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Polysulfated Carbohydrates Analyzed as Ion-paired Complexes with Basic Peptides and Proteins Using Electrospray Negative Ionization Mass Spectrometry

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Electrospray ionization mass spectrometry was used in the negative ion mode to anlayze complexes of sucrose octasulfate, sucrose heptasulfate and sulfated α-, β- and γ-cyclodextrins with synthetically prepared basic peptides, the basic protein ubiquitin and polyamines. The spectra presented demonstrate that complexes with these basic molecules facilitate the analysis of these polysulfated oligosaccharides. Stable (1:1) complexes result from the ion pairing between the protonated basic arginine and lysine residues of the peptide and the anionic sulfate groups of the polysulfated oligosaccharides. Fragmentation of the polysulfated oligosaccharides resulting in the loss of SO_3 could be suppressed by controlling the experimental conditions, such as the nozzle–skimmer voltage, used to obtain the spectra. In the absence of fragmentation, it was possible to obtain data on the purity of sucrose octasulfate and sucrose heptasulfate as well as the distribution of the sulfated cyclodextrins. The confounding presence of sodium counter-ions is also eliminated using this method. Complete chemical sulfation of oligosaccharides is difficult to achieve. Thus, data on sample purity are essential for the characterization of sulfated oligosaccharides used as pharmaceutical agents. © **1997 by John Wiley & Sons, Ltd.**

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INTRODUCTION

Polysulfated compounds, particularly polysulfated carbohydrates, represent an important class of biologically active and pharmaceutically important molecules.[1] Glycosaminoglycans, for example, are highly sulfated, acidic polysaccharides that have a number of important physiological roles.[1] The biological activity of these polyanionic natural products is mediated through their interaction with basic proteins.[1,2] Pharmaceutically important polysulfated compounds include sucrose octasulfate (SOS)[3] and cyclodextrin (CD) sulfates.[4] SOS is prepared through the complete chemical sulfation of the eight hydroxyl groups of sucrose, a neutral disaccharide. The aluminum salt of SOS is the drug sucralfate (Carafate), which is widely used for the treatment of duodenal ulcers.[3] Recently, there has been heightened interest in the sodium salt of SOS and its derivatives, based on reports of its interaction and stabilization of fibroblast growth factor, a basic protein important in wound healing.[5,6] Cyclodextrins, cyclic oligomers of $1 \rightarrow 4$-linked glucose, contain six (α-CD), seven (β-CD) and eight (γ-CD) saccharide units. Chemical sulfation of CD is often incomplete because of the large number of hydroxyl groups (18, 21 and 24 in α-, β- and γ-CD, respectively) and the steric constraints associated with these cyclic oligosaccharides.[7,8] Thus, sulfated CDs are generally mixtures having a range of sulfation.[8] Despite this limitation, sulfated cyclodextrins are under active investigation by the pharmaceutical industry. One important activity associated with sulfated β-CD is its ability to inhibit new vessel growth, angiogenesis, suggesting its application in the treatment of tumors.[8] This activity is believed to be regulated through the interaction of sulfated β-CD with basic growth factors.[6] Sulfated CDs inhibit complement activation[10] and block human immunodeficiency virus (HIV)-I infection,[11] presumably through their interaction with the basic proteins regulating these processes. Sulfated CDs have also been investigated as pharmaceutical excipients, carrying toxic or hydrophobic drugs with low aqueous solubility in the interior cavity of these cyclic oligosaccharides.[12,13]

Methods for the analysis of highly sulfated oligosaccharides, such as sucrose octasulfate and sulfated CDs, prepared through chemical sulfation are critically needed to support their development as phar-

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maceuticals. High-field NMR spectroscopy can generally be used to establish the structure of these molecules.[14] What is lacking, however, is a method to establish the purity of these polysulfated compounds. Specifically, it is extremely difficult to ensure complete chemical sulfation and the absence of minor undersulfated contaminants in pharmaceutical preparations.

The analysis of sulfated oligosaccharides poses a number of challenges. Fractionation of sulfated oligosaccharides relies primarily on taking advantage of the differing levels of negative charge on the various components of a mixture. While high-resolution ion-exchange chromatography[15] and gel[16] and capillary electrophoresis[17–19] can be used to fractionate these highly sulfated oligosaccharides, the absence of chromophores make their detection exceedingly difficult.[15,16,20] Detection often involves conductivity or requires the formation of visible complexes with dyes or metals. The detection sensitivities of these methods are generally inadequate for pharmaceutical analysis, particularly when a polysulfated compound is present in biological samples.

Mass spectrometry offers an alternative, highly sensitive method for the analysis of sulfated oligosaccharides.[21] Fast atom bombardment mass spectrometry (FABMS) in the negative ion mode has been used to analyze the sodium salts of sulfated oligosaccharides, derived from glycosaminoglycans, containing 2–10 saccharide units with 1–15 sulfate units.[22,23] Sulfated cyclodextrins[8] and sulfated cyclodextrin derivatives[19] have also been analyzed by FABMS. This approach affords both molecular ion and fragmentation ($-NaSO_3^- + H^+$) data through the loss of SO_3 and by cleavage at glycosidic linkages.[22,23] The use of FABMS/MS also affords through-ring fragmentation of these molecules, giving additional important structural information.[24] Chromatographic separation prior to FABMS has recently been used for the analysis of glycosaminoglycan-derived disaccharides.[25] Negative ion FABMS has major limitations for the analysis of sulfated oligosaccharides. The relatively low sensitivity of this method, particularly for highly sulfated molecules (requiring nanomole quantities of sample), and the prominence of the fragmentation pathway involving loss of SO_3 make this method less than ideal for pharmaceutical applications. FABMS is also complicated by the exchanges of Na by H, giving a multiplicity of differentially sodiated species.

Electrospray ionization (ESI)-MS in the negative ion mode has been applied to the analysis of sized mixtures of glycosaminoglycan-derived oligosaccharides.[26] These oligosaccharides ranged in size from disaccharides to tetradecasaccharides and had a low level of sulfation ranging from zero to one sulfate group per disaccharide unit. The multiplicity of molecular ions observed for the sulfated oligosaccharides analyzed could have resulted from either the heterogeneity of the analyte or through fragmentation. Recent work has improved the sensitivity for sulfated disaccharides to the level of 100 pmol[27] and has shown the utility of this technique for the analysis of more highly sulfated oligosaccharides.[28] ESI-MS of highly sulfated cyclodextrin derivatives in the positive ion mode gave multiple sodiated molecular ions and sodium chloride adducts.[19] Capillary electrophoresis coupled to ESI-MS was required to establish successfully the purity of these sulfated cyclodextrin derivatives.

