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The effect of substitution of the *N*-acetyl groups of *N*-acetylgalactosamine residues in chondroitin sulfate on its degradation by chondroitinase ABC

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Abstract Chondroitinase ABC is a lyase that degrades chondroitin sulfate, dermatan sulfate and hyaluronic acid into disaccharides. The purpose of this study was to determine the ability of chondroitinase ABC to degrade chondroitin sulfate in which the *N*-acetyl groups are substituted with different acyl groups. The bovine tracheal chondroitin sulfate A (bCSA) was *N*-deacetylated by hydrazinolysis, and the free amino groups derivatized into *N*-formyl, *N*-propionyl, *N*-butyryl, *N*-hexanoyl or *N*-benzoyl amides. Treatment of the *N*-acyl or *N*-benzoyl derivatives of bCSA with chondroitinase ABC and analysis of the products showed that the *N*-formyl, *N*-hexanoyl and *N*-benzoyl derivatives are completely resistant to the enzyme. In contrast, the *N*-propionyl or *N*-butyryl derivatives were degraded into disaccharides with slower kinetics compared to that of unmodified bCSA. The rate of degradation of bCSA derivatives by the enzyme was found to be in the order of *N*-acetyl>*N*-propionyl>>*N*-butyryl bCSA. These results have important implications for understanding the interaction of *N*-acetyl groups of glycosaminoglycans with chondroitinase ABC.

Keywords Chondroitin sulfates · *N*-acetyl group substitution · Chondroitinase ABC degradation

Abbreviations

CS	chondroitin sulfate
C4S	chondroitin 4-sulfate
C6S	chondroitin 6-sulfate
DS	dermatan sulfate
HA	hyaluronic acid
GAG	glycosaminoglycan
CSPG	chondroitin sulfate proteoglycan
DSPG	dermatan sulfate proteoglycan
bCSA	bovine tracheal chondroitin sulfate A
GlcA	glucuronic acid
IdA	iduronic acid
GalNAc	*N*-acetylgalactosamine
GlcNAc	*N*-acetylglucosamine
Gal	galactose
Xyl	xylose
Glc	glucose
IRBCs	infected red blood cells
BD	blue dextran
EDAC	*N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide
MES	2-(*N*-morpholino)ethanesulfonic acid

Introduction

Chondroitin sulfate ABC lyase (EC 4.2.2.4) from *Proteus vulgaris* exhibits broad substrate specificity toward several

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glycosaminoglycans (GAGs). It can quantitatively degrade various chondroitin sulfates (CSs), dermatan sulfate (DS), and hyaluronic acid (HA) into the disaccharide moieties by β-elimination reaction that introduces a double bond between C-4 and C-5 of uronic acid [1]. The enzyme has been widely used for the structural determination of CS and DS chains of proteoglycans and for the preparation and characterization of core proteins of CS and DS proteoglycans (CSPGs and DSPGs). The enzyme has also been used for the generation of unsaturated disaccharide antigenic epitopes on core proteins and immunohistochemical probing of CSPGs and DSPGs in cells and tissues [2–6]. The commonly used commercially available chondroitinase ABC is a mixture of two distinct lyases, chondroitinase ABC I, and chondroitinase ABC II [1, 7]. Both enzymes exhibit similar activity in that they specifically cleave the β(1–4) glycosidic bond between *N*-acetyl-D-galactosamine (GalNAc) and D-glucuronic acid (GlcA) of the GAGs. However, they differ considerably with regard to substrate recognition. Chondroitinase ABC I catalyzes endolytic cleavage of the glycosidic bonds between GalNAc or GlcNAc and GlcA or L-iduronic acid (IdA) residues of CSs, DS and HA to form tetrasaccharides, while chondroitinase ABC II exolytically cleaves nonreducing end disaccharide residues in the oligosaccharides of these GAGs [1].

In animals, GAGs play important biological roles. CS, DS and HA are polysaccharides occur in most organisms. They are abundantly found in connective tissues and tissue matrices [8, 9]. CS also occurs almost ubiquitously on cell surfaces. Structurally, these GAGs share a common feature in that they are linear acidic polysaccharides consisting of alternating residues of uronic acid (GlcA in CS and HA, and IdA in DS) and hexosamine (GalNAc in CS and DS, and GlcNAc in HA). CS and DS consist of, respectively, -4GlcAβ1-3GalNAcβ1- and -4IdAβ1-3GalNAcβ1- disaccharide-repeat units, whereas HA is made up of -4GlcAβ1-3GlcNAcβ1- disaccharide repeat units. Further, while CSs are sulfated at either C-4 and/or C-6 of GalNAc and DS are sulfated at C-4 of GalNAc (in some CSs and DSs additional sulfation occurs at C-2 of GlcA and IdA), HA is nonsulfated [10–12]. In some CSs, a portion of the GalNAc residues are substituted at C-4, while others at C-6, thus forming copolymers with 4-sulfated and 6-sulfated disaccharides moieties [10]. Additionally, in most CSs and DSs, the sulfation of disaccharide moieties is usually not complete and, therefore, they also contain significant levels of nonsulfated disaccharides. The structures of CS and DS from different tissue sources vary enormously with respect to size, level and distribution of sulfate groups, and in the ratio of GlcA and IdA. Thus, differences in the degree of sulfation and levels of GlcA and IdA constitute distinctive CSs and DSs [11, 12]. For example, chondroitin 4-sulfate (C4S, consists of -4GlcAβ1-3GalNAc(4S)β1- repeats), chondroitin 6-sulfate (C6S, made up of -4GlcAβ1-3GalNAc(6S)β1- repeats), chondroitin 2,6-disulfate (C2,6S, with -4GlcA(2S)β1-3GalNAc(6S)β1- repeats), and chondroitin 4,6-disulfate (C4,6S, with -4GlcAβ1-3GalNAc (4,6diS)β1- repeats) [13–16]. In animal tissues, HA occurs exclusively as free ploysaccharide chain, whereas CS and DS are present as conjugates of proteins, such as CSPG and DSPG in which GAG chains are linked to Ser residues of protein *via* a tetrasaccharide core, -4GlcAβ1-3Galβ1-3Galβ1-4Xylβ1- [10–16]. The GAGs play important roles in a number of biological processes, including tissue organization, differentiation and development, embryogenesis, nerve function, bone formation, wound healing, aging, arthritis, tumorogenesis and many other pathological processes [17–22]. Therefore, the structural characterization of the GAGs is important for understanding their functions and, in this regard, chondroitinase ABC has been a very valuable and extensively used tool.

