

Structure and functional impact of glycosaminoglycan modification of HSulf-2 endosulfatase revealed by atomic force microscopy and mass spectrometry

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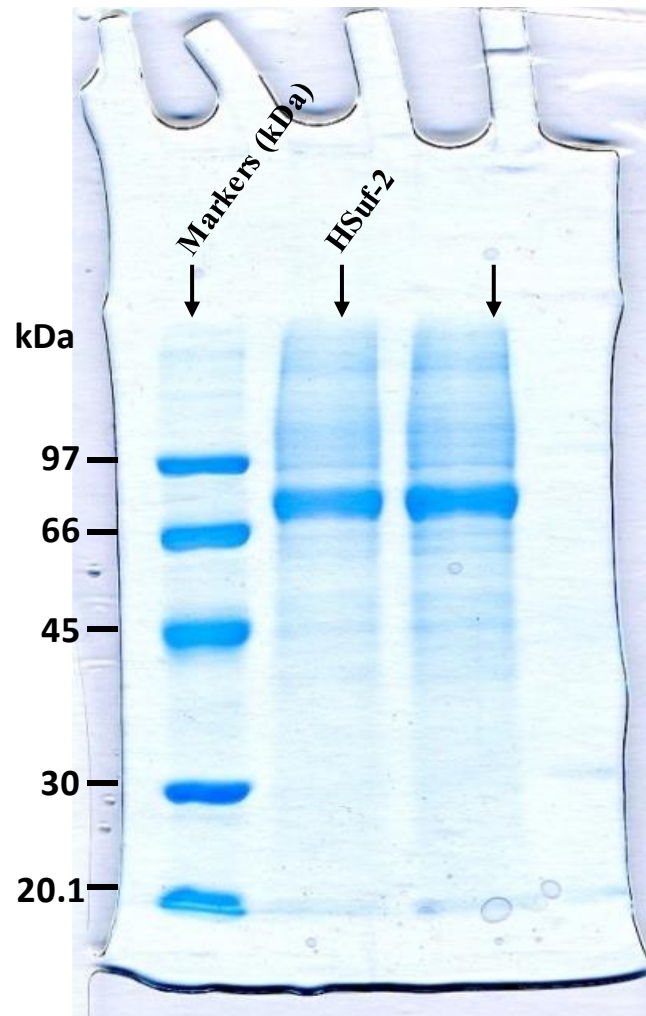


Figure S1. SDS-PAGE analysis of HSulf-2 on 10% polyacrylamide gel and revealed by Coomassie Blue; (Lane 1) Markers, HSulf-2, before (lane 2) and after (lane 3) hydrolysis by chondroitinase ABC (3 μ g HSulf-2/well). LC: long chain. c)

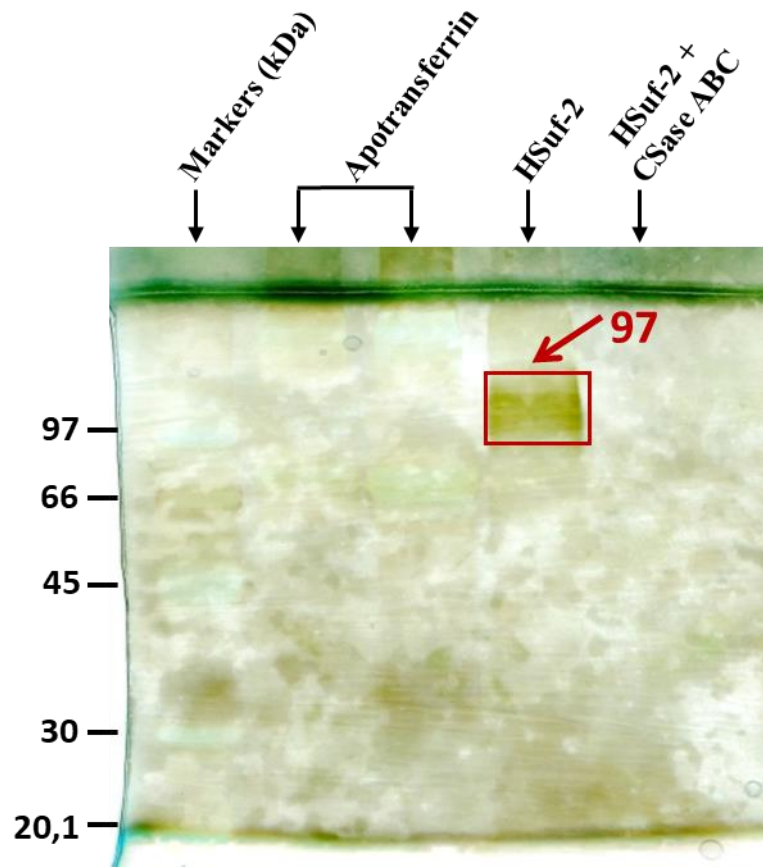


Figure S2. SDS-PAGE analysis of HSulf-2 on 10% polyacrylamide gel and revealed by Alcian blue/silver nitrate on 10% polyacrylamide gel; (Lane 1) Markers, (Lane 2 to 3) Apotransferrin at 3 and 10 μ g as negative control, HSulf-2 treated by chondroitinase ABC, before (lane 4) and after (lane 5) hydrolysis by chondroitinase (3 μ g HSulf-2/well).