Matrix-assisted laser desorption/ionization (MALDI)/MS has been used in the positive ion mode to analyze synthetically prepared monosulfated disaccharides and trisaccharides[29] and sulfated cyclodextrin derivatives.[19] While no fragmentation is observed, sodium adducts of the molecular ions complicate spectral analysis. MALDI/MS in the positive ion mode has also been applied recently to the analysis of sulfated glycosaminoglycan-derived oligosaccharides complexed with basic peptides.[30] This study clearly demonstrates improved sensitivity, particularly of larger highly sulfated oligosaccharides (requiring picomole quantities of sample). However, MALDI/MS, using various carboxylic acids as the matrix, still showed fragmentation through the loss of SO_3 groups and hence could not be used as a quantitative tool to identify undersulfated minor contaminants and to determine sample purity. In more recent work,[31] using 3-hydroxypicolinic acid as matrix, MALDI-MS in the positive ion mode resulted in a considerable reduction or elimination of sulfate loss from oligosaccharides containing up to six sulfate groups in complexes with basic peptides.

In this study, we examined ion-pairing complexes of polysulfated oligosaccharides and basic peptides/proteins and polyamines using electrospray ionization (ESI-MS) in the negative ion mode. The techniques developed were used to determine the purity of the sodiated polysulfated samples without fragmentation of labile sulfate groups and with the suppression of sodium adducts. Quantitation was also demonstrated with selected samples.

EXPERIMENTAL

Sucrose octasulfate (SOS), sodium salt of pharmaceutical purity, was a gift from Bukh-Meditec (Farum, Denmark). The purity of SOS was confirmed by ^{1}H NMR spectroscopy.[14] Sucrose heptasulfate (SHS) (1′,3′,4′,6′-tetra-*O*-sulfo-β-D-fructofuranosyl 2,3,4-tri-*O*-sulfo-γ-D-glucopyranoside, heptasodium salt) was synthesized from sucrose by regiospecific 6-*O*-benzoylation in the presence of dibutyltin oxide, followed by persulfation with trimethylamine–sulfur trioxide complex and de-*O*-benzoylation.[18] The structure of SHS was confirmed by ^{1}H NMR spectroscopy.[18] The sulfated α-, β- and γ-CDs were a gift from American Maize Products (Hammond, IN, USA). The α-CD was prepared as the potassium salt and the β-CD and γ-CD as the soidum salts. The proteins bovine ubiquitin (M_r 8565), horse heart myoglobin (M_r 16 945), the polyamines spermidine (M_r 145.3) and spermine (M_r 202.3) and the synthetic peptides, described in Table 1, were obtained from Sigma Chemical (St Louis, MO, USA). All solvents used were of reagent grade.

ESI-MS

The acidic polysulfated molecules were mixed with basic peptides, proteins or polyamines in water–

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acetonitrile solution to produce strongly bound, ion-paired complexes. These complexes were then analyzed by ESI-MS in the negative ion mode using a Micromass Quattro triple-quadrupole mass spectrometer. The parameters studied included the molar ratios and structures of the ion-pairing reagents, the effect of nozzle–skimmer voltage (as the voltage is lowered the energetics of the interactions are increased for negatively charged species), the effect of solvent composition and the effect of source temperature on the complexes. The negative ion ESI-MS data were compared with positive ion ESI-MS data. The acquired spectra were transformed to a mass scale after median and mean smoothing and baseline subtraction. The peaks of the transformed mass spectra were integrated for sulfate distribution calculations.

The molecular masses are chemically averaged values. The averaged molecular masses for the fully protonated forms of SOS and SHS are 982.2 and 902.7, respectively. The averaged molecular masses of the pepetides studied are given in Table 1. The averaged molecular masses of the sulfated CDs are presented in Table 2. From 100 to 500 pmol of sulfated oligosaccharide sample were used for the ESI-MS analyses. Mixtures of polysulfated oligosaccharides with the peptides, proteins and polyamines were prepared in molar ratios ranging from 1:1 to 1:20 for the ESI-MS analyses.

RESULTS AND DISCUSSION

Polysulfated carbohydrates are known to interact with peptides and proteins primarily through their ion-pairing interaction with basic amino acid residues arginine and lysine.[1,2] This ion-pairing interaction was first exploited in mass spectrometric analysis by Juhasz and Biemann[30] for the analysis of glycosaminoglycan-derived oligosaccharides using MALDI/MS. While this approach facilitated the analysis of highly sulfated oligosaccharides,[30,31] it can also afford fragmentation through loss of SO_3 (depending on the matrix chosen), making the approach not entirely satisfactory for the analysis of sample purity.

ESI-MS has also been successfully applied to the analysis of oligosaccharides having low levels of sulfation.[26] The extension of ESI-MS to highly sulfated oligosaccharides[28] led us to examine its use in the analysis of complexes of basic peptides and polysulfated oligosaccharides.

The effect of the net relative charge and the molar ratio between polysulfated oligosaccharide and basic peptide required for complex formation was first examined. SOS is a pharmaceutically important disaccharide with eight sulfate groups, and SHS (also prepared synthetically) corresponds to a putative, undersulfated contaminant found in pharmaceutical preparations of SOS. When SOS was analyzed by ESI-MS in the negative ion mode using standard conditions (capillary voltage −3.1 kV and nozzle–skimmer voltage −20 V), it produced a very complex spectrum due to multiple, negatively charged molecular ions (containing from three to five negative charges) resulting from multiple additions of sodium ions and multiple losses of SO_3 groups [Figs 1(A) and 2 illustrate the raw and transformed data, respectively]. Thus, the ESI negative ion mass spectrum obtained was of little use in assessing the purity of SOS. Using a higher nozzle skimmer voltage (i.e. −12 V, lower energy), the extent of SO_3 loss could be reduced but extensitve sodium adduct formation resulted. No mass spectrum of SOS could be generated in the positive ion ESI mode.