GAGs have also been shown to mediate cell- and tissue-specific recognition, attachment and invasion of many microorganisms, including the adherence of *Plasmodium falciparum*-infected red blood cells (IRBCs) in human placenta [23]. In the case of *P. falciparum*, very low-sulfated C4S chains of aggrecan family CSPG have been shown as receptors for sequestration of IRBCs in the placenta [24–26]. Thus, adherence of IRBCs in the placenta causes various clinical conditions, including low birth weight, abortion, still birth, and morbidity and mortality of the mother [27–29]. Our laboratory has been investigating the structure-function relationship in the interaction of IRBCs with C4S and gained considerable insight [30–32]. In an extension of these studies, we have been interested in identifying parasite adhesive protein(s), expressed on the IRBC surface, involved in C4S binding. In order to develop specific C4S-based photoaffinity reagents for the identification of IRBC adhesive protein, we studied the roles of key functional groups of C4S [33]. We found that the *N*-acetyl groups of C4S are not required for C4S-IRBC binding, and that the substitution of *N*-acetyl groups of C4S with various *N*-acyl moieties or *N*-benzoyl moiety had no effect in C4S-IRBC binding activity. While attempting to characterize the *N*-acyl or *N*-benzoyl derivatives of C4S using chondroitinase ABC, we noted that some *N*-acyl derivatives of C4S are slowly degraded, while others are completely resistant to the action of the enzyme, suggesting that the *N*-acetyl groups are important either in the binding of GAGs or in the catalysis of the β-elimination reaction by chondroitinase ABC. A survey of the literature showed that no information is available regarding the effect of substitution of the *N*-acetyl groups of CS and related GAGs with different *N*-acyl groups on the ability of chondroitinase ABC to degrade *N*-acyl derivatives. Studies

aimed at understanding the structure-activity relationship of CS and other related GAGs may require modification of their *N*-acetyl groups with other *N*-acyl groups. Because chondroitinase ABC is an important tool for characterization of GAGs, it is important to understand in detail the ability of chondroitinase ABC to recognize and degrade

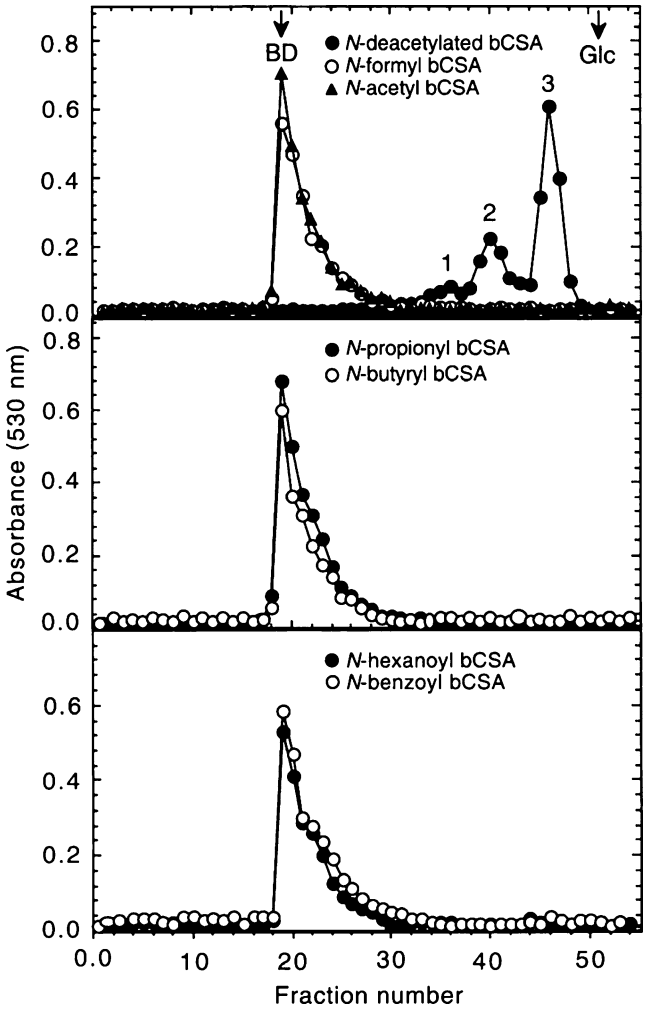

Fig. 1 Analysis of nitrous acid-degradation products of bCSA and its *N*-acyl derivatives by Bio-Gel P-10 column chromatography. bCSA was *N*-deacetylated with anhydrous hydrazine containing 1% hydrazine sulfate and then *N*-acylated using acid anhydrides or by EDAC activated formic acid. The extent of *N*-acylation was determined by treating the *N*-acyl derivatives of bCSA (200 μg) with nitrous acid [37] followed by analysis of products on Bio-Gel P-10 columns (1.5×70 cm) using 0.2 M sodium chloride. In each case, half of the column fractions were dried, dissolved in 200 μl of water, and 50 μl aliquots assayed for uronic acid by the carbazole method [39]. Only *N*-deacetylated bCSA was susceptible (top panel); peaks 1, 2, and 3 correspond to CSA hexa- and tetra- and disaccharides, respectively. None of the *N*-acylated bCSA derivatives was degraded to a noticeable extent by nitrous acid, suggesting that in each case the free amine groups were derivatized completely. Arrows indicate the elution positions of blue dextran (BD, void volume) and glucose (Glc). Note: two disaccharide repeats in the hexasaccharide (peak 1) and one disaccharide moiety in the tetrasaccharide (peak 2) contain *N*-acetyl groups, which were not cleaved during hydrazinolysis