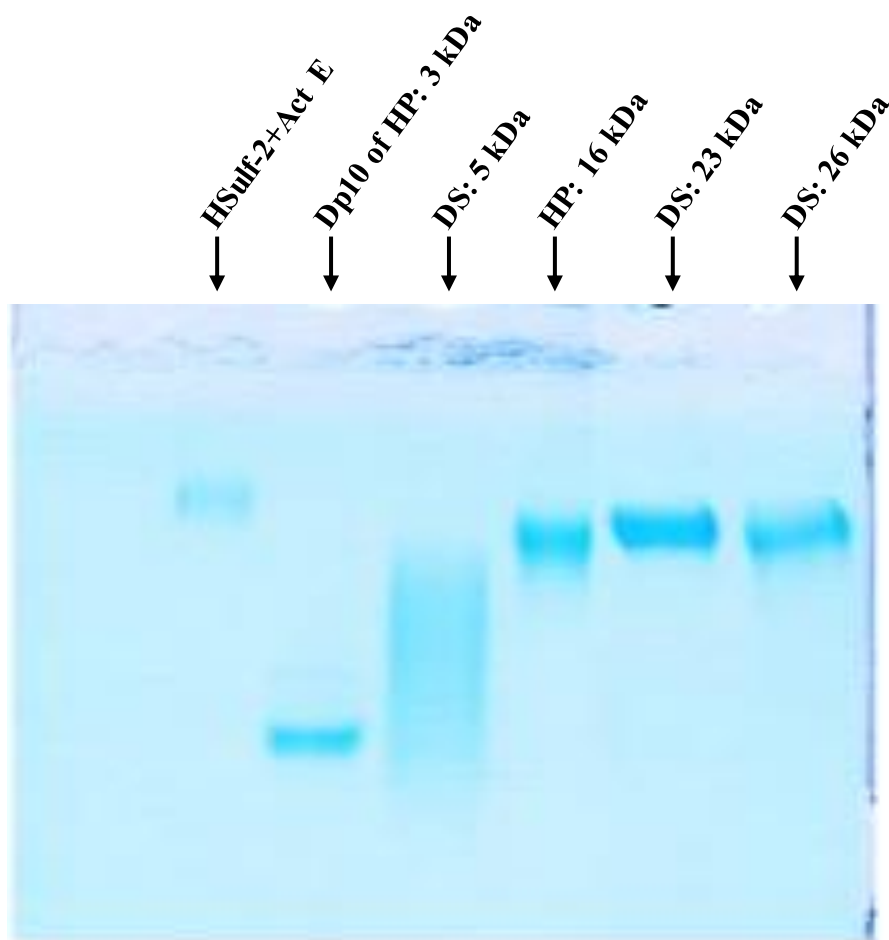


Figure S3. C-PAGE analysis of the CS/DS linked to HSulf-2. (lane 1) HSulf-2 proteolyzed by Actinase E; (lanes 2 to 6) sulfated polysaccharide markers: 3 kDa heparin decasaccharide, 5 kDa dermatan sulfate, 16 kDa heparin, 23 and 26 kDa dermatan sulfate. 27% polyacrylamide gel, Alcian blue staining, 5 μ g HSulf-2/well or 2 μ g sulfated polysaccharide markers/well.

1	FLSHRLKGR	FQDRRNIRP	NIILVLTDDQ	DVELGSMQVM	NKTRRIMEQG	Long Chain
51	GAHFINAFT	TPMCCPSRSS	ILTGKYVHNH	NTYTNNECS	SPSWQAQHE	
101	RTFAVYLNST	GYRTAFFGKY	LNEYNGSYVP	PGWKEWVGLL	KNSRFYNYTL	
151	CRNGVKEKHG	SDYSKDYLTD	LITNDSVSFF	RTSKKMYPHR	PVLMVISHAA	
201	PHGPEDSAPQ	YSRLFPNASQ	HITPSYNYAP	NPDKHWIMRY	TGPMKPIHME	
251	FTNMLQRKRL	QTLMSVDDSM	ETIYNMLVET	GELDNTYIVY	TADHGYHIGQ	
301	FGLVKGKSMP	YEFDIRVPFY	VRGPNVEAGC	LNP HIVLNID	LAPTILDIAG	
351	LDIPADMDGK	SILKLLDTER	PVNRFLHKKK	MRVWRDSFLV	ERGKLLHKRD	
401	NDKVDAQEEN	FLPKYQRVKD	LCQRAEYQTA	CEQLGQKWQC	VEDATGKLKL	
451	HKCKGPMRLG	GSRALSNLVP	KYYGQGSEAC	TCDSGDYKLS	LAGRRKKLFLK	
501	KKYKASYVRS	RSIR				
		SVAIEV	DGRVYHVGLG	DAAQPRNLTK	RHWPGAPEDQ	Short Chain
551	DDKDGGDFSG	TGGLPDYSAA	NPIKVTHRCY	ILENDTVQCD	LDLYKSLQAW	
601	KDHKLHIDHE	IETLQNKIKN	LREVRGHLKK	KRPEECDCHK	ISYHTQHKGR	
651	LKHGSSSLHP	FRKGLQEKDK	VWLLREQKRK	KKLRKLLKRL	QNNDTCSMPG	
701	LTCFTHDNQH	WQTAPFWTLG	PFCAC TSANN	NTYWCMRTIN	ETHNFLFCEF	
751	ATGFLEYFDL	NTDPYQLMNA	VNTLDRDVLN	QLHVQLMELR	SCKGYKQCNP	
801	RTRNMDLGLK	DGGSYEQYRQ	FQRRKWPEMK	RPSSKSLGQL	WEGWEG	

Figure S4. Identification of the band at 50,000 from SDS-PAGE of HSulf-2 treated by chondroitinase ABC. Sequence coverage (%) was determined after in gel trypsin treatment and nanoLC-ESI-MS/MS analysis.

