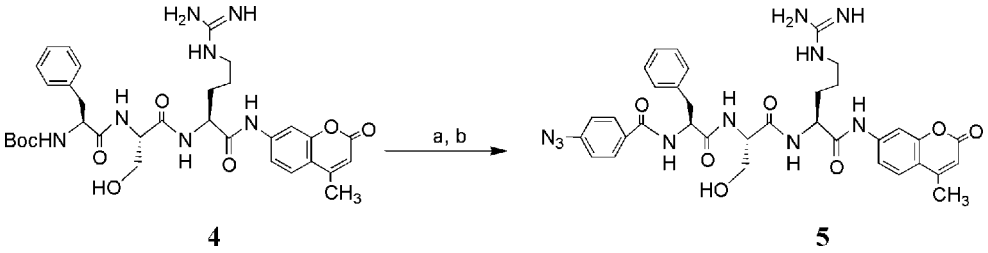
Synthesis and analysis of fluorogenic peptide-ChS **6** and **7**

The click chemistry reaction to ligate fluorogenic peptide **5** with ChS **1** was carried out under mild conditions (room temperature, 3 h) using 10 mM $CuSO_4$ with 10 mM sodium ascorbate as the *in situ* reducing agent to generate the Cu (I) species [30] (Scheme 4). The resulting products were subjected to HPLC (TSK-gel ODS-100 V; RP-column and Showdex OH pack SB-804 HQ; SEC column) (Fig. 2a, b). The RP-column chromatograms showed that the peak of peptide **5** disappeared completely and that a new peak

Scheme 2 Chemical ligation of ChS 1 and azido group-containing core compounds 2



Scheme 3 Synthesis of azido group-containing fluorogenic peptide 5. **a** TFA, CH_2Cl_2, 0°C, 2 h **b** DMT-MM, NMM, MeOH, r.t., 24 h, two steps 65%



4 **5**

appeared at an early elution time, resulting from binding of the highly polar ChS **1** to the peptide **5**. Judging from these results, the yield of fluorogenic peptide-ChS **6** was almost quantitative (Fig. 2a). The molecular weight of the peptide showed a pronounced increase after the click chemistry reaction, as judged from the SEC column chromatogram (Fig. 2b). After purification, **6** was subjected to cellulose acetate membrane electrophoresis and stained with alcian blue, which detects glycosaminoglycans. Compound **6** was clearly stained as a single band (Fig. 3). Furthermore, **6** was digested with chondroitinase ABC (protease-free), which acts on the GlcAβ(1–3)GalNAc sulfate linkage and converts ChS to 4,5-unsaturated disaccharides, except for the reducing-end hexasaccharide (*i.e.,* 4,5-unsaturated GlcA-GalNAc(OSO_3H)-GlcA-Gal-Gal-Xyl) [33] (Scheme 4). After exhaustive digestion, the reaction mixture was subjected to HPLC (Polyamine-II column), and the resulting profile is shown in Fig. 2c. Three peaks (I, II and III) were observed. The fraction corresponding to peak I in the reaction mixture was isolated by HPLC, desalted by dialysis, and subjected to ESI mass spectrometry. The spectrum of peak I showed a peak at *m/z* 1836.0 $[M-3H]^-$. The molecular mass of 1836.0 was in accord with the theoretical value for 4,5-unsaturated GlcA-GalNAc(OSO_3H)-GlcA-Gal-Gal-Xyl-fluorogenic peptide **7** (Fig. 4). Peaks II and III were assigned to 4,5-unsaturated GlcAβ(1–3)GalNAc 6-*O*-sulfate and 4-*O*-sulfate respectively, using an Unsaturated Chondro-Disaccharide Kit. These results indicated that the intact long ChS chain had bound to the core compound efficiently through the click chemistry reaction.