Ion pairing of polysulfated oligosaccharides with peptides

SOS was mixed with six basic peptides containing 2–11 positive charge sites, calculated from the sum of the number of lysines, arginines and amino terminus residues. The SOS:peptide molar ratio was 1:5. The negative ion ESI mass spectra exhibited multiply charged negative ions consistent with 1:1 molar complexes with the peptides containing 4–6 positive sites (Table 1) with almost no evidence for the presence of sodium counterions and the absence of fragmentation (loss of SO_3). [This is illustrated in Fig. 1(B) for the SOS–peptide **5**

Table 1. Peptides evaluated for sucrose octasulfate–peptide ion-paired complexes

Peptide[a]	Peptide structure	Av. M_r	No. of positive sites[b]	p*I*[c]	Charge at pH 7[c]	Stoichiometry of SOS–peptide complex
1	GVVNASCRLA	989.2	2	8.2	+0.9	ND[d]
2	$RRLIEDAEYAARG_{amide}$	1518.7	4	8.9	+0.9	1:1
3	$CSNLYKHVDTGRRY_{amide}$	1710.9	4	9.6	+3.0	1:1
4	$DHLKGILRRRQLYC_{amide}$	1770.1	5	10.3	+4.0	1:1
5	RKRARKE	943.1	6	11.8	+3.9	1:1
6	$CKSVRGKGKGQKRKRKK_{amide}$	1971.5	11	11.9	+10.9	ND

[a] Peptides from Sigma Chemical (peptide No., Cat. No.): **1**, A517-1,2; **2**, A566-3; **3**, A577-1-2; **4**, A571-1-3; **5**, A-8186; **6**, A242-1,2.
[b] Calculated from the sum of the number of arginine (R), lysine (K) and *N*-terminal amino acid residues.
[c] Calculated using Protean v. 3.07a (DNAstar, Madison, WI, USA).
[d] ND, not detected.

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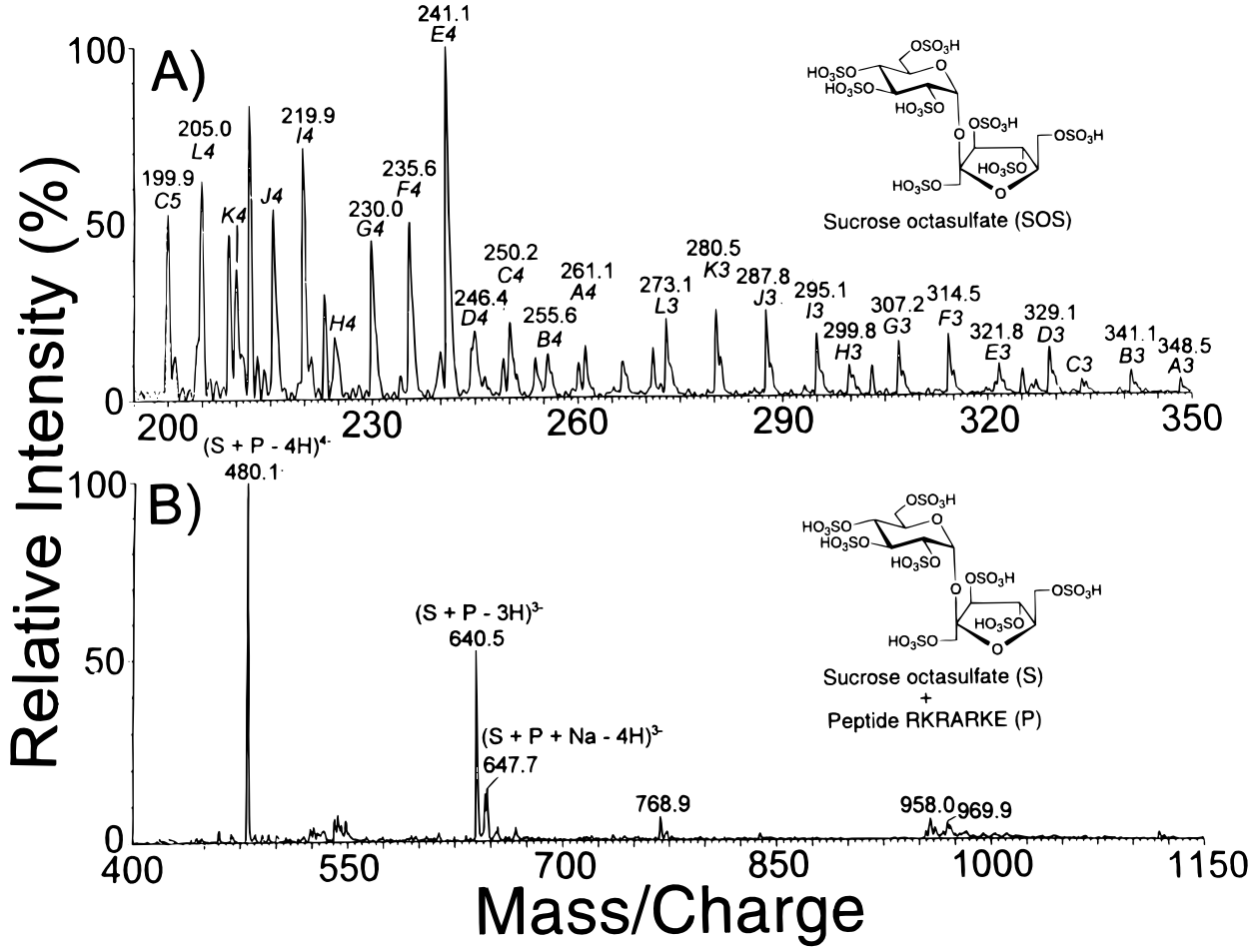

Figure 1. Negative ion ESI mass spectra of SOS and SOS–peptide **5** (1 : 1) complex. (A) SOS using standard conditions (capillary voltage −3.1 kV, nozzle–skimmer voltage −20 V, solvent 1 : 1 (v/v) water–acetonitrile, source temperature 80 °C). Peaks are labeled with an italicized letter (*X*) followed by an italicized number (*q*). These correspond to the chemical series type *X* with observed negative charge *q*, $[M - mSO_3 + n(\text{Na–H}) - q\text{H}]^{-q}$, where *M* is the molecular mass for SOS, *m* is the number of SO_3 groups lost and *n* is the number of sodium ions replacing protons. There are three groups of peaks corresponding to series in which 0, 1 and 2 SO_3 groups were lost. Series *A*, *B* and *C* correspond to $m = 0$, $n = 3$, 2 and 1, respectively. Series *D*, *E*, *F*, *G* and *H* correspond to $m = 1$, $n = 4$, 3, 2, 1 and 0, respectively. Series *I*, *J*, *K* and *L* correspond to $m = 2$, $n = 3$, 2, 1 and 0, respectively. (B) SOS–peptide **5** (1 : 1) complex prepared in a 1 : 5 molar ratio. S and P correspond to SOS and peptide **5**, respectively. The experimental conditions are similar to those in (A), except that the nozzle-skimmer voltage was set to −12 V. In (A) at $m/z > 350$ only noise or very weak signals are observed and in (B) at $m/z < 400$ only background noise is observed.

