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Note

Human skin dermatan sulfate with sulfated and unsulfated non-reducing ends

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Abstract

The non-reducing ends of the preponderant dermatan sulfates of adult human skin (DS_{18} and DS_{28}) can have D-GalNAc, D-GlcA and L-IdoA. D-GlcA of DS_{18} and D-GalNAc of both DS_{18} and DS_{28} are sulfated.

Keywords: Non-reducing termini; Terminal sulfatases; Quail egg white; Dermatan sulfate; Glycosaminoglycan

Dermatan sulfate (DS), an acidic glycosaminoglycan (GAG) ubiquitous in most mammalian tissues, occurs in three forms (DS_{18}, DS_{28} and DS_{35}) in adult human skin [1]. A polymer of L-IdoA and D-GlcA bonded α-(1→3) and β-(1→3), respectively, to D-GalNAc, which in turn is connected to any of these uronic acids via β-(1→4) bonds. DS was first isolated from porcine skin by Meyer and Chaffee [2] and subsequently by other workers [3]. Its chemical structure has been studied extensively [3], but its elucidation has been hampered by its complexity. DS is a polydisperse molecule that in calf ligamentum nuchae appears to have sulfated D-GalNAc, D-GlcA and L-IdoA at its non-reducing termini (M.O. Longas, K. Meyer, unpublished work). Other workers have found sulfated D-GalNAc at the non-reducing ends of cartilage DS [4].

An extracellular component, DS occurs covalently bonded to protein [5]. In its proteoglycan form, it appears to have a variety of biological functions such as retention of water, control of molecular diffusion and filling of the intercellular space [5,6]. Furthermore, the anticoagulant activity of DS has been demonstrated [7,8].

This work was initiated to determine the sugars at the non-reducing termini of the preponderant DSs of adult human skin, DS_{18} and DS_{28} [1]. To this end, these DSs were digested separately with α-L-iduronidase, β-glucuronidase and β-*N*-acetylhexosaminidase. The products were 22.7 and 25.0 nmol of L-IdoA/mg DS_{18} and DS_{28}, respectively (Table 1). The degradation of these glycosaminoglycans (GAGs) with β-glucuronidase resulted in 162.3 nmol D-GlcA/mg DS_{28} and none from

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DS_{18}; their digestion with β-*N*-acetylhexosaminidase did not produce any detectable amounts of D-GalNAc, the expected product (Table 1).

The lack of sensitivity of DS_{18} and DS_{28} to β-*N*-acetylhexosaminidase and of DS_{18} to β-glucuronidase was investigated by allowing these GAGs to react with the sulfatase enzymes of quail egg white in dialysis systems. During this reaction, DS_{18} and DS_{28} lost, respectively, 0.21% (w/w) and 0.55% (w/w) of their sulfate content (Table 2). These partially desulfated GAGs were digested by β-*N*-acetylhexosaminidase, and the product was D-GalNAc, 11.25 nmol/mg DS_{18} and 2.85 nmol/mg DS_{28} (Table 1). The reaction of partially desulfated DS_{18} with β-glucuronidase yielded 3.80 nmol D-GlcA/mg of GAG (Table 1).

The low amounts of sulfate lost suggest limited enzymatic desulfation, perhaps confined to the sulfate esters at the non-reducing termini (Table 2). A total of 68% of the 22 nmol of sulfate lost from DS_{18} during the desulfation reaction was recovered as terminal non-reducing D-GlcA (3.80 nmol) and D-GalNAc (11.25 nmol) (Table 1). Of the 57 nmol of sulfate lost by DS_{28} during the same reaction, only 2.85 nmol were recovered as terminal non-reducing D-GalNAc (Table 1). Although the removal of the indicated sugars with the corresponding exoglycosidases was conducted under optimal conditions, the results suggest that not all of the desulfated terminal sugars were successfully removed by the exoglycosidases enzymes.

Most of the sulfate lost during desulfation was recovered as free sulfate in the dialysates. Only the controls displayed no free or bonded sulfate in the dialysates. The lack of detectable free or bonded sulfate in the dialysates of the controls that contained DS plus egg white, preheated at 37 °C for 48 h, indicates the absence of sulfatase or chondroitinase B activity [9–11] in the quail egg white treated under these conditions. It should be pointed out that inactivation of these enzymes was carried out at 37 °C rather than at higher temperatures to avoid precipitation of albumin. Egg white was

Table 1
Uronic acids and GalNAc at dermatan sulfate non-reducing ends

DS type [a]	D-IdoA [b]		D-GlcA [b]		D-GalNAc [b]		SD [c]
	Before	After	Before	After	Before	After	
DS_{18}	22.7	ND	ND	3.80	ND	11.25	0.78
DS_{28}	25.0	ND	162.3	ND	ND	2.85	0.18

[a] The subscripts represent the percentage of ethanol used to precipitate DS during purification [1]. See text for additional information.
[b] Expressed as nmol/mg DS before and after desulfation, using the averages of two different determinations conducted in triplicate. See text for experimental conditions. ND, not detectable.
[c] Standard deviation of the mean.