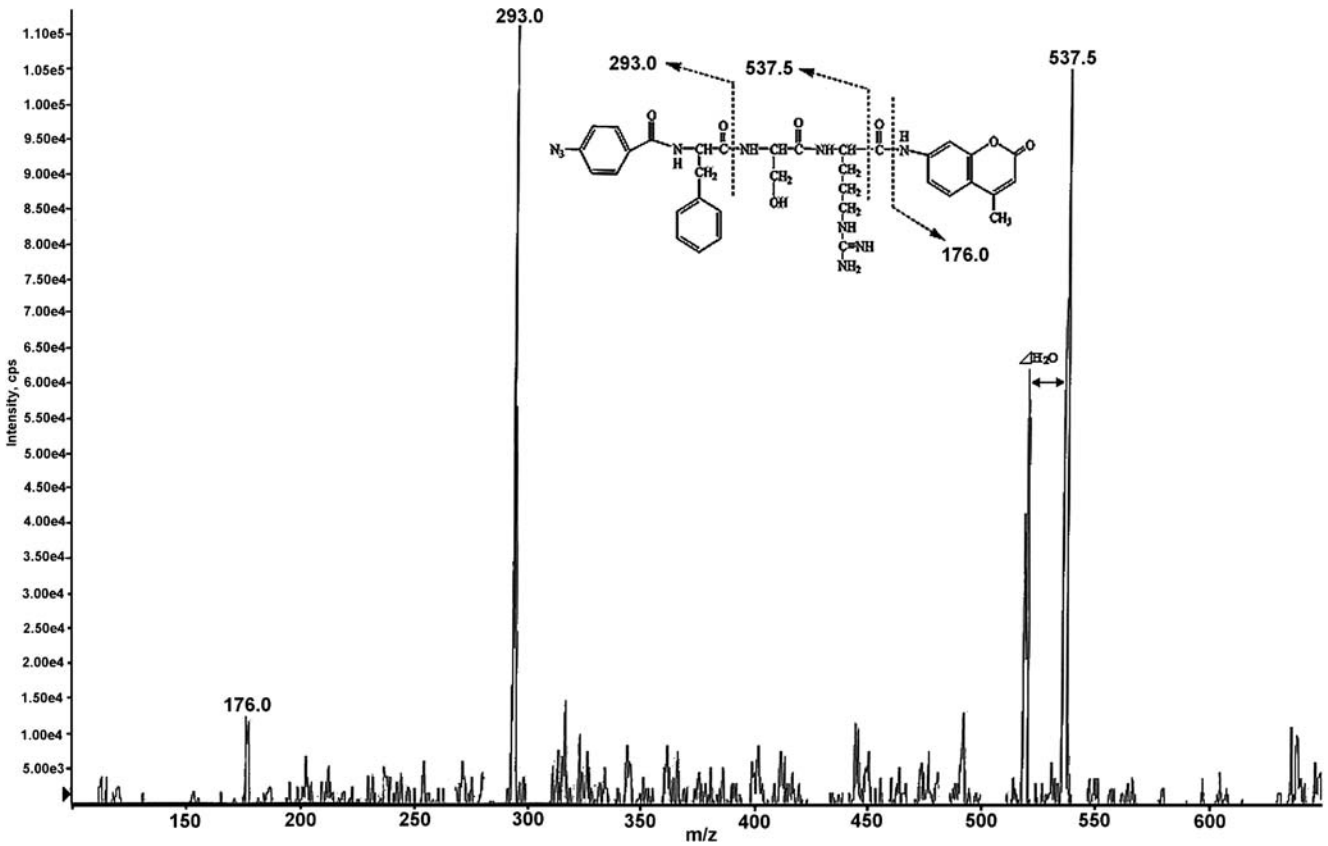
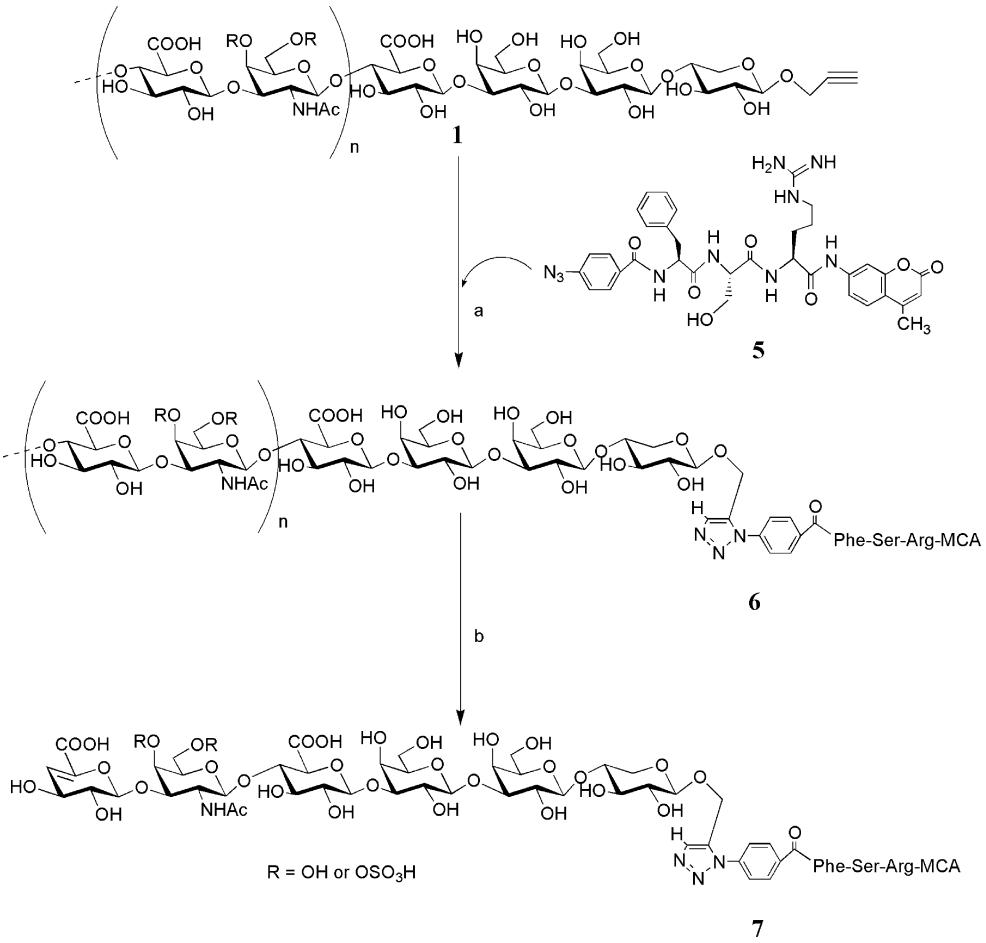