(1 : 1) complex; note the simplicity of this spectrum compared with the ESI mass spectrum for pure SOS produced under similar ESI-MS conditions, Fig. 1(A)]. These SOS–peptide (1 : 1) complexes were observed by ESI-MS only when the number of negative sites in SOS exceeded the number of positive sites in the peptide by 2–4 negative charges (Fig. 3). This result was expected because a 1 : 1 complex has a net charge of −2 to −4 while a 1 : 2 (or higher complex) would be positively charged. The positive ion ESI mass spectra of SOS–peptide mixtures exhibited only ions corresponding to the multiply charged peptide (+1 to +4).

Peptide **5** was chosen for use in subsequent studies because of its moderate number of charge sites and its relatively low molecular mass (943.1 Da). The transformed negative ion ESI mass spectra for 1 : 1 complexes of SOS–peptide **5** and SHS–peptide **5** demonstate the purity of these polysulfated disaccharides [Fig. 4(A) and (B), respectively]. While pharmaceutical SOS contains trace amounts of SHS, SHS was exeedingly pure, showing no hexasulfated or octasulfated material. A 1 : 1 molar mixture of SOS and SHS was next analyzed with a 10 molar excess of peptide **5** [Fig. 4(C)]. Only two peaks are observed, corresponding to the two 1 : 1 ion-pairing complexes of SOS–peptide **5** and SHS–peptide **5**. The absence of multiply sodiated species is particularly noteworthy. Hence this ion-pairing complexation method can be used to determine the relative purity of this class of polysulfated disaccharides. In addition, since these SOS and SHS samples are very pure, they can be used as calibration standards for quantitation. Table 2 summarizes the signal-to-noise ratios, absolute area counts and relative responses for the SOS–peptide **5** (1 : 1) and SHS–peptide **5** (1 : 1) complexes based upon the transformed ESI-MS data illustrated in Fig. 4(C). The unusual feature of these quantitation measurements is that the SHS–peptide **5** complex has about half the response of the SOS–peptide **5** complex when prepared in an SOS : SHS : peptide **5** molar ratio of 1 : 1 : 10, and concentration of 50 pmol μl^{-1} of SOS.

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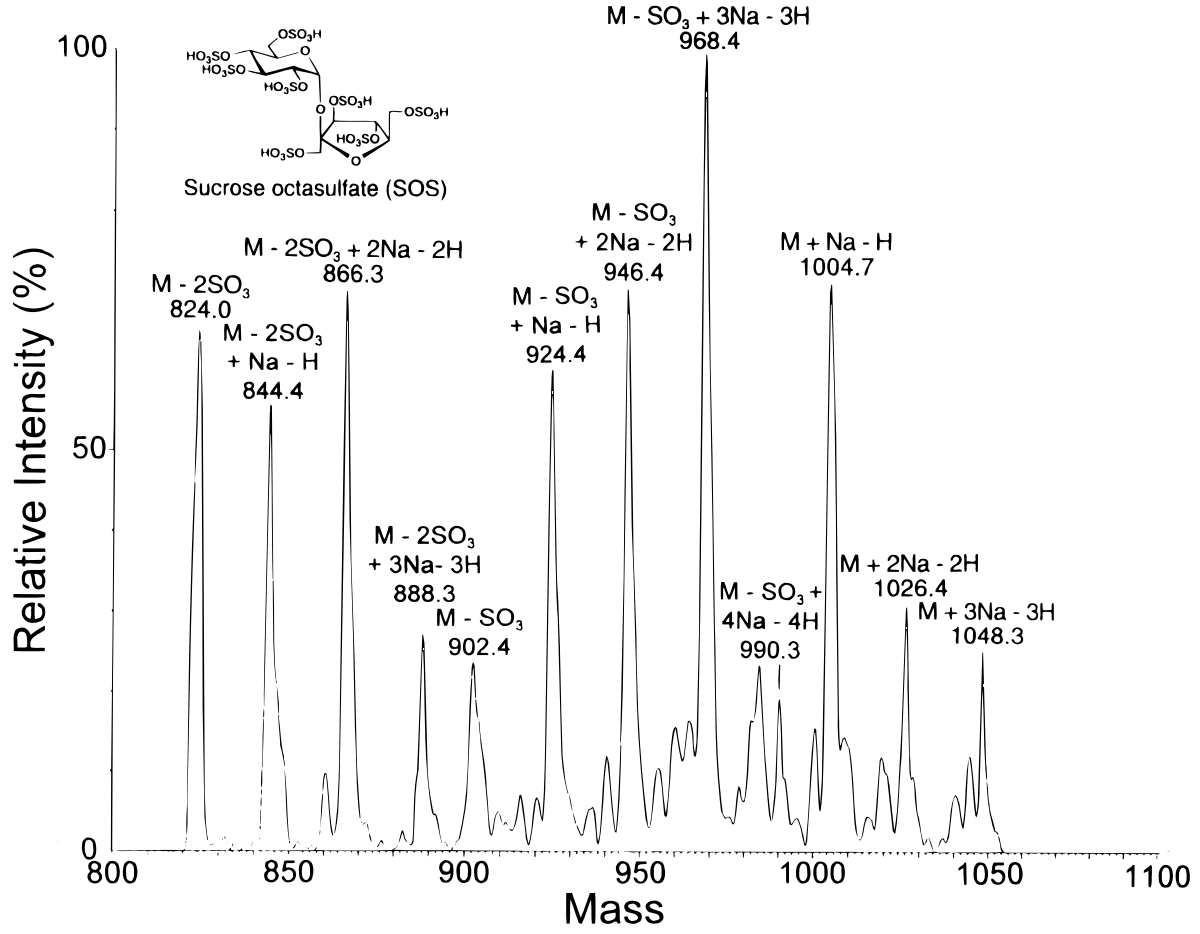

Figure 2. Transformed negative ion ESI mass spectrum of SOS. The average molecular mass of the acid form of SOS is 982.8. The nozzle–skimmer voltage was −20 V, the solvent was 1 : 1 (v/v) water–acetonitrile and the source temperature was 80 °C.