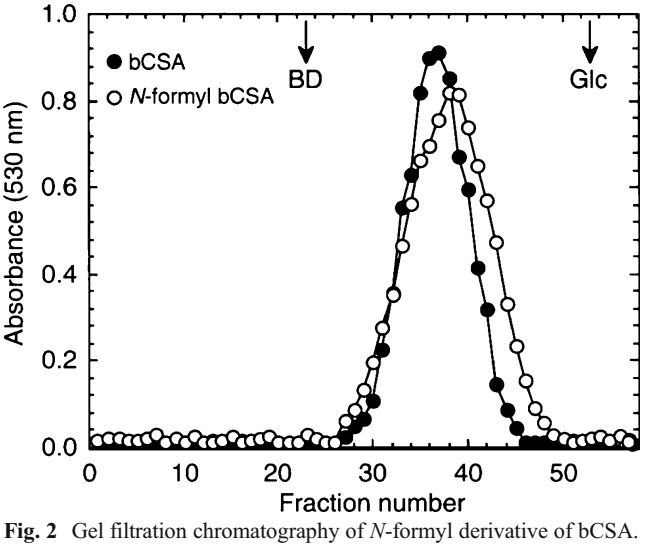

Fig. 2 Gel filtration chromatography of *N*-formyl derivative of bCSA. The *N*-deacetylated bCSA was *N*-formylated using EDAC-activated formic acid. The *N*-formyl CSA and unmodified CSA (200 μg each) were chromatographed separately on Sepharose CL-6B column (1× 48 cm) using 0.2 M NaCl at a flow rate of 16 ml/h. Fractions (0.67 ml) were collected and aliquots analyzed by the carbazole method [39]. Arrows indicate the elution positions of blue dextran (BD, void volume) and glucose (Glc)

GAGs modified with various *N*-acyl groups. Thus, in this study, we determined the effect of substitution of the *N*-acetyl groups of GalNAc in CS with either shorter (formyl) or bulkier (propionyl, butyryl, hexanoyl or benzoyl) *N*-acyl groups in the degradation of CS derivatives by chondroitinase ABC. Our results showed that the *N*-formyl, *N*-hexanoyl and *N*-benzoyl derivatives of CS are completely resistant to chondroitinase ABC, whereas the *N*-propionyl and *N*-butyryl derivatives undergo degradation, albeit at much slower rates.

Materials and methods

Materials

Proteus vulgaris chondroitinase ABC (110 units/mg) was purchased from Seikagaku America (Falmouth, MA). bCSA, anhydrous hydrazine, hydrazine sulfate, EDAC, formic acid, and acetic, propionic, butyric, hexanoic, and benzoic anhydrides were obtained from Sigma-Aldrich (St. Louis, MO). Sepharose CL-6B, and blue dextran were from Amersham-GE, (Piscataway, NJ). Bio-Gel P-10 was from Bio-Rad (Hercules, CA).

N-Deacetylation

N-Deacetylation of CSA was carried out according to Shaklee et al. [34]. Freshly dried bCSA (70 mg) was dissolved in anhydrous hydrazine (1.2 ml) containing 1%

hydrazine sulfate in a glass ampoule and heated under nitrogen atmosphere at 105°C. After 5 h, the reaction mixture was cooled, freeze-dried, and dissolved in 0.25 M iodic acid (1 ml). The released iodine was removed by extraction with chloroform, and pH of the solution adjusted to 7.5 with 1 M sodium hydroxide. The product was dialyzed and lyophilized.

N-Acylation or *N*-benzoylation

Prior to *N*-acylation, the *N*-deacetylated bCSA (2 mg) in PBS (500 μl) was dialyzed extensively against saturated sodium bicarbonate in water. *N*-Acylation was carried out by adding, at 20 min intervals, 3×50 μl of a 10% solution of respective anhydrides (acetic, propionic, butyric, hexanoic and benzoic) in acetone at room temperature [35]. The reaction products were recovered by dialysis and lyophilization. The extent of modification was determined by nitrous acid treatment followed by the analysis of the products on Bio-Gel P-10 columns. In the case of bulky anhydrides such as hexanoic and benzoic anhydrides, the *N*-acylation reaction was repeated two times to achieve complete derivatization.

N-Formylation

Since the anhydride of formic acid is not available, *N*-formylation was carried out using formic acid and EDAC