Fig. 1 ESI-MS-MS spectrum of azido group-containing fluorogenic peptide 5



Scheme 4 Synthesis of fluorogenic peptide-ChS 6 and 7. **a** 10 mM $CuSO_4$, 10 mM sodium ascorbate, r.t., 3 h, quant. The yield depended on peptide 5. **b** chondroitinase ABC (0.5 U), 0.4 M Tris-HCl buffer (pH 8.0), 0.4 M sodium acetate, 37°C, 24 h

Km values of trypsin for the three synthetic compounds **5**, **6** and **7**

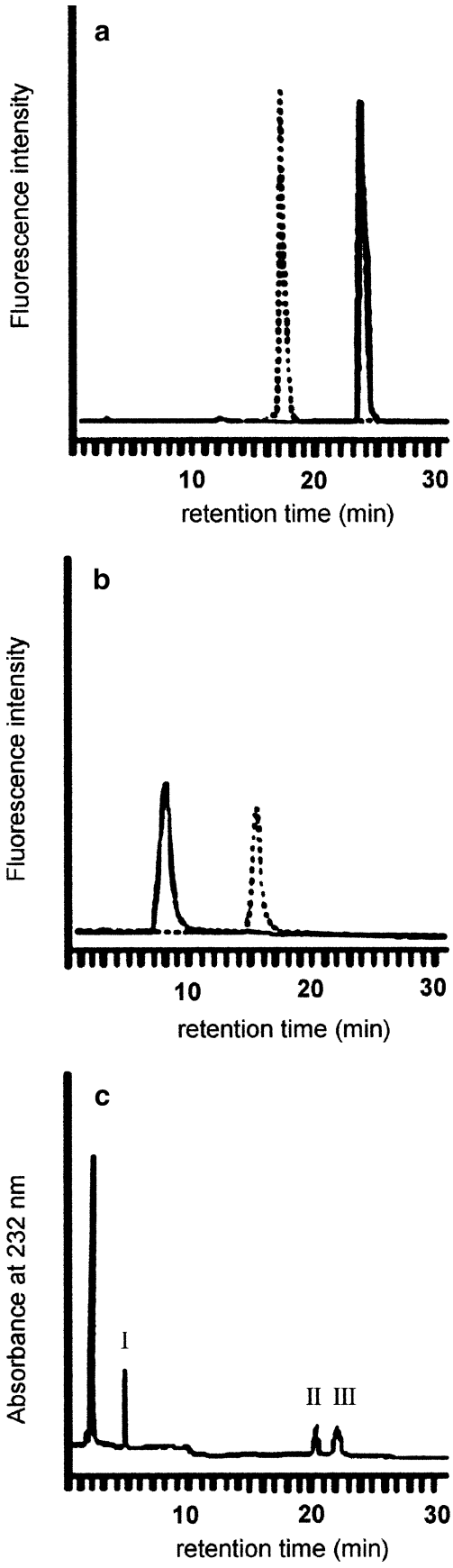
The Km values for the three synthetic compounds **5**, **6** and **7** were calculated as 8.4×10^{-5}M, 5.7×10^{-4}M and 3.8×10^{-4}M, respectively (Table 1). The *Km* values of trypsin for these synthetic compounds decreased with the increasing attached saccharide chain length of the peptide. These results suggested that introduction of the long ChS into the peptide largely decreased the affinity for trypsin.