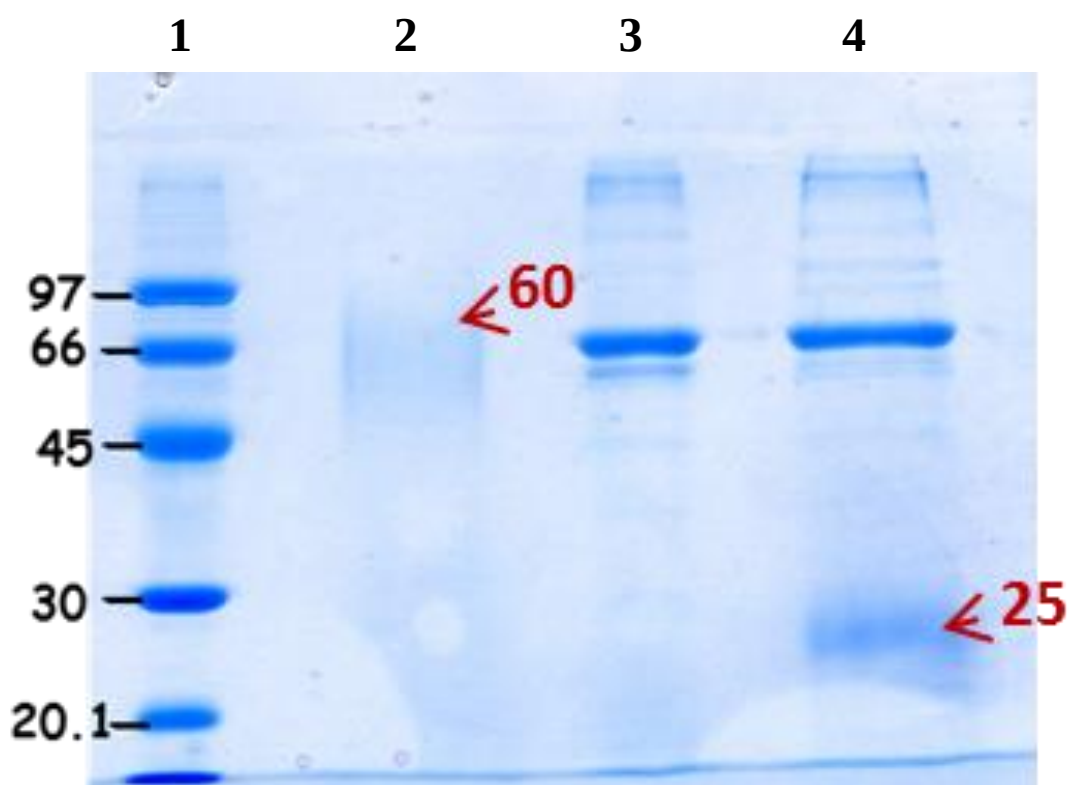
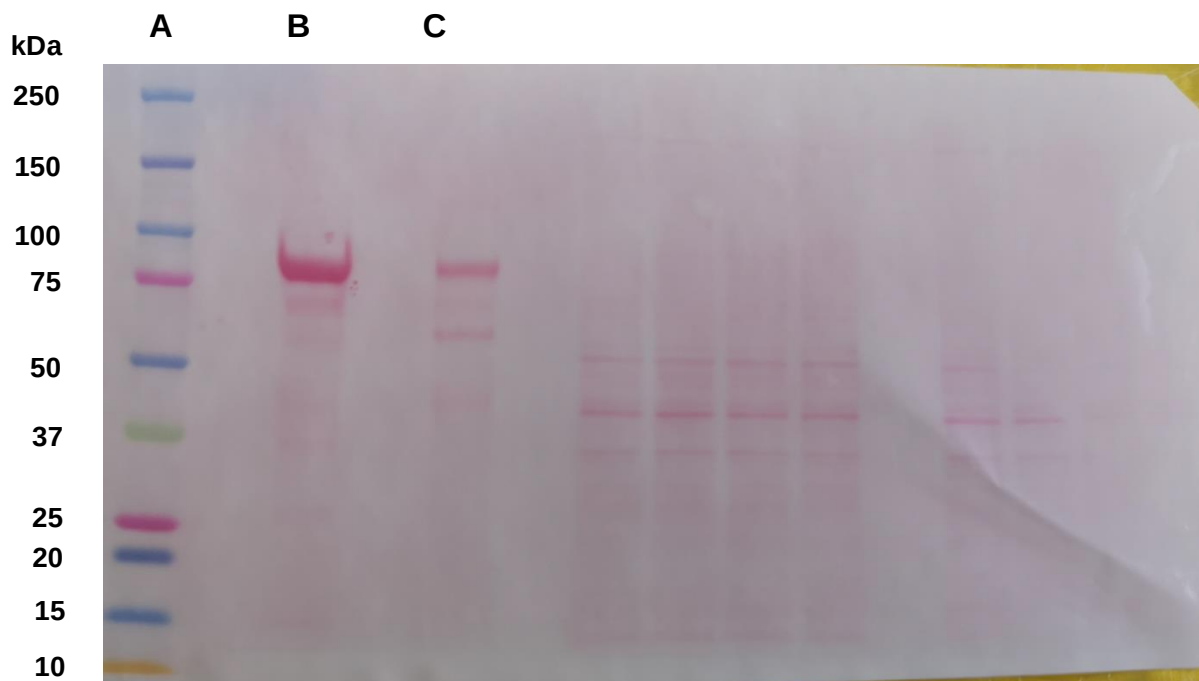


Figure S5. SDS-PAGE analysis of Endocan by Coomassie Blue staining on 12% polyacrylamide gel. Lane 1: molecular weight markers; Lane 2: Endocan (2µg); Lane 3: chondroitinase ABC; Lane 4: Endocan after action of the chondroitinase ABC (10 mU) during 24h at 37°C.

a)



b)

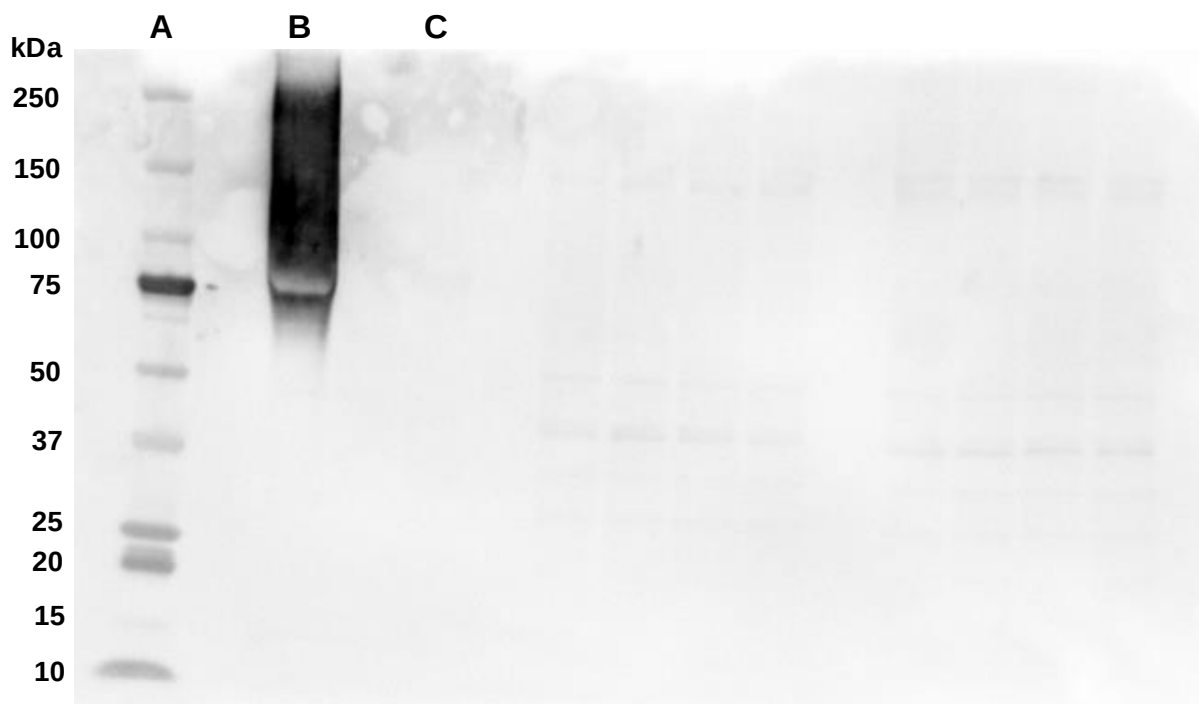


Figure S6. Western Blot of HSulf-2 revealed by anti-chondroitin sulfate antibody. a) Ponceau staining and b) Immunodetection of HSulf-2 by the anti-chondroitin antibody CS-56 before (lane B) and after action of the chondroitinase ABC (lane C). Lane A: molecular weight markers.