Table 2
Effect of quail egg white sulfatases on the sulfate composition of dermatan sulfate

DS type [a]	% Sulfate [b]		% Sulfate lost during reaction	SD [c]	% Sulfate removed by sulfatases
	Before	After			
DS_{18}	14.4	14.0	0.36	0.00	
DS_{18} (control)	14.4	14.2	0.15	0.00	0.21
DS_{28}	19.9	19.1	0.76	0.02	
DS_{28} (control)	19.9	19.7	0.21	0.01	0.55

[a] For subscript definition refer to Table 1.
[b] Expressed as μg SO_4^{2-}/100 μg DS before and after desulfation. Values represent the mean of three different determinations done in triplicate.
[c] Standard deviation of the mean.

Table 3
Recovery of exogenous dermatan sulfate from the quail egg white used for desulfation

Sample description [a]	DS recovered (mg)	% Yield [b]
DS_{18} + quail egg white	56	52
DS_{18} + quail egg white previously incubated at 37 °C for 48 h	55	51
DS_{18} + buffer	106	99
quail egg white	ND	ND
DS_{28} + quail egg white	26	24
DS_{28} + quail egg white previously incubated at 37 °C for 48 h	60	56
DS_{28} + buffer	105	98
quail egg white	ND	ND

[a] Subscript description as in Table 1.
[b] Based on 108 mg DS used per sample. DS reaction with quail egg white and reisolation were carried out twice; the average results are reported. ND, not detectable.

incubated at the temperature indicated andassayed for terminal sulfatase and chondroitinase B, until they were no longer detectable.

Exogenous DS was recovered in the precipitate that formed in the aqueous–organic interphase during centrifugation of the mixture of non-dialysable liquid of the desulfation reaction with the chloroform–amyl alcohol reagent. It displayed one spot at the position of the standard on cellulose polyacetate membranes (12) and gave an FTIR spectrum characteristic of standard DS [13] (not shown), suggesting no depolymerization.

Although DS depolymerization could not be established from sulfate recovery, the yields of exogenous DS_{18} and DS_{28} reisolated from quail egg white were only 52 (w/w) and 24% (w/w), respectively (Table 3). The unrecovered DS could have been trapped in the various precipitates formed during purification. The rather low yields of exogenous DS_{18} and DS_{28} from the controls devoid of detectable dermatanase B activity, which were 51 (w/w) and 56% (w/w), respectively, support this assumption. Notice that $\geq$ 98% DS was recovered from the controls devoid of quail egg white, while none was obtained from the controls that contained quail egg white in buffer (Table 3).

The lack of detectable DS in the controls containing quail egg white suggests that if this GAG is present in this specimen, larger amounts of starting material are needed to isolate a detectable concentration. In the present work we started with 275 mL of quail egg white, the final product was reconstituted in 100 μL of water, and 1–10 μL was used for electrophoresis under conditions that detected at least 0.2 μg of DS [12]. Furthermore, no reports on quail egg white DS were found in the literature.

This work presents the first conclusive evidence for the sulfation of human skin DS at its non-reducing termini, and confirms data from other workers who have found sulfated non-reducing terminal D-GalNAc in cartilage DS [4] and in chondroitin sulfate of rat chondrosarcoma cultures [14]. In addition to corroborating the existence of the known quail egg white sulfatase activity that removes sulfates bonded to C-4 of terminal non-reducing D-GalNAc [9], these results indicate that quail egg white contains sulfatase(s) capable of cleaving sulfate esters at C-2 or C-3 of terminal non-reducing D-GlcA of DS. Because of the various sugars (D-GalNAc, D-GlcA and L-IdoA) detected at the non-reducing ends, the data also confirm the polydispersity of DS.