Stability of four synthetic compounds **4**, **5**, **6** and **7** against protease digestion

We investigated the resistance of the ChS-bound peptide against protease (trypsin) digestion. To evaluate the stabilization of the peptide, we employed four types of substrate for trypsin (**4**, **5**, **6** and **7**) (Scheme 4). Each substrate was digested by trypsin, and the released 7-amino-4-methylcoumarin (AMC) was measured on a spectorofluorometer. The relative activity toward compound **4** is shown in Fig. 5. In this case, the low activity indicated that the compound was resistant to trypsin digestion. Compound **5** was promptly digested by trypsin, but **6**, bearing a ChS, showed marked resistance to trypsin digestion. **7**, which contained hexasaccharide, also showed resistance to trypsin digestion, although not to such a marked extent as substance **6**. Nevertheless, the resistance was significant in comparison with **5**. On the other hand, the mixed ChS **1** and peptide **5** that were not linked to each other did not show marked resistance to digestion in comparison with the ChS-linked peptide (Fig. 6).

Discussion

We have developed a chemoenzymatic method for introduction of intact ChS into target materials. The method consists of three simple steps and can efficiently provide purpose-made ChS conjugate compounds. In our previous study, we succeeded in constructing ChS-bound polyethylene glycol and BSA, but did not conduct biological assays [25]. Our present objective is to clarify the relative stabilities of core materials in relation to the attached long ChS chain and oligosaccharide. To achieve this purpose, it is necessary to develop novel ChS-bound probes. Accordingly, we have synthesized fluorogenic peptide **5**, fluorogenic peptide-ChS



◀ **Fig. 2** HPLC profiles of compound 5, 6 and 7. **a** Reversed-phase HPLC (TSKgel ODS 100 V) profile of compound 5 was shown by the solid line and 6 was shown by the dotted line. **b** Size exclusion chromatography HPLC (Shodex OHpak SB-804 HQ) profile of compound 5 was shown by the dotted line and 6 was shown by the solid line. **c** HPLC (YMC Pollyamine II) profile of chondroitinase ABC digestion products of compound 6. Peaks marked with I, II and III indicate the compound 7, 4,5-unsaturated GlcAβ(1–3) GalNAc 6-O-sulfate and 4,5-unsaturated GlcAβ(1–3)GalNAc 4-O-sulfate respectively

6 and **7**, whose relative stabilities can be easily evaluated. The use of these synthetic probes has allowed us for the first time to clarify the relative stabilities of core materials to protease digestion. We found that a long ChS chain decreased the affinity of the substrate to protease. This was enough to stabilize the peptide against protease digestion, compared with a short saccharide chain (*i.e.,* hexasaccharide), and demonstrated the importance of direct binding of the ChS chain to the peptide for effective protection against protease digestion. In addition, we prepared a 4,5-unsaturated GlcA-Gal-Gal-Xyl-fluorogenic peptide by digestion of **7** with chondroitinase AcII [33]. We then investigated the susceptibility of the fluorogenic peptide-tetrasaccharide against protease digestion. There was no significant difference in comparison with **7**. These results have important implications for controlling the stability of core materials.