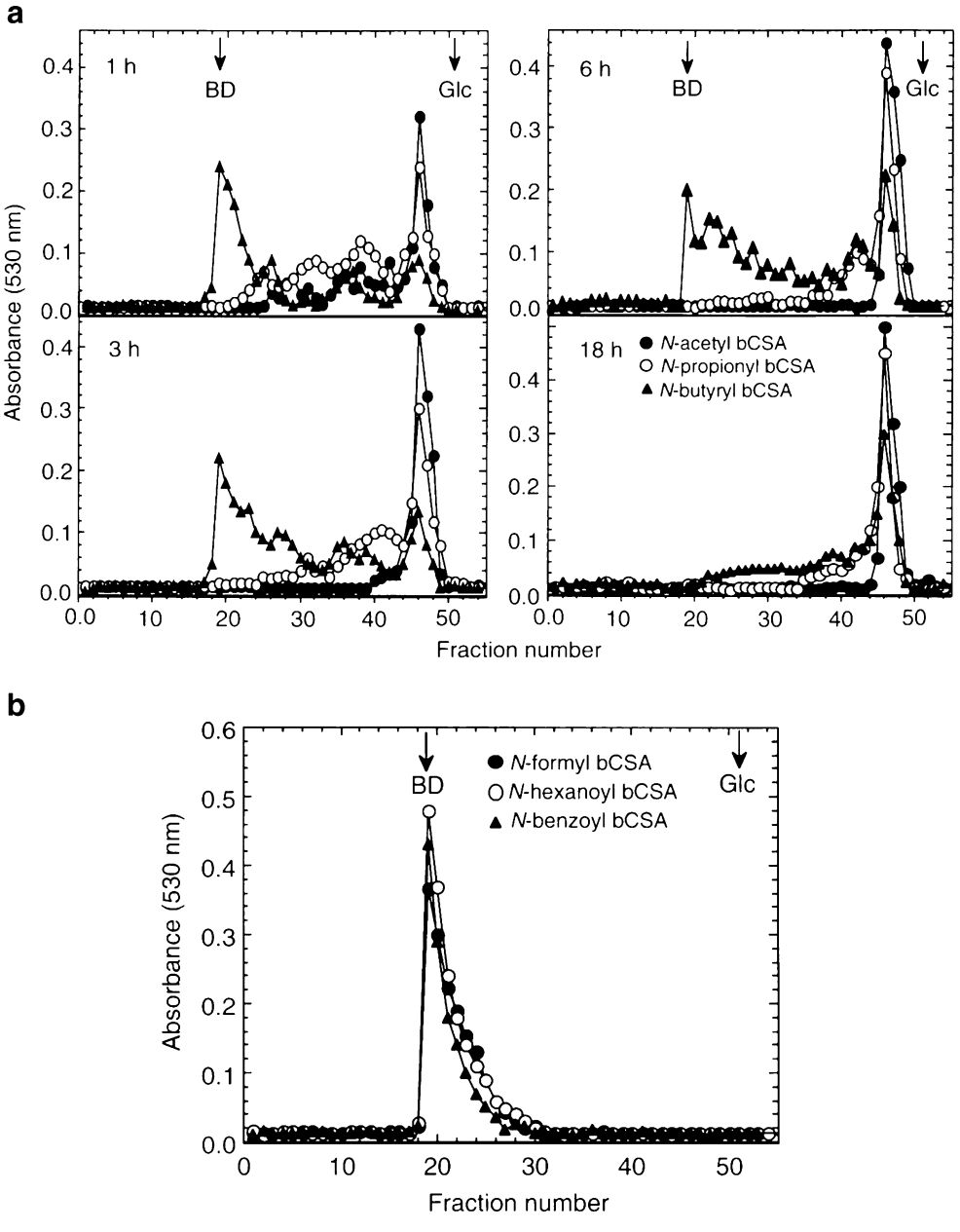

Fig. 3 Analysis of chondroitinase ABC-degradation products of bCSA and its *N*-acyl derivatives by Bio-Gel P-10 column chromatography. Panel **a**: bCSA and the *N*-acyl derivatives of bCSA (100 μg each) were treated with chondroitinase ABC (50 mU) at 37°C for 1, 3 or 6 h. For an aliquot of each, after 6 h incubation additional chondroitinase ABC (50 mU) was added and incubated for 12 h. The products were chromatographed on Bio-Gel P-10 columns (1.5×70 cm) using 0.1 M pyridine and 0.1 M acetic acid mixture, pH 5.5. In each case, fractions (2 ml) were collected, dried, dissolved in 100 μl water, and 25 μl aliquots assayed for uronic acid by the carbazole method [39]. Symbols for different curves in all four panels of Fig. 3**a** are as shown in the last panel. Arrows indicate the elution positions of blue dextran (BD, void volume) and glucose (Glc). Panel **b**: The indicated derivatives of bCSA were incubated with chondroitinase ABC for 6 h and the products were analyzed as outlined in Panel **a**

[36]. To a solution of formic acid (~60 mg) in 500 µl of 0.05 M MES buffer, pH 4.5, solid EDAC (50 mg) was added with continuous stirring and maintaining pH at 4.5 by the addition 1 M of NaOH. After 3 h at room temperature, the *N*-deacetylated bCSA (2 mg) was added and the reaction mixture was stirred further for 2 h, dialyzed against water, and lyophilized. The above steps were repeated twice, on the recovered product, to achieve complete derivatization. The resulting *N*-formyl bCSA derivative was treated with 0.1 M NaOH under nitrogen atmosphere at room temperature for 12 h to hydrolyze any carboxyl groups esterified with EDAC, dialyzed and lyophilized. About 200 µg of *N*-formyl bCSA was analyzed by gel filtration on Sepharose CL-6B.

Determination of the degree of *N*-acylation or *N*-benzoylation

The *N*-deacetylated, *N*-acylated or *N*-benzoylated bCSA (200 µg), was dissolved in 200 µl of 40 mM sodium acetate buffer, pH 3.8, and incubated with an equal volume of 1 M sodium nitrite at room temperature for 24 h [37]. Excess nitrous acid was destroyed by the addition of 250 µl of 1 M glycine. The reaction products were analyzed on Bio-Gel P-10 columns (1.5×70 cm).

Degradation of C4S derivatives by chondroitinase ABC

The *N*-acyl and *N*-benzoyl bCSA (~100 µg each) were digested with chondroitinase ABC (50 mU) in 50 µl of 100 mM Tris-HCl, pH, 8.0, containing 30 mM NaOAc and 0.01% bovine serum albumin at 37°C for 1, 3 and 6 h [38]. After 6 h additional 50 mU of the enzyme was added and incubated for further 12 h.

Size-exclusion chromatography

The unmodified and *N*-acetyl group-substituted bCSA (200 µg each) were analyzed on Sepharose CL-6B (1× 48 cm) columns using 0.2 M sodium chloride at a flow rate of 16 ml/h. The nitrous acid- or chondroitinase ABC-degradation products of bCSA and its *N*-acyl derivatives were chromatographed on Bio Gel P-10 columns (1.5× 70 cm) with 0.1 M pyridine and 0.1 M acetic acid buffer, pH 5.5. Fractions (0.67 ml in the case of Sepharose CL-6B column or 2 ml in the case of Bio-Gel P-10 column) were collected and aliquots analyzed for uronic acid content by the carbazole assay [39].

HPLC analysis

The unsaturated disaccharides isolated by chromatography on Bio-Gel P-10 column (fractions 45–48 in a run similar to that shown in Fig. 3a) after digestion of *N*-acyl derivatives of chondroitin sulfates with chondroitinase ABC for 6 h were analyzed on an amine-bonded silica PA03 column (YMC Inc, Milford, MA) using a 600E HPLC system (Waters, Milford, MA) [40]. Approximately 10 µg of disaccharides were injected to the HPLC column and eluted with a linear gradient of 16–530 mM NaH_2PO_4 over a period of 70 min at room temperature at a flow rate of 1 ml/min. The elution of disaccharides was monitored by measuring the absorption at 232 nm using a Waters model 484 variable wavelength UV detector. The data was processed with the Millennium 2010 chromatography manager using NEC PowerMate 433 data processing system.