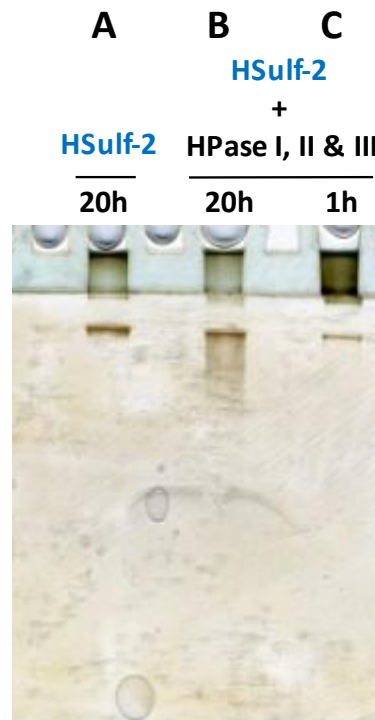


Figure S7. C-PAGE analysis of HSulf-2 with or without treatment by heparinase I, II and III. HSulf-2 without any enzyme treatment incubated at 37°C during 20h (A) or incubated at 37°C with heparinase I, II and III (5mU of each) during 20 (B) and 1h (C). 27% polyacrylamide gel, Alcian blue/silver nitrate staining, 3µg HSulf-2/well.

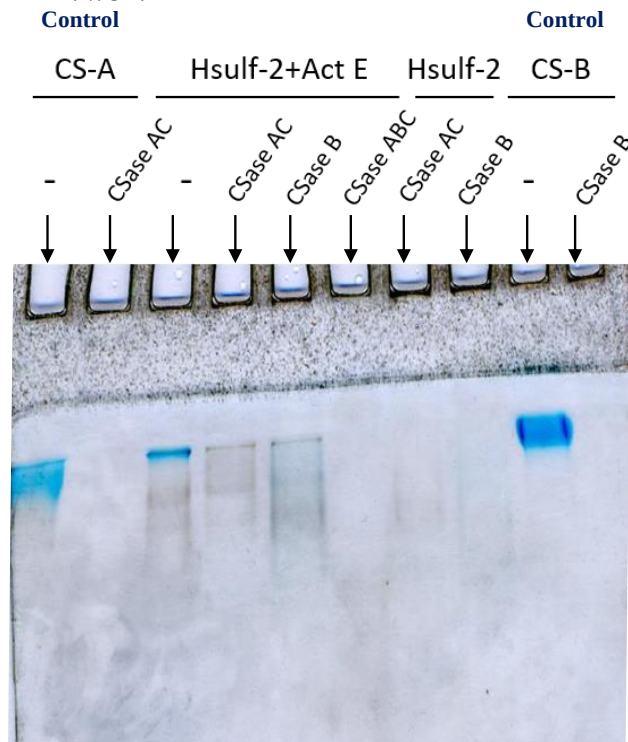
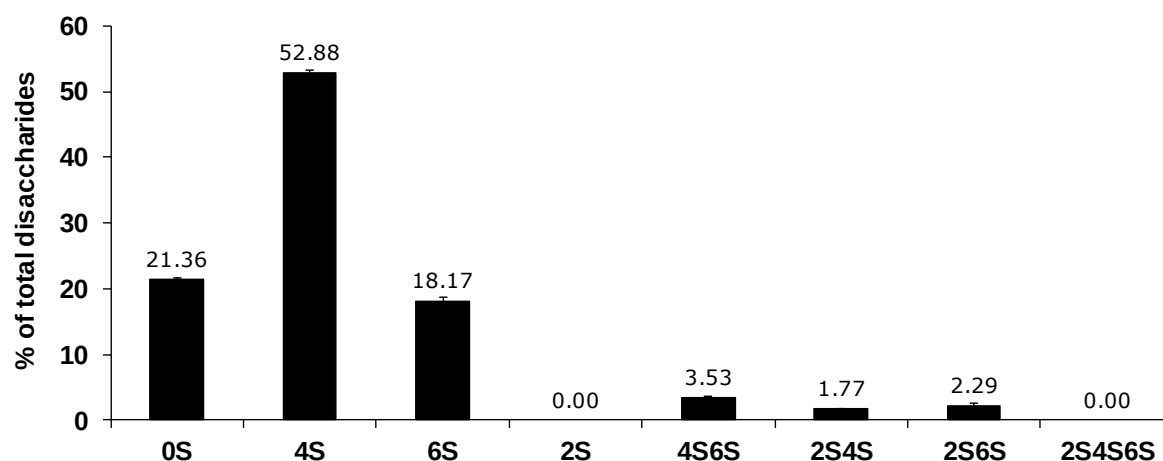


Figure S8. C-PAGE analysis of the depolymerization of the CS/DS linked to HSulf-2 by chondroitinases. HSulf-2 proteolyzed by Actinase E without chondroitinase (4), with CSase AC (5), and with CSase B (6); non-proteolyzed HSulf-2 with CSase ABC (7), with CSase AC (8), and with CSase B. Controls: CS-A (1), CS-A with CSase AC (2), CS-B (9), CS-B with CSase B (10). 27% polyacrylamide gel, Alcian blue/silver nitrate staining, 3µg HSulf-2/well, 2 µg CS-A, -B/well.

a)



b)