As another approach, to obtain alkyne-containing glycosaminoglycan (GAG) oligosaccharides, we tried to transglycosylate GAG oligosaccharides to propargyl alcohol utilizing the transglycosylation activity of bovine testicular hyaluronidase [34]. However, no alkyne-containing GAG oligosaccharides were obtained. Therefore it appears that propargyl alcohol is not an appropriate acceptor for the transglycosylation reaction of hyaluronidase.

In conclusion, our present method has made it possible to manipulate long intact glycosaminoglycans with predesigned properties. This proteoglycan glycotechnology will be applicable for comprehensive research on biological phenomena that involve glycosaminoglycans. For instance, it would be useful for high-throughput analytical techniques such as carbohydrate microarrays, surface plasmon resonance analysis (SPR), and quartz crystal microbalance (QCM). In addition, our results suggest that ChS-conjugated therapeutic peptides and proteins can be made to acquire resistance to proteolysis, which would improve their performance as medicines from two viewpoints. First, their resistance to proteolysis would extend their biological half-life, and second, their binding to specific tissues would enable them to deliver drugs precisely to a target site. Thus, addition of ChS would improve the biopharmaceutical properties of drugs. The approach proposed here will be broadly applicable for utilization of ChS.

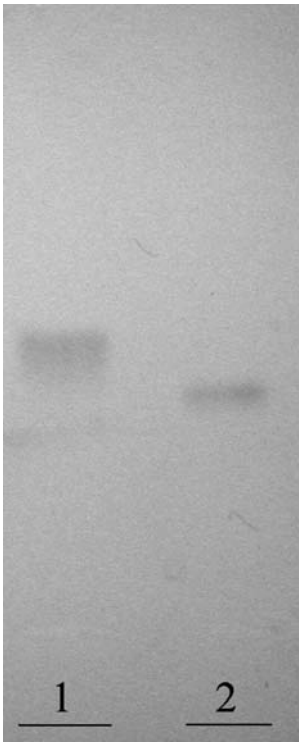


Fig. 3 Electrophoretogram of compound 6 on cellulose acetate membrane. The staining of chondroitin sulfate on the cellulose acetate membrane was carried out with 0.05% alcian blue in 70% ethanol. Lane 1; chondroitin 6-O-sulfate from shark cartilage. Lane 2; compound 6

Experimental section

General methods

Endo-β-xylosidase was isolated from *Patnopecten* mid-gut gland, as described by our previous report [22]. Peptide-ChS (donor) and alkyne containing ChS **1** (from salmon cartilage, average molecular mass, 8100) were prepared from proteoglycan using the procedure described in our previous reports [25, 26]. Chondroitinase ABC protease-free (from *Proteus vulgaris*), chondroitin 6-*O*-Sulfate (from shark cartilage, average molecular mass, 64000) and unsaturated Chondro-Disaccharide Kit were purchased from Seikagaku Kogyo Co. (Tokyo Japan). Trypsin (from bovine pancreas, 10,000 BAEE units/mg protein) was purchased from Sigma-Aldrich (St. Louis, MO). All chemicals used were obtained from commercial sources.

^{1}H and ^{13}C NMR spectra were recorded with a Buruker AV500 spectrometer ^{1}H (400 Hz) and ^{13}C (100 Hz). The values of δ (ppm) are given relative to Me_4Si as the internal standard.

ESI-MS and MS-MS spectra were measured using a PE SCIEX, API 100 and 300. The samples were dissolved in 0.1% formic acid-containing acetonitrile (50 : 50) and injected at 2 μL/min with a micro-HPLC syringe pump.

Amino acids were analyzed by a Hitachi L-8500 amino acid analyzer.

HPLC A high-performance liquid chromatography (Hitachi L-6200, Hitachi Co., Tokyo, Japan) connected to a fluorescence detector (Hitachi F-1050) or a UV-detector (Hitachi L-4200) was used. Purification and analysis of the synthetic peptide and peptide-ChS were carried out using reverse-phase chromatography TSKgel ODS-100 V (150×4.6 mm, TOSOH Co., Tokyo, Japan) and size exclusion chromatography Shodex OH pack SB-804 HQ (300×8.0 mm, Showa Denko