Results and discussion

In the present study, bCSA consisting of 52% 4-sulfated, 39% 6-sulfated, and 9% non-sulfated disaccharides was used. To determine the effect of substitution of the *N*-acetyl groups of GalNAc in the binding and degradation of CS by chondroitinase ABC, the acetyl groups of bCSA were replaced with other acyl or benzoyl groups. *First*, the acetyl groups were removed by *N*-deacetylation with hydrazine in the presence of 1% hydrazine sulfate. The extent of *N*-deacetylation by hydrazinolysis was assessed by chromatography of the products formed by the nitrous acid

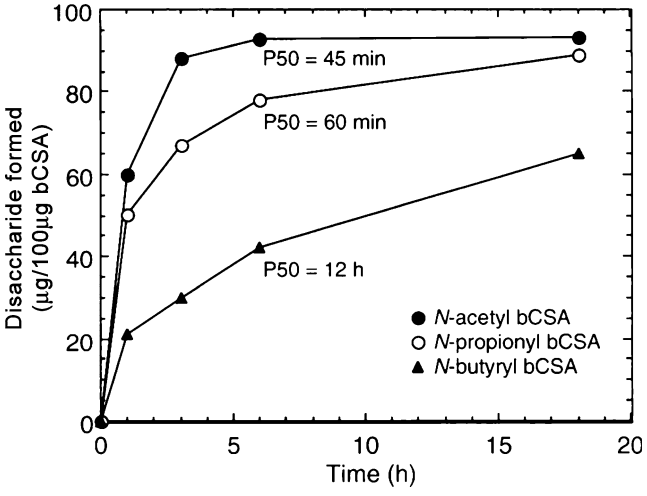

Fig. 4 Determination of the rate of disaccharide formation from *N*-acyl bCSA derivatives treated with chondroitinase ABC. Unmodified bCSA, and *N*-propionyl and *N*-butyryl bCSA (100 µg each) were treated with chondroitinase ABC for various time periods as described under "Materials and Methods." The products were chromatographed on Bio-Gel P-10 columns (see Fig. 3). The amount of disaccharide formed under each incubation condition was estimated by the carbazole method [39] and the values were plotted against the incubation time. The time required for the conversion of 50% of bCSA or bCSA derivative into respective disaccharides (P50) is indicated for each curve

degradation of the *N*-deacetylated bCSA on Bio-Gel P-10. As expected, nitrous acid degraded the *N*-deacetylated bCSA predominantly into disaccharides (peak 3 in Fig. 1; major). Significant levels of tetra- and hexasaccharides, respectively, peaks 2 and 1 in Fig. 1, were also formed. The formation of tetra- and hexasaccharides is due to the presence of *N*-acetyl groups that were not removed by hydrazinolysis. Since nitrous acid cleaves the bond between GalN (formed by *N*-deacetylation) and GlcA residues, but not that between GalNAc and GlcA residues, the tetra- and hexasaccharides should contain, respectively, one and two disaccharide moieties with GalNAc. Considering this and the areas under the peaks 1, 2, and 3, it is estimated that about 85% of the *N*-acetyl groups of bCSA were removed by hydrazinolysis and ~15% were resistant (Fig. 1). Re-*N*-acetylation of the hydrazine-treated bCSA and analysis on Sepharose CL-6B showed that hydrazinolysis did not cause significant depolymerization (not shown).

Second, the free amino groups of *N*-deacetylated bCSA were converted into various amide groups by *N*-acylation using either acyl anhydrides or by carbodiimide-activated coupling of formic acid. Thus, *N*-propionyl, *N*-butyryl, *N*-hexanoyl and *N*-benzoyl derivatives were prepared by treatment of the *N*-deacetylated bCSA with the respective acid anhydrides using a procedure similar to that used for *N*-acetylation of primary amine groups [35]. With propionic and butyric anhydrides, a single treatment step in three aliquots was sufficient to quantitatively *N*-acylate the amino groups in *N*-deacetylated bCSA. In the case of *N*-acylation with hexanoic and benzoic anhydrides, the partially *N*-acylated product on Bio-Gel P-10 was isolated and the treatment was repeated twice to achieve quantitative derivatization of the amino groups. In the case of *N*-formylation, because the respective anhydride is not available, formic acid was coupled by converting the carboxyl groups into activated ester groups with EDAC [36]. Upon gel filtration chromatography, the product, *N*-formylated bCSA, was eluted at volume similar to that of unmodified bCSA and no detectable higher molecular weight product was observed (Fig. 2). These results suggested that the derivatization procedure using EDAC did not cause intermolecular cross-linking of *N*-deacetylated bCSA by amide bond formation between amine and carboxyl groups. The degree of *N*-acylation or *N*-benzoylation, in the above bCSA derivatives, was