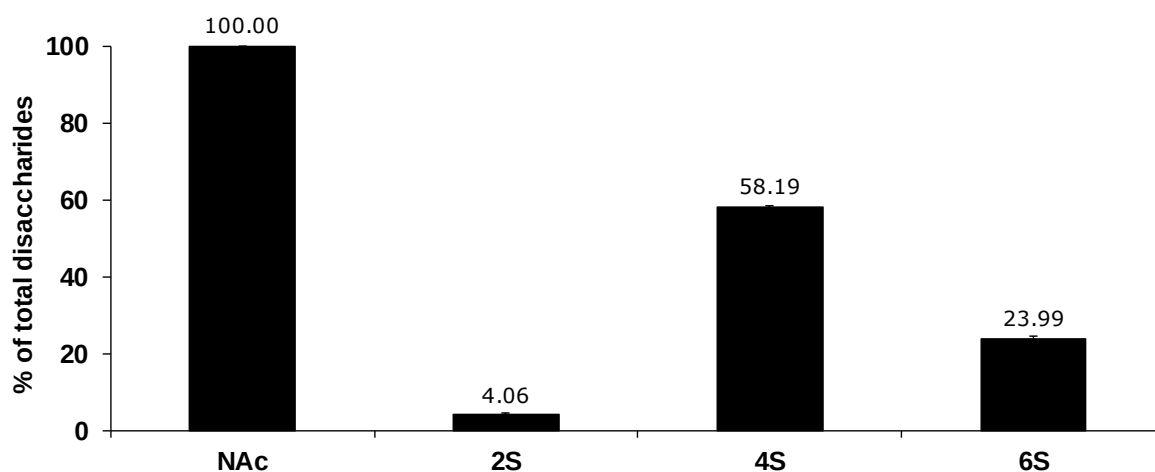


Figure S9. Disaccharide analysis of the CS/DS GAG chain attached to HSulf-2. CS/DS disaccharide composition was determined by RPIP HPLC. a) The relative amount of the different disaccharides. 0S, Δ UA-GalNAc; 4S, Δ UA-GalNAc(4S); 6S, Δ UA-GalNAc(6S); 2S, Δ UA(2S)-GalNAc; 4S6S, Δ UA-GalNAc(4S,6S); 2S4S, b) Percentage of disaccharides with different modifications calculated from the data in a).

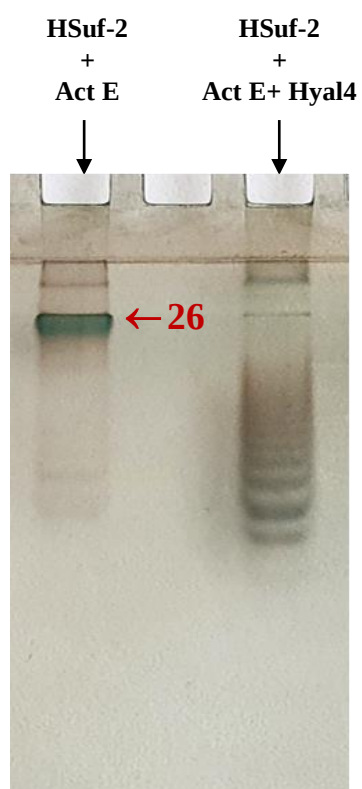


Figure S10. C-PAGE analysis of the depolymerization of the CS/DS linked to HSulf-2 by hyaluronidase. CS/DS chain released from HSulf-2 by Actinase E (1), and subsequent depolymerization by hyaluronidase-4 (2). 27% polyacrylamide gel, Alcian blue/silver nitrate staining.

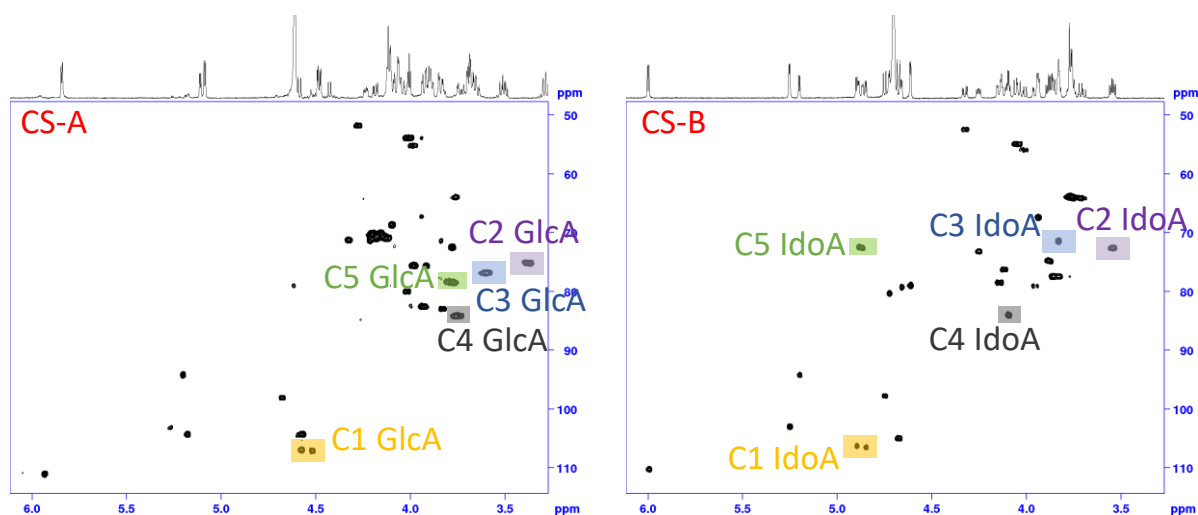


Figure S11. 2D ^1H - ^{13}C HSQC spectra of CS-A and CS-B tetrasaccharides at 30°C. GlcA and IdoA residue signals of CS-A and CS-B, respectively, are labelled.