Four highly sulfated cyclodextrins (Table 3) were examined when mixed in a 1 : 5 molar ratio with peptide **5**, which has six positive charge sites, using ESI-MS in the negative ion mode [Fig. 5(A)–(D)]. Clusters of peaks were observed in each mass spectrum, with each peak 80 Da apart within the cluster, corresponding to a distribution of molecular ions containing additional sulfate groups. This technique is uniquely capable of obtaining the distribution of multiply sulfated species, not possible using other mass spectrometric methods. This distribution of species is consistent with the highly heterogeneous nature of sulfated CDs.[8] The clusters observed in Fig. 5 could be assigned to sulfated cyclodextrin–peptide complexes of stoichiometry 1 : 0, 1 : 1, 1 : 2 and 1 : 3 (see Table 3). The α-CD, having 8–11 sulfates per molecule, showed primarily a 1 : 1 complex with a very minor amount of 1 : 2 complex [Fig. 5(A)]. β-CD 1 had an unexpectedly low level of sulfation ranging from two to seven sulfate groups. Thus, a sizable percentage of the sample failed to give a sufficiently stable complex with peptide **5** and instead afforded a prominent cluster of peaks associated with the uncomplexed (1 : 0) sample and a minor amount of 1 : 1 complex [Fig. 5(B)] demonstrating incomplete

Table 2. Relative ESI-MS responses for SOS–peptide 5 and SHS–peptide 5 complexes

Parameter	SOS–peptide **5** (1 : 1)	SHS–peptide **5** (1 : 1)	Peptide **5**
Number of pmol used of sulfated disaccharide or peptide **5**	500	500	5000
Signal-to-noise ratio	60[a]	33[b]	—
Area count	9.084×10^6 [c]	5.348×10^6 [c]	—
Relative response (%)	62.9	37.1	—

[a] Based on the $[SOS–peptide\ \mathbf{5} - 4H]^{4-}$ peak in the negative ion ESI mass spectrum raw data.
[b] Based on the $[SHS–peptide\ \mathbf{5} - 3H]^{3-}$ peak in the negative ion ESI mass spectrum raw data.
[c] Based on the transformed ESI mass spectrum (Fig. 4(C)) of SOS–peptide **5** and SHS–peptide **5** complexes.

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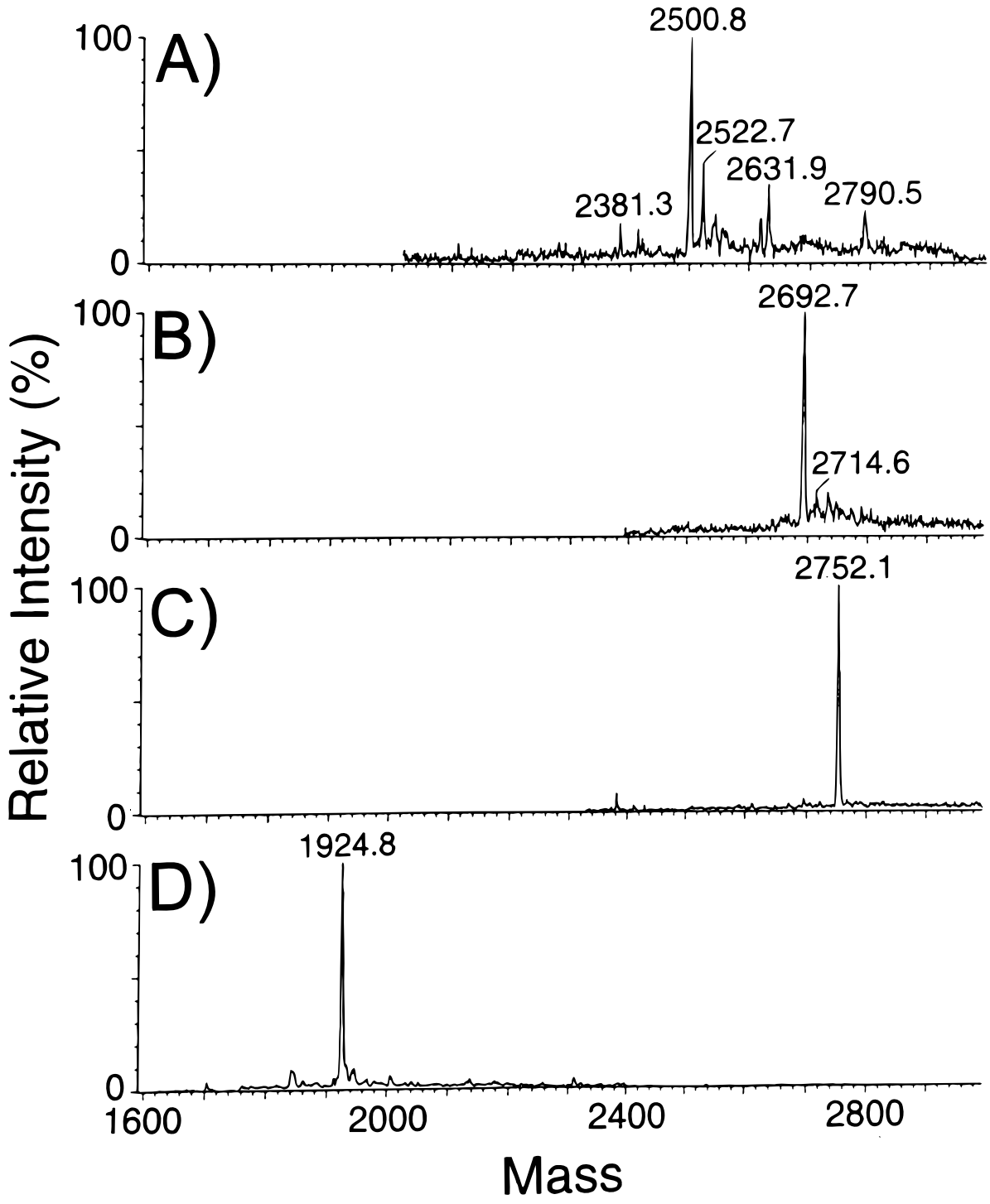

Figure 3. Transformed negative ion ESI mass spectra of SOS–peptide complexes. The average molecular mass of the acid form of SOS and peptides (A) **2**, (B) **3**, (C) **4** and (D) **5** are 982.8, 1518.7, 1710.9, 1769.0 and 943.1, respectively. The nozzle–skimmer voltage was −20 V, the solvent was 1 : 1 (v/v) water–acetonitrile and the source temperature was 80 °C.

chemical sulfation of this batch of β-CD. The β-CD 2 showed only complexed sample, with approximately equal intensities of 1 : 1 and 1 : 2 complex, consistent with higher levels of sulfation from 11 to 15 sulfate groups per molecule [Fig. 5(C)]. The larger γ-CD, having 11–16 sulfate groups per molecule, gave primarily a 1 : 2 complex with a very low abundance 1 : 3 complex. Hence the stoichiometry of the complex appears to depend on both the size and net charge of the sulfated oligosaccharide component. In the positive ion ESI mode, only peptide molecular ions were observed without sodium or potassium adducts.