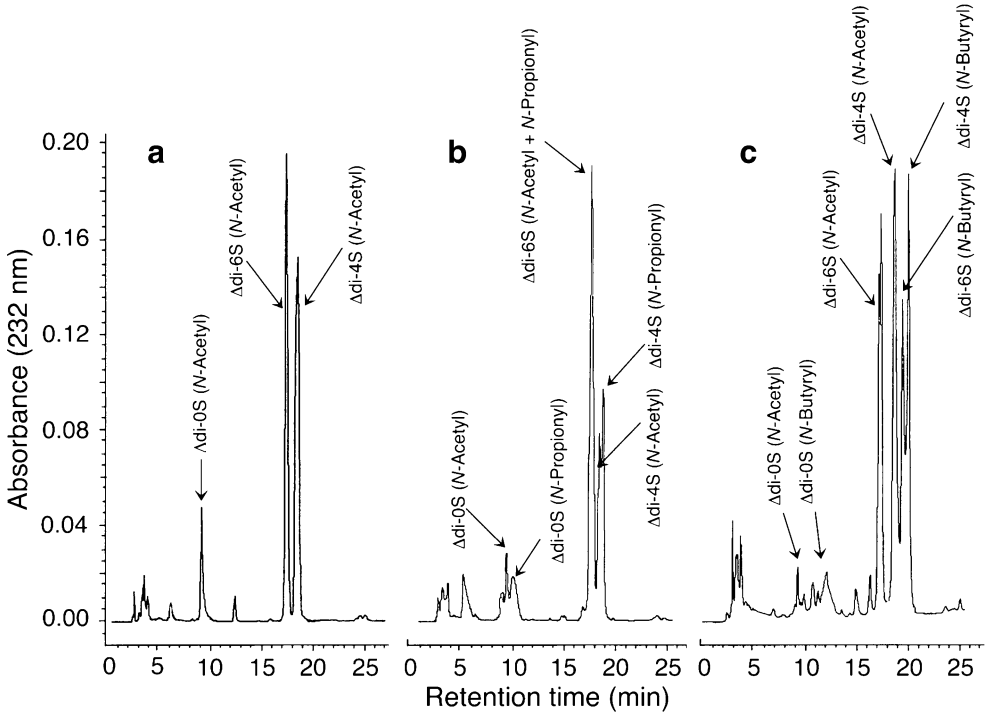

Fig. 5 HPLC analysis of disaccharides formed by the degradation of *N*-acyl derivatives of bCSA by chondroitinase ABC. The unmodified bCSA, and *N*-propionyl and *N*-butyryl bCSA (100 μg each) were treated with chondroitinase ABC for 6 h and products chromatographed on Bio-Gel P-10 as outlined in Fig. 3 using 0.1 M pyridine and 0.1 M acetic acid mixture, pH 5.5, as eluent. In each case, fractions corresponding to disaccharide peak (fractions 45–48) were pooled and dried. About 10 μg of disaccharide samples were analyzed on an amine-bonded silica PA03 column by HPLC using a linear gradient of 16–530 mM NaH_2PO_4 over a period of 70 min at room temperature at a flow rate of 1 ml/min. The elution of the disaccharides was monitored by measuring absorption at 232 nm. Panel **a**, disaccharides formed from unmodified bCSA; panel **b**, disaccharides from *N*-propionyl bCSA; panel **c**, disaccharides from *N*-butyryl bCSA. Note: Δdi-0S=unsaturated disaccharide with non-sulfated *N*-acylgalactosamine; Δdi-6S=unsaturated disaccharide with 6-sulfated *N*-acylgalactosamine; Δdi-4S=unsaturated disaccharide with 4-sulfated *N*-acylgalactosamine

assessed by degradation with nitrous acid. In contrast to the quantitative degradation of *N*-deacetylated bCSA, no measurable levels of di-, tetra- or higher oligosaccharides were formed from the *N*-acyl derivatives of bCSA, revealing that almost all amino groups were converted into the respective amides (Fig. 1).

Third, the ability of chondroitinase ABC to degrade variously *N*-acylated or *N*-benzoylated derivatives of bCSA was analyzed. Chondroitinase ABC could quantitatively degrade the re-*N*-acetylated product of *N*-deacetylated bCSA into disaccharides (Fig. 3a, closed circles). In contrast, the *N*-formyl, *N*-hexanoyl, and *N*-benzoyl derivatives of bCSA were resistant to the action of the enzyme; no noticeable levels of di-, tetra- or higher oligosaccharides were formed (Fig. 3b). In the case of *N*-propionylated and *N*-butyrylated bCSA, the enzyme could degrade the polysaccharide derivative, but at much slower rates compared to *N*-acetylated bCSA (Fig. 3a).

To determine the rate of degradation of *N*-acyl derivatives that are susceptible to chondroitinase ABC, we measured the amount of disaccharide formed from 100 μg of each *N*-acyl bCSA at various time points (Fig. 4). Under the enzyme incubation condition used, 50% of *N*-acetyl bCSA was converted into disaccharides in 45 min (P50= 45 min), whereas in the case of *N*-propionyl bCSA and *N*-butyryl bCSA, 50% of the substrates converted into disaccharides at 60 min and 12 h (P50=60 min and 12 h), respectively. Incubation for further 12 h with an additional equal amount of enzyme added after 6 h resulted in the conversion of ~90 and ~70% of *N*-propionyl and *N*-butyryl bCSA derivatives into disaccharides; in each case, remainder of bCSA derivative was degraded into larger oligosaccharides (Fig. 3a). These results suggest that *N*-propionyl and *N*-butyryl groups cause significant steric constraints for binding of the substrate and/or catalysis of glycosidic bond cleavage by the enzyme.

HPLC analysis of the disaccharides released by chondroitinase ABC treatment showed that disaccharides released from *N*-propionyl and *N*-butyryl bCSA are distinctly different (strikingly different in the case of *N*-butyryl bCSA) from those of unmodified bCSA as revealed by the significant differences in the retention time of disaccharides (Fig. 5, compare panels **b** and **c** with panel **a**). In both *N*-propionyl and *N*-butyryl bCSA, significant amounts of disaccharides with GalNAc residues were present; these disaccharides correspond to the disaccharide repeats with *N*-acetyl groups, which were not removed during hydrazinolysis. In the case of *N*-propionyl derivative of bCSA, while Δdi-0S and Δdi-4S with *N*-propionyl groups were partially separated from the corresponding disaccharides with *N*-acetyl groups, Δdi-6S with *N*-propionyl groups were not resolved from those with *N*-acetyl groups (compare panel **b** with panel **a** in Fig. 5). In *N*-butyryl bCSA, Δdi-0S, Δdi-6S and Δdi-6S with *N*-acetyl groups were resolved from the corresponding oligosaccharides with *N*-butyryl groups (compare panel **c** with panel **a** in Fig. 5). The proportion of disaccharides with *N*-acetyl groups relative to those with *N*-butyryl groups is considerably higher than that expected (~15% of the disaccharides) if the *N*-butyryl derivative were to be completely degraded into disaccharides. This is due to the preferential cleavage of disaccharide repeat units with *N*-acetyl groups compared to those with *N*-butyryl groups by chondroitinase ABC. Taken together, these results show, for the first time, that the *N*-acetyl groups are involved in the binding and/or catalysis of the degradation of GAGs by chondroitinase ABC.