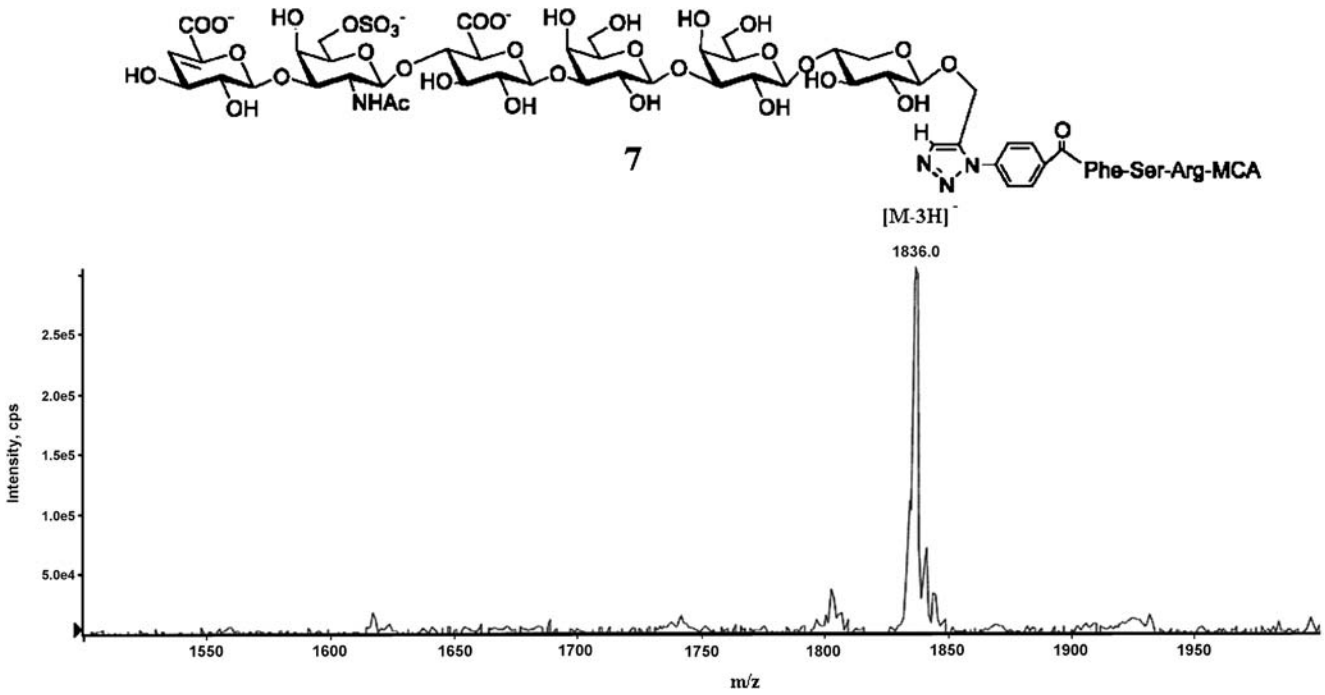


Fig. 4 ESI-MS spectrum of compound 7

Table 1 *Km* values of trypsin for three substrates 5, 6 and 7

Substrate	Km (M)
Fluorogenic peptide 5	8.4×10^{-5}
Fluorogenic peptide-ChS 6	5.7×10^{-4}
Fluorogenic peptide-hexasaccharide 7	3.8×10^{-4}

Co., Kawasaki, Japan). The flow rate was 1 ml/min and column temperature 40°C. The eluates were monitored at excitation and emission wavelengths of 330 and 400 nm, respectively. The elution conditions were described as follows: TSKgel ODS=100 V column, elution buffer A: 0.1% TFA in H_2O; elution buffer B: 0.1% TFA in MeCN. The linear gradient was A : B=80 : 20 to A : B=50 : 50 after 30 min. SB-804 HQ column was eluted with 0.2 M NaCl : MeOH=80 : 20.

Analysis and purification of chondroitinase ABC digestion products were carried out with a Polyamine II column (250×4.6 mm, YMC Co., Tokyo, Japan) at a flow rate of 1 ml/min and a column temperature of 40°C. The eluates were monitored by absorbance at 232 nm. Elution buffer A: 10 mM NaH_2PO_4; elution buffer B: 1 M NaH_2PO_4. The linear gradient was A : B=100 : 0 to A : B=60 : 40 after 40 min.