The distributions for the CD–peptide **5** complexes can be calculated from the integrated intensities of the peaks appearing in the transformed negative ion ESI mass spectra [Fig. 5(A)–(D)]. Since no standards for any of the individual CD–peptide **5** components are available, the relative responses for each of the components were assumed to be equivalent. Table 4 lists the distributions (relative abundances) for the components

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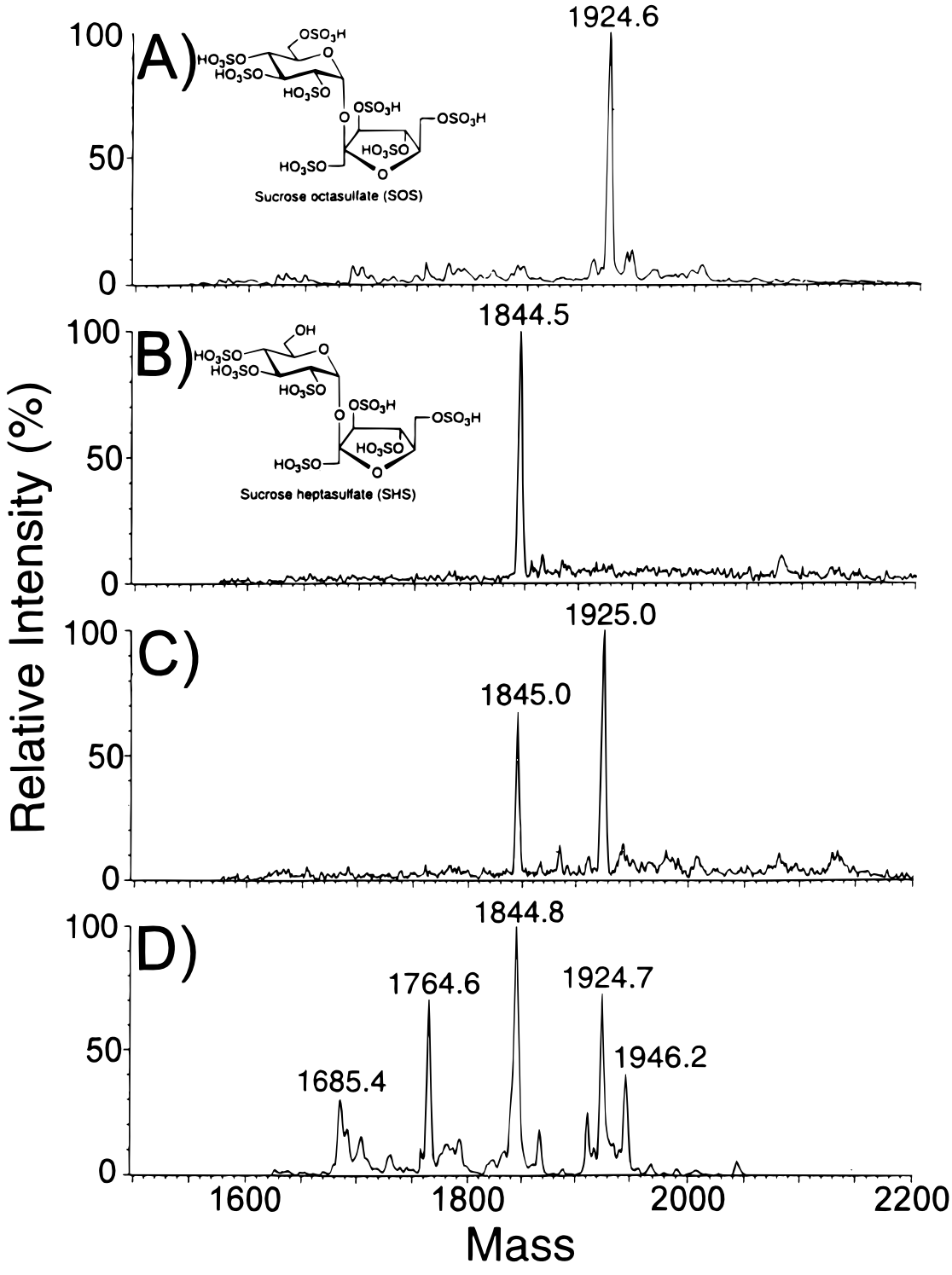

Figure 4. Transformed negative ion ESI mass spectra of 1 : 1 SOS and SHS complexes with peptide **5**. The average molecular masses of SOS, SHS (acid forms) and peptide **5** are 982.8, 902.7 and 943.1, respectively. (A) 1 : 1 SOS–peptide **5** complex; (B) 1 : 1 SHS–peptide **5** complex; and (C) equimolar mixture of 1 : 1 SOS–peptide **5** complex and 1 : 1 SHS–peptide **5** complex. The nozzle–skimmer voltage for the spectra shown in (A)–(C) was −12 V; (D) 1 : 1 SOS–peptide **5** complex at a nozzle–skimmer voltage of −30 V. The solvent used in these experiments was 1 : 1 (v/v) water–acetonitrile and the source temperature was 80 °C.

appearing in the negative ion ESI mass spectra for each of the CD samples. The weighted average number of sulfate groups for each CD was calculated from the distributions (Table 4) and correlates reasonably well with the values for the average degree of sulfation (Table 3) supplied by the manufacturer of the CDs.

Optimization of negative ion ESI-MS ion-pairing conditions

A number of experimental parameters had to be varied to optimize the signal-to-noise ratio in the negative ion

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Table 3. Properties of sulfated cyclodextrins and sulfated cyclodextrin–peptide 5 complexes

Sulfated cyclodextrin[a]	No. of saccharides	Desulfated cyclodextrin av. M_r	Maximum possible No. of sulfates[b]	Fully sulfated cyclodextrin av. M_r	Polysulfated cyclodextrin–peptide **5** complexes observed[c]	Range of sulfate groups observed	Average degree of sulfation[d]
α-CD	6	972.8	18	2394.3	1:1 s, 1:2 vw	8–12	12
β-CD 1	7	1135.0	21	2793.4	1:0 s, 1:1 vw	2–7	4
β-CD 2	7	1135.0	21	2793.4	1:1 s, 1:2 s	11–15	14
γ-CD	8	1297.2	24	3192.5	1:2 s, 1:3 vw	11–16	15

[a] Sulfated cyclodextrins from American Maize Products (type, lot number): α-CD, D8048; β-CD 1, F8050; β-CD 2, F8029; γ-CD, D8047.
[b] Total number of hydroxyl groups in the cyclodextrin precursor.
[c] Complex stoichiometry; s is strong peak, vw is very weak peak.
[d] Provided by manufacturer.