1. Experimental

Materials.—Post-surgical breast skin (obtained at area hospitals) was examined by the pathologist in service, depleted of its subcutaneous fat and frozen 2–3 h after surgery. Quail eggs were purchased from Carolina Biochemicals, Inc., NC and used within 2 days of hatching. DS was purified and characterized as indicated previously [15], with the modifications described [1]; its chemical and spectroscopic characteristics were also as indicated previously, since standard human skin DS was used in the present studies [1]. Rabbit anti-chicken ovalbumin (1.3 mg antibody/mL) was a product of ICN Biochemicals, Inc. CNBr-activated Sepharose 4B, β-glucuronidase (EC 3.2.1.31), β-*N*-acetylhexosaminidase (EC 3.2.1.52), protease (pronase) type XIV from *Streptomyces griseus* and chondroitinase B (dermatanase) (EC 4.2.2) of *Flavobacterium heparinum* were purchased from Sigma. Rabbit anti-chicken ovalbumin-Sepharose 4B was prepared as indicated [16]. α-L-Iduronidase of dog

testis was a gift from Dr Elizabeth F. Neufeld, UCLA Biological Chemistry, School of Medicine. Nanopure water was prepared by passing it through a series of cartridges that included charcoal and mixed-bed ion exchangers from Millipore Corp., followed by distillation. Freshly distilled water collected in glass was used in all of the experiments. A microplate reader spectrophotometer model 450, which was purchased from Bio-Rad, was employed to determine the absorbance in all microassays. All other chemicals were reagent grade of the highest quality commercially available.

Removal of D-*GlcA from dermatan sulfate non-reducing ends.*—Dermatan sulfate (1.8 mg) and β-glucuronidase (200 U) were dissolved in 210 μL of 0.10 M sodium acetate, pH 4.5, containing 0.071 mM maleic acid (an α-L-iduronidase inhibitor [17]) and 0.1 mM D-GalNAc (a β-*N*-acetylhexosaminidase inhibitor [17]). The reaction mixture was flushed with N_2 gas for 1 min and sealed tightly; reaction was conducted at 37 °C for 2.5 h and terminated by the addition of 100 μL of 30% (v/v) aq HCl. Unreacted DS and enzyme were precipitated with 1.5 mL of absolute EtOH at 4 °C overnight, removed by centrifugation at 10,000*g* and 4 °C for 20 min, washed twice with 0.5 mL of 80% (v/v) aq chilled EtOH and stored frozen. The EtOH supernatant and washes were combined and evaporated to dryness using a vacuum oven at 60 °C, and the dry sample was reconstituted in 500 μL of H_2O, filtered through a 0.220 nm filter and lyophilized. The final product was redissolved in 210 μL of H_2O and 5 μL was used for triplicate D-GlcA assays, using a μscale modification of conventional methods [18,19]. One control contained all the reagents except the enzyme; the second control contained all reagents, except DS.

Removal of L-*IdoA from dermatan sulfate non-reducing ends.*—Dermatan sulfate (3.9 mg) and α-L-iduronidase (0.138 U) were dissolved in 100 μL of 0.4 M sodium formate–0.5 mM NaCl, pH 3.5, containing 0.1 mM D-GlcNAc (a β-*N*-acetylhexosaminidase inhibitor [17]) and bovine serum albumin (20 μg). NaCl at the concentration used was a β-glucuronidase inhibitor [17]. The reaction was carried out under N_2 at 37 °C for 3 h and terminated by lowering the temperature to 23 °C. Unreacted DS and enzyme were precipitated with 500 μL of absolute EtOH, using the conditions described in the preceding experiment. Ethanol supernatant and washes were treated as indicated above. The final product was dissolved in 80 μL of H_2O, and 5 μL was employed for triplicate L-IdoA assays, using the orcinol method [20] scaled down to the μL level. The controls were also as described in the preceding enzymatic digestion of DS.

Removal of D-*GalNAc from dermatan sulfate non-reducing ends.*—Dermatan sulfate (1.3 mg) and β-*N*-acetylhexosaminidase (0.07 U) were dissolved in 190 μL of 0.5 M sodium citrate, pH 4.3 (adjusted with HCl) and allowed to react under N_2 at 37 °C for 4.5 h. The mixture was diluted four-fold with absolute EtOH and refrigerated overnight to stop the reaction. Precipitated matter was removed by centrifugation as indicated above. Ethanol was evaporated to dryness under reduced pressure at 60 °C. D-GalNAc was removed from the dry EtOH residue by extraction with absolute EtOH, using three extractions of 100 μL each. The EtOH extracts were combined and filtered through a 0.2 μm Nylon-66 filter from Rainin Instruments Co. The filtrate was evaporated to dryness as indicated, redissolved in 100 μL of H_2O, and 15 μL was used for triplicate D-GalNAc assays, using the Morgan–Elson reaction [21] scaled down to the μL level indicated below. The final product (5 μL) was also subjected to triplicate assays for D-GlcA and L-IdoA. The controls were as described above. The enzyme for this experiment was pre-dialyzed thoroughly against 0.50 M sodium citrate, pH 4.3.

Assay of quail egg white terminal sulfatase with synthetic substrate.—Quail egg white was homogenized in a Waring blender at 0 °C for 15 s, adjusted to pH 7.0 with 0.066 M sodium acetate, and tested for sulfatase activity with *p*-nitrocatechol sulfate as substrate [9].