Cellulose acetate membrane electrophoresis was carried out using Separax (6×22 cm, Jookoo Co., Tokyo, Japan) in formic acid-pyridine buffer (0.1 M, pH 3.0) at 1 mA/cm for 10 min. Staining of ChS on the cellulose acetate membrane was carried out with 0.05% alcian blue in 70% ethanol.

Azido group-containing fluorogenic peptide **5**

To a solution of **4** (15.4 mg, 23.9 μM) in CH_2Cl_2 (1.2 mL) was added trifluoro acetic acid (0.4 mL) at 0°C. The reaction mixture was stirred for 2 h at 0°C, then 10% TEA

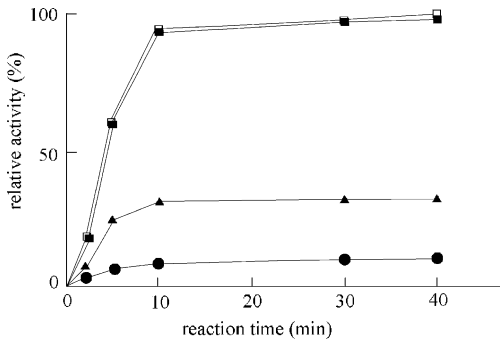


Fig. 5 The relative activity of trypsin toward compound 4, 5, 6 and 7. The activity shows the relative rate of the 7-amino-4-methylcoumarin (AMC) when the compound 4 was completely digested by trypsin is 100%. Compound 4, open squares; compound 5, closed squares; compound 6, closed circles; compound 7, closed triangles

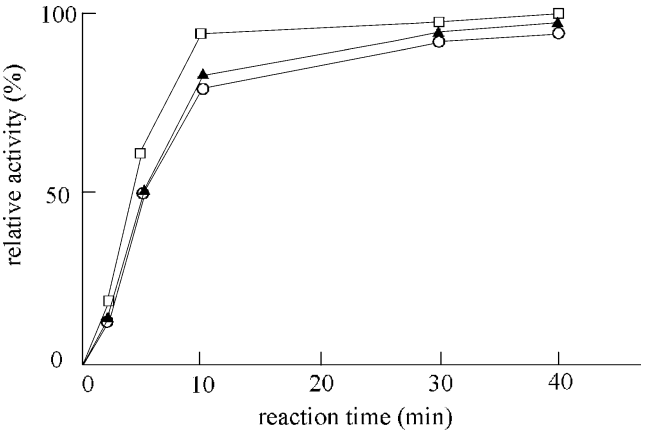


Fig. 6 The relative activity of trypsin toward compound 5 in the presence of different concentrations of ChS 1. 0 w% ChS, open squares; 0.5 w% ChS, closed triangles; 1 w% ChS, open circles

in CH_2Cl_2 (1 mL) was added to the reaction mixture. The reaction mixture was concentrated, the residual syrup was treated with 4-azido benzoic acid (4.67 mg, 28.6 μM), DMT-MM (15.7 mg, 5.71 μM) and NMM (4.5 μL, 34.3 μM) in MeOH (1.1 mL), and the mixture was stirred for 24 h at room temperature under a N_2 atmosphere, and then concentrated. The obtained residue was purified on HPLC (TOSO TSKgel ODS-100 V) to yield **5** (11.0 mg, 65%) as an amorphous mass. RP-HPLC: t_R=23.02 min (Fig. 2a: solid line) and SEC-HPLC: t_R=15.62 min (Fig. 2b: dotted line). H^1 NMR 400 MHz (CD_3OD): δ=7.84–7.09 (12 H, Ar), 6.27 (s, 1 H), 4.79 (m, 1 H), 4.61 (m, 1 H), 4.44 (m, 1 H), 3.97 (m 1 H), 3.85 (dd, 1 H, *J*=4.8 and 8.8 Hz), 3.28–3.24 (m, 2 H), 3.14 (dd, 1 H, *J*=7.6 and 10.8 Hz), 2.47 (s, 3 H), 2.13 (m, 1 H), 1.87 (m, 1 H), 1.76 (m, 2H). C^{13} NMR 100 Hz (CD_3OD): δ=174.45, 172.81, 172.26, 163.10, 155.32, 155.28, 155.21, 143.06, 138.59, 130.40, 130.26, 129.56, 127.90, 126.68, 119.92, 117.46, 117.27, 113.79, 108.98, 108.26, 108.19, 93.29, 62.64, 57.33, 57.22, 54.90, 41.96, 41.60, 38.06, 34.86, 29.78, 26.35, 18.50. ESI MS calcd. for $C_{35}H_{38}N_{10}O_7$ $[M + H]^+$ 711.3, found 711.4. Amino acid analysis by hydrolysis with 6 M HCl at 150°C for 2 h: $Ser_{1.01}Phe_1Arg_{1.14}$.