Recently, the active site of chondroitinase ABC I has been characterized by the crystallographic studies of the enzyme-CS complex [41, 42]. The results show that the carboxyl group and C-5 hydrogen interact with the basic amino acids of the enzyme in the active site via hydrogen bond formation. It has been predicted that Arg500 interact with the carboxyl groups, and His501, Arg560 and Glu653 with the C-5 hydrogen. These interactions have been proposed to involve proton abstraction, leading to the cleavage of β(1–4) glycosidic bond between GalNAc and GlcA, forming the 4,5-unsaturated GlcA residue. In those studies it was not clear whether the *N*-acetyl groups of CS play a role in the β-elimination reaction that cleaves the β(1–4) bond between GalNAc and GlcA [41, 42]. However, our observations that chondroitinase ABC cannot degrade the *N*-formyl derivative of bCSA and the bCSA derivatives with bulky *N*-hexanoyl or *N*-benzoyl groups (Fig. 3b) strongly suggest that the *N*-acetyl groups do interact with the enzyme. It is possible that the *N*-acetyl groups are involved in either maintaining a critical configuration of the active site or in positioning the substrate for the efficient β-elimination reaction. Alternatively, it is possible that the *N*-acetyl groups have no role in the interaction with or catalysis by chondroitinase ABC I, but play critical role in the catalysis by chondroitinase ABC II. Further comprehensive studies are required to understand the role the *N*-acetyl groups in the degradation of CSs by chondroitinase ABC.

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References

1. Hamai, A., Hashimoto, N., Mochizuki, H., Kato, F., Makiguchi, Y., Horie, K., Suzuki, S.: Two distinct chondroitin sulfate ABC lyases. An endoeliminase yielding tetrasaccharides and an exoeliminase