ESI-MS analyses of sulfated oligosaccharide–peptide ion-paired complexes. Both the fragmentation (loss of SO_3) and the sodium adduct formation also needed to be minimized. The experimental parameters optimized were the nozzle skimmer voltage, the molar ratio of ion-pairing reagent, the solvent composition and the source temperature. SOS and peptide **5** were chosen for these studies.

The effect of the nozzle–skimmer voltage on poly-sulfated oligosaccharide fragmentation in 1:1 poly-sulfated disaccharide–peptide complexes was examined. When low nozzle–skimmer voltages (−10 to −12 V) were used, the negative ion ESI mass spectra of peptide **5** complexes of SOS and SHS contained essentially single peaks, demonstrating the high purity of the SOS and SHS samples [Fig. 4(A) and (B), respectively]. The nozzle–skimmer voltage was then decreased (increased energy) from −12 to −20, −30 and −40 V. A series of fragment ions appeared in the ESI negative ion mass spectra of SOS below −12 V and became abundant at

Table 4. Sulfate distributions for sulfated cyclodextrins ion paired with peptide 5 as measured by ESI-MS in the negative ion mode

Sample (Fig.)	Mass[a]	Distribution Relative abundance (%)	Molar ratio[b] [CD]:[P]:[#S]	Weighted average No. of sulfate groups[c] (#S)
α-CD	2555	15.8	1:1:8	
[Fig. 5(A)]	2635	34.9	1:1:9	
	2715	34.0	1:1:10	
	2795	9.1	1:1:11	
	2887	6.2	1:1:12	9.6
β-CD 1	1294	5.0	1:0:2	
[Fig. 5(B)]	1374	21.0	1:0:3	
	1455	33.7	1:0:4	
	1535	34.4	1:0:5	
	1614	5.8	1:0:6	4.2
β-CD 2	2958	10.2	1:1:11	
[Fig. 5(C)]	3037	23.1	1:1:12	
	3118	32.3	1:1:13	
	3198	22.1	1:1:14	
	3277	10.2	1:1:15	
	3356	2.1	1:1:16	13.1
	3980	13.8	1:2:12	
	4060	31.7	1:2:13	
	4140	38.3	1:2:14	
	4221	16.2	1:2:15	13.6
α-CD	4061	10.9	1:2:11	
[Fig. 5(D)]	4141	19.5	1:2:12	
	4220	19.9	1:2:13	
	4303	20.3	1:2:14	
	4383	17.1	1:2:15	
	4463	12.2	1:2:16	13.5

[a] Transformed ESI masses (Fig. 5).
[b] [CD]:[P]:[#S] = sulfated cyclodextrins:peptide:number of sulfates molar ratio. Note that for β-CD and γ-CD there is only a 2 Da difference between [β-CD = 1]:[P = 1]:[n #S] and [γ-CD = 1]:[P = 1]:[(n − 2) #S].
[c] Weighted average #S = [Σ(%)(#S)]/[Σ(%)].

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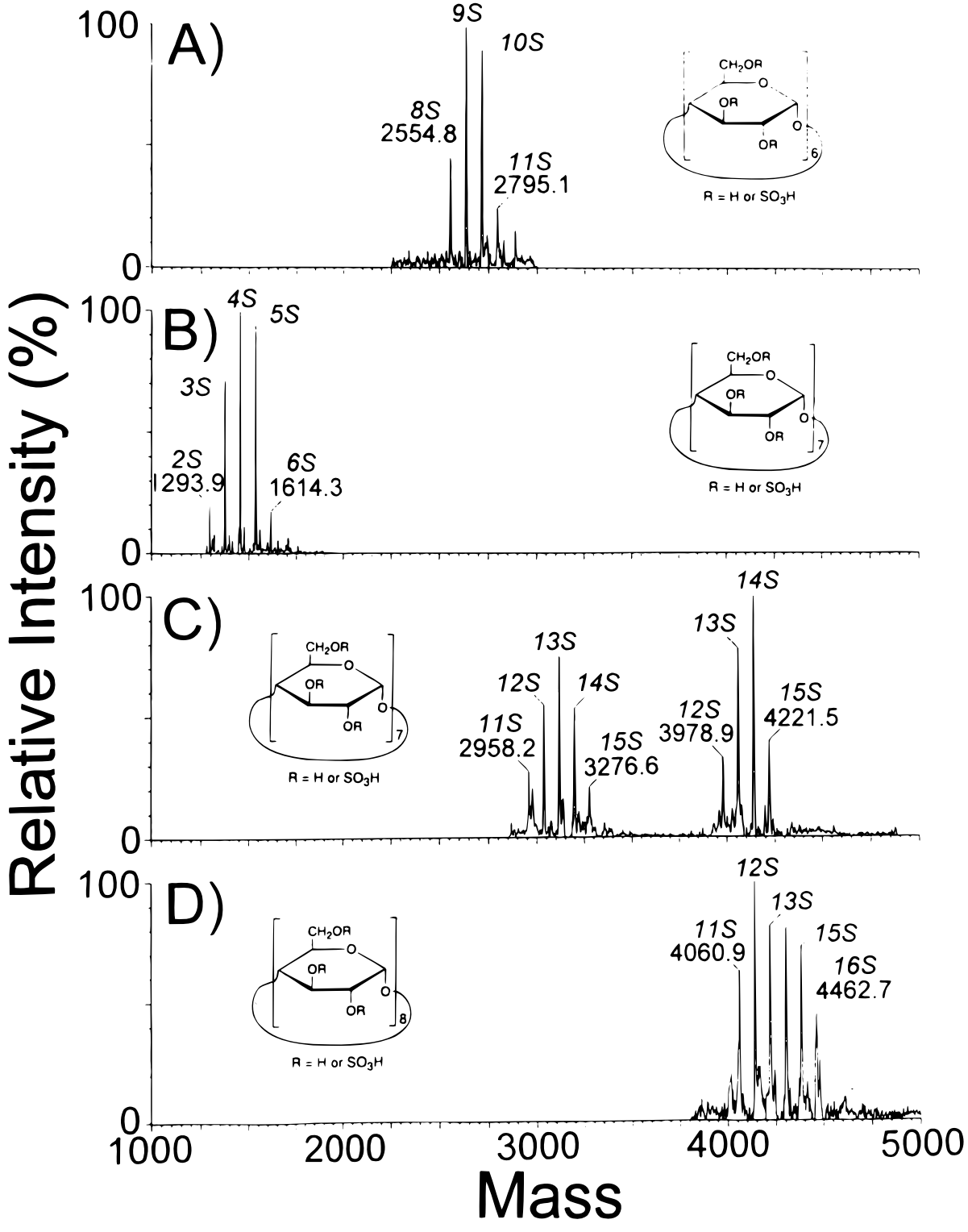