Fluorogenic peptide-ChS **6**

To a solution of **1** (3 mg) in H_2O (300 μL) were added **5** (20 μL; 10 mM in DMSO), $CuSO_4$ (40 μL; 10 mM) and sodium ascorbate (40 μL; 10 mM). The reaction mixture was vortexed and allowed to react at 37°C for 3 h under a N_2 atmosphere. Column chromatography (H_2O : EtOH=9 : 1) of the residue on Sephadex G-25 gave the crude target molecule, and this was purified using a C18-Sep-Pac cartridge (MeOH : H_2O=0 : 100 to 50 : 50) to afford **6** (1.6 mg, quant. Based on peptide **5**). Compound **6** was analyzed by RP-HPLC (t_R=16.31 min, Fig. 2a: dotted line)

and SEC-HPLC (t_R=7.18 min, Fig. 2b: solid line). Electrophoresis of **6** was carried out on a cellulose acetate membrane and stained with alcian blue. The conditions and results are summarized in Fig. 3.

H^1 NMR 400 MHz (DMSO-d_6): δ=7.01 (br–d), 5.48 (m), 5.03 (m), 4.88 (m), 4.76 (br–d), 4.56 (d, *J*=4.4 Hz), 4.51 (d, *J*=4 Hz), 3.65–3.16 (m), 3.06 (m), 2.08 (s, AcN).

Fluorogenic peptide-hexasaccharide **7**

To a solution of **6** (0.8 mg) in H_2O (75 μL) were added 400 mM Tris-HCl buffer (pH 8.0, 10 μL), 400 mM AcONa buffer (pH 5.0, 10 μL), 0.1% BSA in H_2O solution (10 μL) and chondroitinase ABC (0.5 U) at 37°C. The reaction mixture was incubated at 37°C for 24 h with gentle agitation. The reaction was terminated by immersion in a boiling water bath for 3 min, followed by filtration. The resulting solution was purified by HPLC (YMC-Pack Polyamine II) affording **7**. HPLC: t_R=4.77 min (Fig. 2c). ESI-MS calcd. for $C_{75}H_{97}N_{11}O_{41}S$ $[M\text{-}3H]^-$ 1836.5, found 1836.0.

Measurement of protease (trypsin) activity on three synthetic compounds **5**, **6** and **7**

Fluorogenic assay was performed in 0.4 mL of 10 mM Tris-HCl buffer, pH 8.3, containing 0.1 mM $CaCl_2$, using a Hitachi F-1050 spectrometer. Fluorogenic peptide was added to a cuvette thermo stated at 37°C, and the liberated 7-amino-4-methylcoumarin (AMC) was measured. The enzyme activity was expressed as of nmol of AMC liberated per min. The *Km* values were determined by a double-reciprocal plot of the data.

Protease (trypsin) digestion of **4**,**5**, **6** and **7**

The substrates **4**, **5**, **6** and **7** were added to a different cuvette, respectively, in 0.1 M Tris-HCl buffer (pH 8.3), containing 0.1 mM $CaCl_2$ at 37°C, each at a final concentration of 6.68 μM, and the reaction was started by addition of trypsin (66 ng). The 7-amino-4-methylcoumarin (AMC) liberated from these fluorogenic substrates was then quantified for 30 s, 5 min, 10 min, 30 min, and 40 min with excitation at 380 nm and emission at 460 nm using a fluorescence spectrophotometer.

Protease (trypsin) digestion of **5** in the presence of different concentrations of free ChS **1**

The substrate **5** (6.68 μM final concentration) and free ChS **1** (0 wt%, 0.5 wt% and 1 wt% final concentration) were added to a different cuvette, respectively, in 0.1 M Tris-HCl buffer (pH 8.3), containing 0.1 mM $CaCl_2$, in final volumes of 0.4 mL. The subsequent procedures were the same as those used in the prior experiment.

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