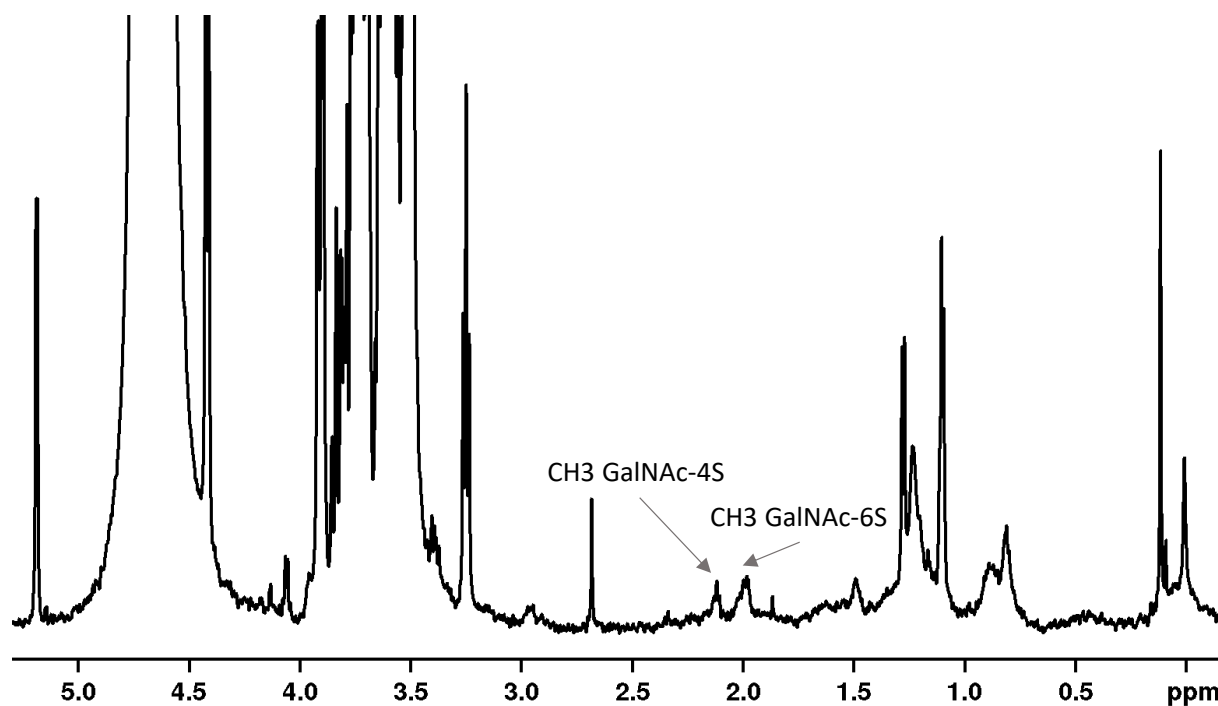


Figure S12. 1D ^1H NMR spectrum of the CS/DS oligosaccharides released from HSulf-2 after hyaluronidase depolymerization. Spectrum give clear evidences of the presence of both 4-O-, and 6-O-sulfated GalNAc residues in CS/DS oligosaccharides.

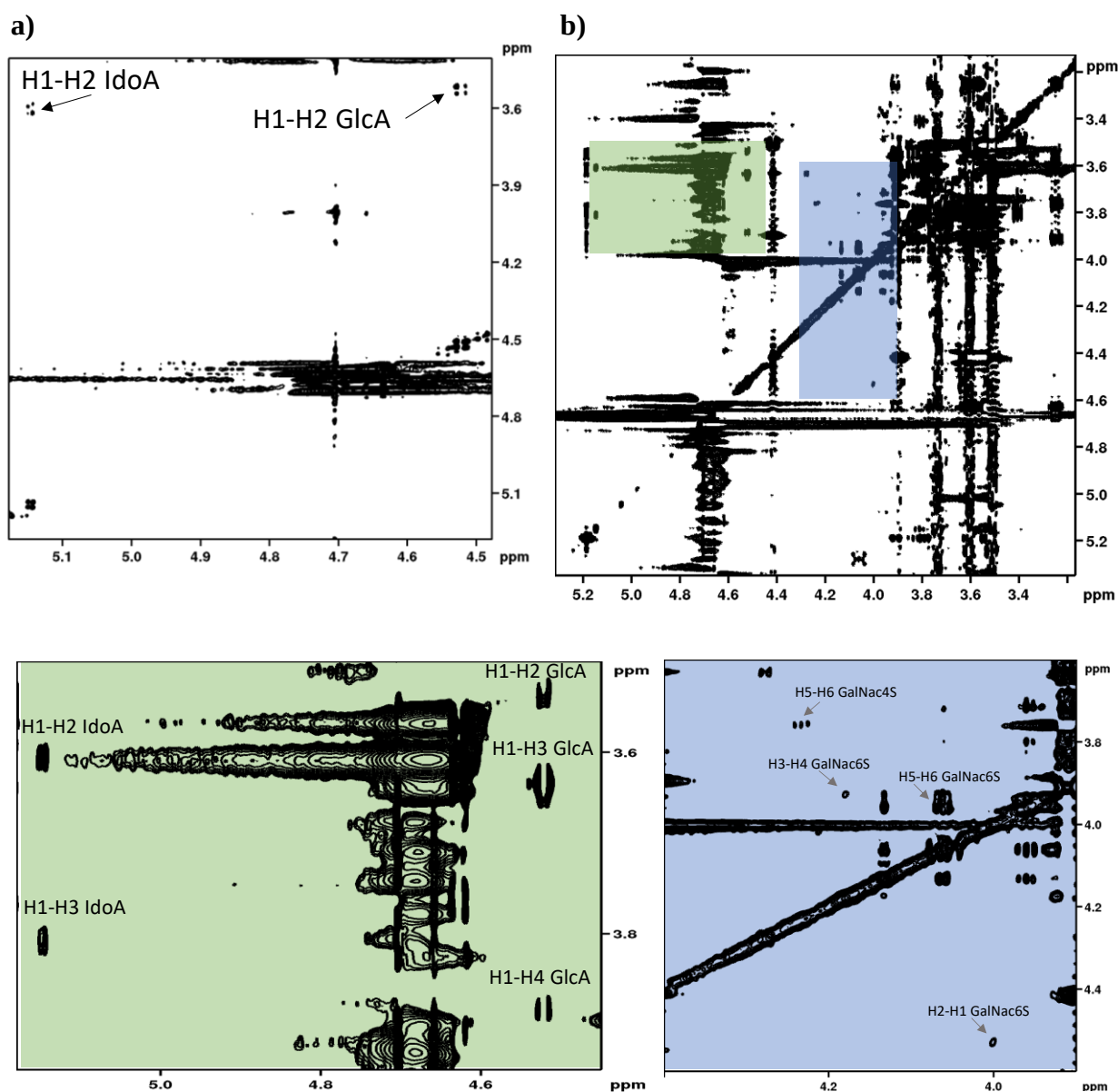


Figure S13. 2D ^1H - ^1H COSY (a) and TOCSY (b) NMR spectrum of the spectrum of the CS/DS oligosaccharides released from HSulf-2 after hyaluronidase depolymerization. Analysis achieved at 30°C . Right inserts in green and blue correspond to zooms on the TOCSY spectrum.

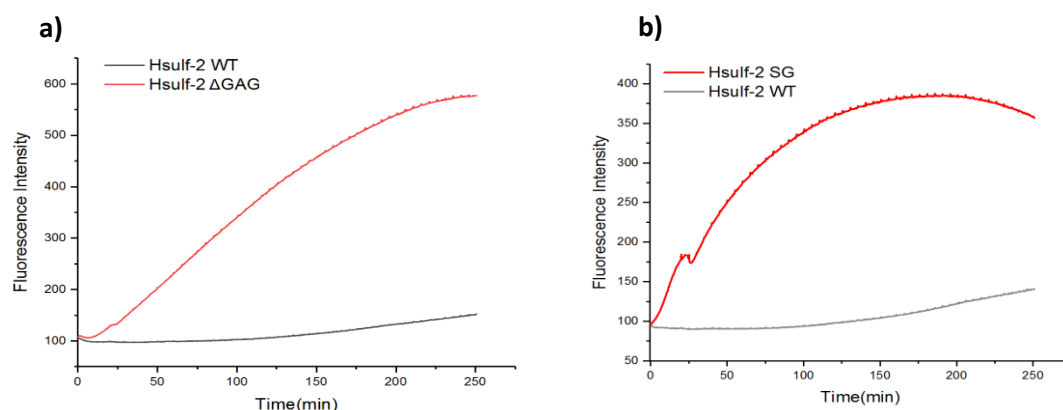


Figure S14. Arylsulfatase activity of HSulf-2 measured on the synthetic substrate 4-MUS. The arylsulfatase of HSulf-2 by comparing (A) the activity of HSulf-2WT (black) to the activity of HSulf-2 Δ GAG (red) whose has been GAG digested by chondroitinase ABC, or (B) of HSulf-2 SG mutant (red) lacking the CS GAG. Arylsulfatase assay at 37°C with 4 mM 4-MUS in 100 μ L 50 mM Tris buffer containing 10 mM $MgCl_2$, pH 7.5. The enzyme desulfation of 4-MUS to 4-MU was measured by fluorescence emission at 465 nm.