Ovalbumin was removed from egg white by batch adsorption with rabbit anti-chicken ovalbumin-Sepharose 4B. In a typical experiment, quail egg white (550 mL) was mixed with rabbit anti-chicken ovalbumin-Sepharose

4B (50 mL packed), shaken gently at 30 °C for 30 min, and centrifuged to remove Sepharose beads agglutinated with albumin. Egg white depleted of ovalbumin was adjusted to pH 5.6 using AcOH, and reassayed for sulfatase activity with the synthetic substrate indicated [9]. Using 290 μL of quail egg white and 0.312 nmol of *p*-nitrocatechol sulfate in a total reaction volume of 300 μL, the net A_{490} was 0.718. This represented an 18.4-fold increment of sulfatase activity. The specific activity using *p*-nitrophenol as the substrate was 0.3 nmol of phenolate/30 min reaction at 37 °C [9].

Desulfation of dermatan sulfate by the terminal sulfatases of quail egg white.—Ovalbumin-depleted egg white (275 mL) was adjusted to 3 mM maleic acid, 1 mM D-GlcA, 1 mM D-GlcNAc, 1 mM D-GalNAc to inhibit, respectively, α-L-iduronidase, β-glucuronidase, and β-*N*-acetylhexosaminidase [17]; DS (108 mg) was dissolved in this solution, placed in a dialysis membrane of 12,000 molecular weight cut-off and immersed in 1 L of 0.066 M sodium acetate, pH 5.6, containing the glycosidase inhibitors described. Dialysis was carried out overnight under N_2 at 37 ± 1 °C. The dialysate was lyophilized and assayed for free sulfate [22].

The non-dialysable materials of all of the reaction mixtures containing egg white displayed large white precipitates that were removed by centrifugation and discarded after they proved to be undigestable with pronase. The liquid portions of the non-dialysable materials were used to repurify DS. Reaction conditions for the controls were those of the experimental samples; one control had egg white without DS; another control contained DS without egg white; and a third control had DS plus egg white whose chondroitinase B and sulfatase activities had been destroyed by heating under N_2 at 37 °C for 48 h.

Purification of exogenous dermatan sulfate from the quail egg white used for its desulfation.—The non-dialysable liquid of the enzymatic desulfation of DS described above was combined with an equal volume of 4:1 $CHCl_3$–amyl alcohol, stirred vigorously at 30 °C for 30 min, and centrifuged at 1000*g* and room temperature for 10 min. The top (aqueous) layer was separated from the bottom (organic) layer by a white precipitate that was employed to reisolate DS_{18} and DS_{28} as follows.

The white precipitate was homogenized in 0.15 M tris(hydroxymethyl)aminoethane–0.15 M sodium acetate–0.15 M calcium chloride, pH 7.8, adjusted with acetic acid. The homogenate was adjusted with buffer to a final volume of 325 mL, protease (pronase) (0.30 g) was added, and the reaction was conducted under N_2 at 37 ± 1 °C overnight; two batches of the same amount of enzyme were added, each followed by a 12 h incubation period.

The reaction mixture was cooled to 4 °C and allowed to stand at this temperature for 4 h. The precipitate that formed was filtered through a cotton pad and discarded. The liquid phase, which had a natural pH of 6.15, was combined with 1.5 volumes of 95% (v/v) aq EtOH to precipitate DS at 4 °C overnight [1,15]. DS was removed by centrifugation at 3020*g* and 5 °C for 20 min, washed once with 80% (v/v) chilled, aq EtOH, and dissolved in a minimum volume of 0.60 M sodium acetate–0.50 M AcOH, pH 3.0. Five parts of the latter solution was combined with one part of the $CHCl_3$–amyl alcohol reagent indicated and centrifuged as described above.

The aqueous layer obtained upon centrifugation of the latter reaction mixture was diluted three-fold with absolute EtOH and refrigerated overnight. The precipitate that formed was removed by centrifugation, washed as indicated, dissolved in a minimum volume of water, and tested for GAGs using electrophoresis on cellulose polyacetate membranes [12].

Quantitation of sulfate removed by quail egg white terminal sulfatases.—Sulfate was quantified using the Dodgson–Price method scaled down to a 360 μL total reaction volume [22]. Specifically, DS that had been repurified from the non-dialysable liquid material of the desulfation reaction was hydrolyzed in formic acid (24.64%, v/v) at 100 ± 2 °C for 24 h. Excess acid was removed under reduced pressure, and the sulfate was allowed to react with $BaCl_2$ and was quantified by A_{490}. Free sulfate in the dialysate was also quantified using the same procedure.

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