preferentially acting on oligosaccharides. J. Biol. Chem. **272**, 9123–9130 (1997)
2. Sugahara, K., Ohi, Y., Harada, T., de Waard, P., Vliegenthart, J.F. G.: Structural studies on sulfated oligosaccharides derived from the carbohydrate-protein linkage region of chondroitin 6-sulfate proteoglycans of shark cartilage: I. Six compounds containing 0 or 1 sulfate and/or phosphate residues. J. Biol. Chem. **267**, 6027–6035 (1992)
3. Schmalfeldt, M., Dours-Zimmermann, T., Winterhalter, K.H., Zimmermann, D.R.: Versican V2 is a major extracellular matrix component of the mature bovine brain. J. Biol. Chem. **273**, 15758–15764 (1998)
4. Valiyaveettil, M., Achur, R.N., Muthusamy, A., Gowda, D.C.: Chondroitin sulfate proteoglycans of the endothelia of human umbilical vein and arteries and assessment for the adherence of *Plasmodium falciparum*-infected erythrocytes. Mol. Biochem. Parasitol. **134**, 115–126 (2004)
5. Christner, J.E., Caterson, B., Baker, J.R.: Immunological determinants of proteoglycans. Antiobodies against the unsaturated oligosaccharide products of chondroitinase ABC-digested cartilage proteoglycans. J. Biol. Chem. **255**, 7102–7105 (1980)
6. Valiyaveettil, M., Achur, R.N., Muthusamy, A., Gowda, D.C.: Characterization of chondroitin sulfate and dermatan sulfate proteoglycans of extracellular matrices of human umbilical cord blood vessels and Wharton's Jelly. Glycoconj. J. **21**, 361–375 (2004)
7. Yoshida, K., Arai, M., Kohno, Y., Maeyama, K., Miyazono, H., Kikuchi, H., Morikawa, K., Tawada, A., Suzuki, S.: In: Scott, J.E. (ed.). Dermatan Sulfate Proteoglycans: Chemistry, Biology, Chemical Pathology, pp. 55–70. Portland, London (1993)
8. Hascall, V.C., Hascall, G.K.: Proteoglycan. In: Hay, E.D. (ed.) Cell Biology of Extracellular Matrix, pp. 39–63. Plenum, New York (1981)
9. Iozzo, R.V.: Matrix proteoglycans: from molecular design to cellular function. Annu. Rev. Biochem. **55**, 539–567 (1986)
10. Bhavanandan, V.P., Davidson, E.A.: Proteoglycans: structure, synthesis, function. In: Allen, H.J., Kisailus, E.C. (eds.) Glycoconjugates: Composition, Structure, and Function, pp. 167–202. Marcel Dekker, New York (1992)
11. Sugahara, K., Kitagawa, H.: Recent advances in the study of the biosynthesis and functions of sulfated glycosaminoglycans. Curr. Opin. Struct. Biol. **10**, 518–527 (2000)
12. Sugahara, K., Mikami, T., Uyama, T., Mizuguchi, S., Nomura, K., Kitagawa, H.: Recent advances in the structural biology of chondroitin sulfate and dermatan sulfate. Curr. Opin. Struct. Biol. **13**, 612–620 (2003)
13. Casu, B.: Structural features and binding properties of chondroitin sulfates, dermatan sulfate and heparan sulfate. Semin. Thromb. Hemost. **17**, 9–14 (1991)
14. Lauder, R.M., Huckerby, T.N., Nieduszynski, I.A.: A fingerprinting method for chondroitin/dermatan sulfate and hyaluronan oligosaccharides. Glycobiology **10**, 393–401 (2000)
15. Silbert, J.E. Sugumaran, G.: Biosynthesis of chondroitin/dermatan sulfate. IUBMB Life **54**, 177–186 (2002)
16. Kresse, H., Hausser, H., Schonherr, E., Bittner, K.: Biosynthesis and interactions of small chondroitin/dermatan sulphate proteoglycans. Eur. J. Clin. Chem. Clin. Biochem. **32**, 259–264 (1994)
17. Templeton, D.M.: Proteoglycans in cell regulation. Crit. Rev. Clin. Lab. Sci. **29**, 141–184 (1992)
18. Volpi, N.: Advances in chondroitin sulfate analysis: application in physiological and pathological states of connective tissue and during pharmacological treatment of osteoarthritis. Curr. Pharm. Des. **12**, 639–658 (2006)
19. Carulli, D., Laabs, T., Geller, H.M., Fawcett, J.W.: Chondroitin sulfate proteoglycans in neural development and regeneration. Curr. Opin. Neurobiol. **15**, 116–120 (2005)
20. Morgenstern, D.A., Asher, R.A., Fawcett, J.W.: Chondroitin sulphate proteoglycans in the CNS injury response. Prog. Brain. Res. **137**, 313–332 (2002)
21. Wegrowski, Y., Maquart, F.X.: Involvement of stromal proteoglycans in tumor progression. Crit. Rev. Oncol./Hematol. **49**, 259–268 (2004)
22. Volpi, N.: The pathobiology of osteoarthritis and the rationale for using the chondroitin sulfate for its treatment. Current Drug Targets. Immune, Endocrine, & Metabolic Disorders **4**, 119–127 (2004)
23. Gowda, D.C.: Role of chondroitin 4-sulfate in pregnancy-associated malaria. Adv. Pharmacol. **53**, 375–400 (2006)
24. Fried, M., Duffy, P.E.: Adherence of *Plasmodium falciparum* to chondroitin sulfate A in the human placenta. Science **272**, 1502–1504 (1996)
25. Achur, R.N., Valiyaveettil, M., Alkhalil, A., Ockenhouse, C.F., Gowda, D.C.: Characterization of proteoglycans of human placenta and identification of unique chondroitin sulfate proteoglycans of the intervillous spaces that mediate the adherence of *Plasmodium falciparum*-infected erythrocytes to the placenta. J. Biol. Chem. **275**, 40344–40356 (2000)
26. Fried, M., Nosten, F., Brockman, A., Brabin, B.J., Duffy, P.E.: Maternal antibodies block malaria. Nature **395**, 851–852 (1998)
27. Brabin, B.J., Romagosa, C., Abdelgalil, S., Menendez, C., Verhoeff, F.H., Mc Gready, R., Fletcher, K.A., Owens, S., D'Alessandro, U., Nosten, F., Fisher, P.R., Ordi, J.: The sick placenta—the role of malaria. Placenta **25**, 359–378 (2004)
28. Menendez, C., Ordi, J., Ismail, M.R., Ventura, P.J., Aponte, J.J., Kahigwa, E., Font, F., Alonso, P.L.: The impact of placental malaria on gestational age and birth weight. J. Infect. Dis. **181**, 1740–1745 (2000)
29. Ismail, M.R., Ordi, J., Menendez, C., Ventura, P.J., Aponte, J.J., Kahigwa, E., Hirt, R., Cardesa, A., Alonso, P.L.: Placental pathology in malaria: a histological, immunohistochemical, and quantitative study. Human Pathol. **31**, 85–93 (2000)
30. Alkhalil, A., Achur, R.N., Valiyaveettil, M., Ockenhouse, C.F., Gowda, D.C.: Structural requirements for the adherence of *Plasmodium falciparum*-infected erythrocytes to chondroitin sulfate proteoglycans of human placenta. J. Biol. Chem. **275**, 40357–40364 (2000)
31. Muthusamy, A., Achur, R.N., Valiyaveettil, M., Madhunapantula, S.V., Kakizaki, I., Bhavanandan, V.P., Gowda, C.D.: Structural characterization of the bovine tracheal chondroitin sulfate chains and binding of *Plasmodium falciparum*-infected erythrocytes. Glycobiology **14**, 635–645 (2004)
32. Achur, R.N., Valiyaveettil, M., Gowda, D.C.: The low sulfated chondroitin sulfate proteoglycans of human placenta have sulfate group-clustered domains that can efficiently bind *Plasmodium falciparum*-infected erythrocyts. J. Biol. Chem. **278**, 11705–11713 (2003)
33. Gowda, A.S.P., Madhunapantula, S.V., Achur, R.N., Valiyaveettil, M., Bhavanandan, V.P., Gowda, D.C.: Structural basis for the adherence of *Plasmodium falciparum*-infected erythrocytes to chondroitin 4-sulfate and design of novel photoactivable reagents for the identification of parasite adhesive proteins. J. Biol. Chem. **282**, 916–928 (2007)
34. Shaklee, P.N., Conrad, H.E.: Hydrazinolysis of heparin and other glycosaminoglycans. Biochem. J. **217**, 187–197 (1984)
35. Finne, J., Krusius, T.: Preparation and fractionation of glycopeptides. Methods Enzymol. **83**, 269–277 (1982)
36. Danishefsky, I., Siskovic, E.: Conversion of carboxyl groups of mucopolysaccharides into amides of amino acid esters. Carbohydr. Res. **16**, 199–205 (1971)
37. Ferguson, M.A.J.: Chemical and enzyme analysis of glycosylphosphatidylinositol anchors. In: Hooper, N.M., Turner, A.J. (eds.) Lipid Modification of Proteins: A Practical Approach, pp. 191–231. IRL, New York (1992)

38. Oike, Y., Kimata, K., Shinomura, T., Nakazawa, K., Suzuki, S.: Structural analysis of chick-embryo cartilage proteoglycan by selective degradation with chondroitin lyases (*chondroitinases*) and endo-β-D-galactosidase (*keratanase*). Biochem. J. **191**, 193–207 (1980)
39. Dische, Z.: A new specific color reaction to hexuronic acids. J. Biol. Chem. **167**, 189–198 (1947)
40. Sugahara, K., Shigeno, K., Masuda, M., Fujii, N., Kurosaka, A., Takeda, K.: Structural studies on the chondroitinase ABC-resistant sulfated tetrasaccharides isolated from various chondroitin sulfate isomers. Carbohydr. Res. **255**, 145–163 (1994)
41. Prabhakar, V., Raman, R., Capila, I., Bosques, C.J., Pojasek, K., Sasisekharan, R.: Biochemical characterization of the chondroitinase ABC I active site. Biochem. J. **390**, 395–405 (2005)
42. Prabhakar, V., Capila, I., Bosques, C.J., Pojasek, K., Sasisekharan, R.: Chondroitinase ABC I from *Proteus vulgaris*: cloning, recombinant expression and active site identification. Biochem. J. **386**, 103–112 (2005)