Table S1. Putative sequences extracted from MALDI-TOF MS analyses of oligosaccharides issued from chondroitinase ABC depolymerization of the HSulf-2-bound GAG. *Sequences in italics represent the least abundant reported in the whole literature.*

dp	Experimental <i>m/z</i>	Theoretical <i>m/z</i>	Putative sequences
2	582.0	581.9	Δ UA-GalNAc4S6S
	480.0	480.0	Δ UA-GalNAc4S/6S
4	1085.0	1085.0	(Δ UA-GalNAc4S/6S)-(GlcA/IdoA-GalNAc4S6S) (Δ UA-GalNAc4S6S)-(GlcA/IdoA-GalNAc4S/6S)
	983.1	983.1	(Δ UA-GalNAc4S/6S) ₂ <i>(ΔUA-GalNAc4S6S)-(GlcA/IdoA-GalNAc)</i> <i>(ΔUA-GalNAc)-(GlcA/IdoA-GalNAc4S6S)</i>
	881.1	881.1	<i>(ΔUA-GalNAc4S/6S)-(GlcA/IdoA-GalNAc)</i> <i>(ΔUA-GalNAc)-(GlcA/IdoA-GalNAc4S/6S)</i>
6	1588.0	1588.0	(Δ UA-GalNAc4S6S)-(GlcA/IdoA-GalNAc4S/6S) ₂ (Δ UA-GalNAc4S/6S)-(GlcA/IdoA-GalNAc4S6S)-(GlcA/IdoA-GalNAc4S/6S) (Δ UA-GalNAc4S/6S) ₂ -(GlcA/IdoA-GalNAc4S6S) <i>(ΔUA-GalNAc4S6S)₂-GlcA/IdoA-GalNAc)</i> <i>(ΔUA-GalNAc4S6S)-(GlcA/IdoA-GalNAc)-(GlcA/IdoA-GalNAc4S6S)</i> <i>(ΔUA-GalNAc)-(GlcA/IdoA-GalNAc4S6S)₂</i>
	1486.1	1486.1	(Δ UA-GalNAc4S/6S) ₃ <i>(ΔUA-GalNAc4S6S)-(GlcA/IdoA-GalNAc4S/6S)-(GlcA/IdoA-GalNAc)</i> <i>(ΔUA-GalNAc4S6S)-(GlcA/IdoA-GalNAc)-(GlcA/IdoA-GalNAc4S/6S)</i> <i>(ΔUA-GalNAc4S/6S)-(GlcA/IdoA-GalNAc4S6S)-(GlcA/IdoA-GalNAc)</i> <i>(ΔUA-GalNAc)-(GlcA/IdoA-GalNAc4S6S)-(GlcA/IdoA-GalNAc4S/6S)</i> <i>(ΔUA-GalNAc)-(GlcA/IdoA-GalNAc4S/6S)-(GlcA/IdoA-GalNAc4S6S)</i> <i>(ΔUA-GalNAc4S/6S)-(GlcA/IdoA-GalNAc)-(GlcA/IdoA-GalNAc4S6S)</i>

Table S1. *continued*

dp	Experimental m/z	Theoretical m/z	Putative sequences
8	1989.1	1989.1	$(\Delta\text{UA-GalNAc4S/6S})_4$ $(\Delta\text{UA-GalNAc4S/6S})_2-(\text{GlcA/IdoA-GalNAc4S6S})-(\text{GlcA/IdoA-GalNAc})$ $(\Delta\text{UA-GalNAc4S/6S})_2-(\text{GlcA/IdoA-GalNAc})-(\text{GlcA/IdoA-GalNAc4S6S})$ $(\Delta\text{UA-GalNAc4S/6S})-(\text{GlcA/IdoA-GalNAc4S6S})-(\text{GlcA/IdoA-GalNAc4S/6S})-(\text{GlcA/IdoA-GalNAc})$ $(\Delta\text{UA-GalNAc4S/6S})-(\text{GlcA/IdoA-GalNAc4S6S})-(\text{GlcA/IdoA-GalNAc})-(\text{GlcA/IdoA-GalNAc4S/6S})$ $(\Delta\text{UA-GalNAc4S/6S})-(\text{GlcA/IdoA-GalNAc})-(\text{GlcA/IdoA-GalNAc4S6S})-(\text{GlcA/IdoA-GalNAc4S/6S})$ $(\Delta\text{UA-GalNAc4S/6S})-(\text{GlcA/IdoA-GalNAc})-(\text{GlcA/IdoA-GalNAc4S6S})-(\text{GlcA/IdoA-GalNAc4S/6S})$ $(\Delta\text{UA-GalNAc4S/6S})-(\text{GlcA/IdoA-GalNAc})-(\text{GlcA/IdoA-GalNAc4S/6S})-(\text{GlcA/IdoA-GalNAc4S6S})$ $(\Delta\text{UA-GalNAc4S6S})_2-(\text{GlcA/IdoA-GalNAc})_2$ $(\Delta\text{UA-GalNAc4S6S})-(\text{GlcA/IdoA-GalNAc})_2-(\text{GlcA/IdoA-GalNAc4S6S})$ $(\Delta\text{UA-GalNAc4S6S})-(\text{GlcA/IdoA-GalNAc})-(\text{GlcA/IdoA-GalNAc4S6S})-(\text{GlcA/IdoA-GalNAc})$ $(\Delta\text{UA-GalNAc})-(\text{GlcA/IdoA-GalNAc4S6S})_2-(\text{GlcA/IdoA-GalNAc})$ $(\Delta\text{UA-GalNAc})-(\text{GlcA/IdoA-GalNAc4S6S})-(\text{GlcA/IdoA-GalNAc})-(\text{GlcA/IdoA-GalNAc4S6S})$ $(\Delta\text{UA-GalNAc})_2-(\text{GlcA/IdoA-GalNAc4S6S})_2$ $(\Delta\text{UA-GalNAc4S6S})-(\text{GlcA/IdoA-GalNAc4S/6S})_2-(\text{GlcA/IdoA-GalNAc})$ $(\Delta\text{UA-GalNAc4S6S})-(\text{GlcA/IdoA-GalNAc4S/6S})-(\text{GlcA/IdoA-GalNAc})-(\text{GlcA/IdoA-GalNAc4S/6S})$ $(\Delta\text{UA-GalNAc4S6S})-(\text{GlcA/IdoA-GalNAc})-(\text{GlcA/IdoA-GalNAc4S/6S})_2$ $(\Delta\text{UA-GalNAc})-(\text{GlcA/IdoA-GalNAc4S/6S})_2-(\text{GlcA/IdoA-GalNAc4S6S})$ $(\Delta\text{UA-GalNAc})-(\text{GlcA/IdoA-GalNAc4S/6S})-(\text{GlcA/IdoA-GalNAc4S6S})-(\text{GlcA/IdoA-GalNAc4S/6S})$ $(\Delta\text{UA-GalNAc})-(\text{GlcA/IdoA-GalNAc4S6S})-(\text{GlcA/IdoA-GalNAc4S/6S})_2$

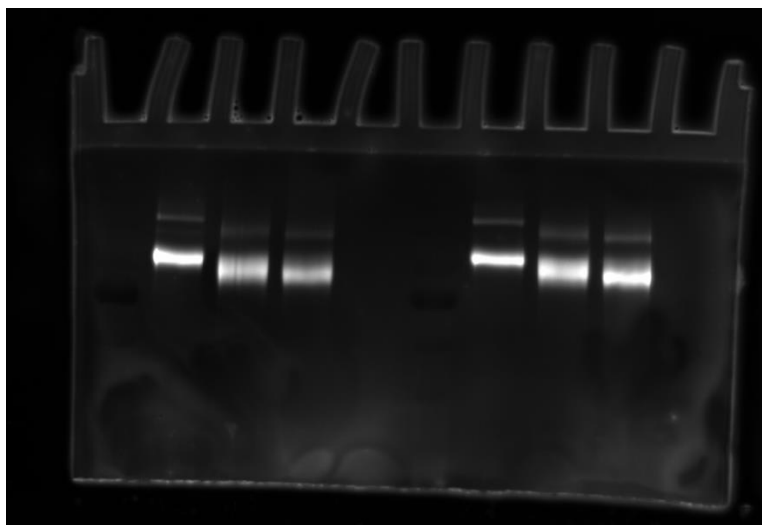
Table S2. Identification of the five *N*-glycans located within glycopeptides at Asn88, Asn125, Asn174, and Asn217 on the long chain and at Asn537 on the short chain of HSulf-2.

<i>T</i> (min)	<i>Glycopeptides</i>				<i>N</i> -glycan	
	<i>Exp.</i> <i>m/z</i> (4+)	<i>Calc.</i> <i>m/z</i> (4+)	Δm (ppm)	<i>Ascribed sequence</i> (<i>Asn</i> residue number in red)	<i>Deducted mass</i>	<i>Composition</i>
12.60	1202.2285	1202.2282	0.2	YVHNHNTYTNNE ^{N88} CSSPSWQAQHESR	1702.5842	HexNAc(2)Hex(8)
13.03	998.7252	998.7243	0.9	VYHVGLGDAAQPR ^{N537} LTKR	1996.7869	HexNAc(6)Hex(3)Fuc(2)
20.57	1 274.3281	1274.3297	1.3	LFP ^{N217} ASQHITPSYNYAPNPDKHWIMR	1996.7810	HexNAc(6)Hex(3)Fuc(2)
25.18	1039.2092	1039.2087	0.5	EKHGSDYSKDYLTLIT ^{N174} DSVSFFR	1216.4248	HexNAc(2)Hex(5)
28.68	1120.7775	1120.7753	2.0	TAFFGKYLNEY ^{N125} GSYVPPGWKEWVGLLK	1216.4316	HexNAc(2)Hex(5)

Table S3. List of the most abundant glycopeptides containing Asn residue issued from trypsin and PNGase F treatment and detected after CID/ETD analysis. Each site of glycosylation was confirmed by the detection of deamidation introduced by PNGase F treatment (+0.984 mass increment on the MS/MS spectra of de-glycosylated peptides). Exact composition and relative abundance of each precursor were estimated using Byologic Protein Metric software (see experimental section) and manually confirmed.

Identified peptide sequence	Chain
SSILTGKYVHNHNTYTNNE ^{N88} CSSPSWQAQHESR	Short
YVHNHNTYTNNE ^{N88} CSSPSWQAQHESR	
TAFFGKYLNEY ^{N125} GSYVPPGWKEWVGLLK	
YLNEY ^{N125} GSYVPPGWK	
YLNEY ^{N125} NGSYVPPGWKEWVGLLK	
YLNEY ^{N125} GSYVPPGWKEWVGLLKNSR	
DYLTDLIT ^{N174} DSVSFFR	
EKHGSDYSKDYLTLIT ^{N174} DSVSFFR	
HGSDYSKDYLTLIT ^{N174} DSVSFFR	
LFP ^{N217} NASQHITPSYNYAPNPDKHWIMR	
P ^{N217} ASQHITPSYNYAPNPDKHWIMR	
GLGDAAQPR ^{N537} LTKR	Long
GRVYHVGLGDAAQPR ^{N537} LTK	
HVGLGDAAQPR ^{N537} LTK	
HVGLGDAAQPR ^{N537} LTKR	
SVAIEVDGRVYHVGLGDAAQPR ^{N537} LTK	
VYHVGLGDAAQPR ^{N537} LTK	
VYHVGLGDAAQPR ^{N537} LTKR	

a)



b)

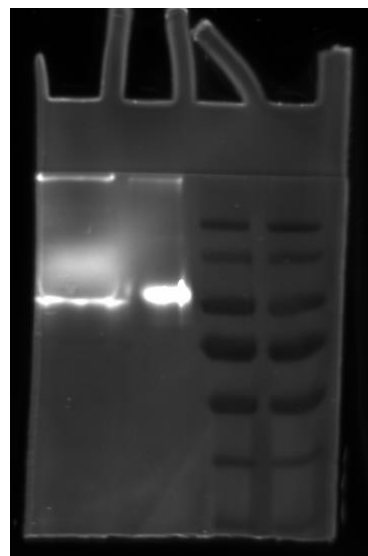


Figure S15. Uncropped, full-length SDS-PAGE gels corresponding to (a) the SDS-PAGE analysis shown in figure 6A and (b) the SDS-PAGE analysis shown in figure 6C., detection with the SNAP-Vista Green.