Figure 5. Transformed negative ion ESI mass spectra of cyclodextrin ion-paired complexes with peptide **5**. The average molecular masses of α-CD, β-CD 1, β-CD 2, γ-CD and peptide **5** are 972 + (80 × *n*), 1134 + (80 × *n*), 1134 + (80 × *n*), 1297 + (80 × *n*) (*n* = number of sulfate groups) and 943.1, respectively. Numbers followed by an S indicate the number of sulfate groups. See Table 4 for the complete mass assignments. (A) 1 : 1 α-CD–peptide **5** ion-paired complex; (B) 1 : 0 β-CD 1–peptide **5** ion-paired complex; (C) 1 : 1 and 1 : 2 β-CD 2–peptide **5** ion-paired complexes; and (D) 1 : 2 γ-CD–peptide **5** ion-paired complex. The nozzle–skimmer voltage was −12 V, the solvent was 1 : 1 (v/v) water–acetonitrile and the source temperature was 80 °C.

−30 V. These fragment ions were 80 Da apart, originating from the multiple loss of SO_3 from the sulfate groups [Fig. 4(D)]. The low abundance peaks 22 Da higher than the polysulfated components correspond to the replacement of the labile acidic H with Na. SHS behaved similarly (data not shown). These negative ion ESI-MS data demonstrate the stability of the 1 : 1 polysulfated oligosaccharide–peptide complexes and the

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ability to control the lability of the sulfate groups by adjusting the nozzle–skimmer voltage.

Mixtures of SOS and peptide **5** were prepared with molar ratios from 1:1 to 1:10. Negative ion ESI mass spectra (nozzle–skimmer voltage −12 V) were obtained and optimum sensitivity was observed using a 1:5 molar ratio mixture. This molar ratio gave exclusively 1:1 complexes without the formation of sodium adducts and without fragmentation (loss of SO_3). Low levels of uncomplexed SOS were also detected as sodium salts with multiple losses of SO_3. The negative ion ESI mass spectra of SOS–peptide **5** mixtures, prepared in 1:1 and 1:2 molar ratios, also afforded abundant sodiated adducts of the 1:1 complex containing Na–H and 2(Na–H).

The effect of solvent composition was then examined. When the solvent volume ratio of water–acetonitrile was changed from either 50:50 or 10:90 to 25:75, the sensitivity of peak detection for the SOS–peptide **5** (1:1) complex increased approximately twofold. Optimum sensitivity is achieved at a 25:75 water:acetonitrile solvent volume ratio. Next, the effect of ESI source temperature on the sensitivity of 1:1 polysulfated oligosaccharide–peptide complexes was examined. When the ESI source temperature was lowered from 80 to 60 °C, a drastic loss of sensitivity was incurred; however, the relative abundance of the SOS–peptide **5** (1:1) complex increased approximately twofold with a concurrent dramatic decrease in sulfate fragmentation.

Ion pairing of polysulfated oligosaccharides with proteins

The formation of 1:1 polysulfated oligosaccharide–protein complexes was also studied by negative ion ESI-MS. Ubiquitin (UB), a slightly basic protein (p*I* 7.16) of molecular mass 8565 is known to complex polyanions.[27] Complexes (1:1) of ubiquitin with SOS, SHS and polysulfated α-CD were observed in negative ion ESI mass spectra with sodiated adducts (Na–H) of low abundance (Fig. 6). Optimized spectra were obtained when the complex was prepared from a 1:2 molar ratio mixture of polysulfated oligosaccharide and ubiquitin, at a low capillary voltage of −2.4 kV and with a nozzle–skimmer voltage of −20 to −50 V. Figure 6(A) and (B) show the transformed ESI mass spectra of SOS and SHS in complexes with ubiquitin. Free ubiquitin is observed with a molecular mass of 8563 in both spectra. Figures 6(C) and 5(A) show the transformed negative ion ESI mass spectra of 1:1 complexes of sulfated α-CD with ubiquitin and peptide **5**, respectively. The similarity in the sulfate distributions observed and the average number of sulfate group complexes for the protein (Table 5) and peptide complexes (Table 4) further validates the ESI-MS method for the analysis of polysulfated oligosaccharides. The additional minor peaks (marked with asterisks) observed in the ESI mass spectrum of the ubiquitin–α-CD complex [Fig. 6(C)] are potassium adducts (K–H). Likewise, note the similarity in the sulfate distribution and the average number of sulfate groups for the α-CD complexes with ubiquitin and ubiquitin + (K–H) (Table 4). A similar sample of ubiquitin and α-CD in the positive ion ESI mode produced spectra consistent with the presence of polypotassiated (K–H) ubiquitin and showed no α-CD–ubiquitin complex. Horse heart myoglobin, a large neutral protein (M_r 16 945) was examined to determine whether complex formation between polysulfated oligosaccharide and larger proteins could be observed using ESI-MS. No α-CD–horse heart myoglobin complexes were observed in either the negative or positive ion ESI mass spectra. This result again suggests that an appropriate protein structure and a balance between excess negative and positive charges within the complex are required for the analysis of a complex by ESI-MS.

Table 5. Sulfate distribution for sulfated α-cyclodextrin ion paired with ubiquitin as measured by ESI-MS

Sample (Fig.)	Mass[a]	Distribution relative abundance (%)	Molar ratio[b] [CD]:[P]:[#S]	Weighted average No. of sulfate groups[c] (#S)
α-CD:ubiquitin	10 013	2.4	1:1:6	
[Fig. 6(C)]	10 095	2.9	1:1:7	
	10 176	14.1	1:1:8	
	10 256	29.6	1:1:9	
	10 335	25.0	1:1:10	
	10 414	18.7	1:1:11	
	10 493	5.3	1:1:12	
	10 573	2.0	1:1:13	9.6
α-CD:ubiquitin + K–H	10 138	10.6	1:1:7	
	10 214	7.8	1:1:8	
[Fig. 6(C)]	10 293	20.6	1:1:9	
	10 373	27.6	1:1:10	
	10 452	22.1	1:1:11	
	10 531	8.2	1:1:12	
	10 609	3.1	1:1:13	9.8

[a] Transformed ESI masses (Fig. 6).
[b] [CD]:[P]:[#S] = sulfated cyclodextrin:peptide:number of sulfates molar ratio.
[c] Weighted average #S = $[\Sigma(\%)(\#S)]/[\Sigma(\%)]$.

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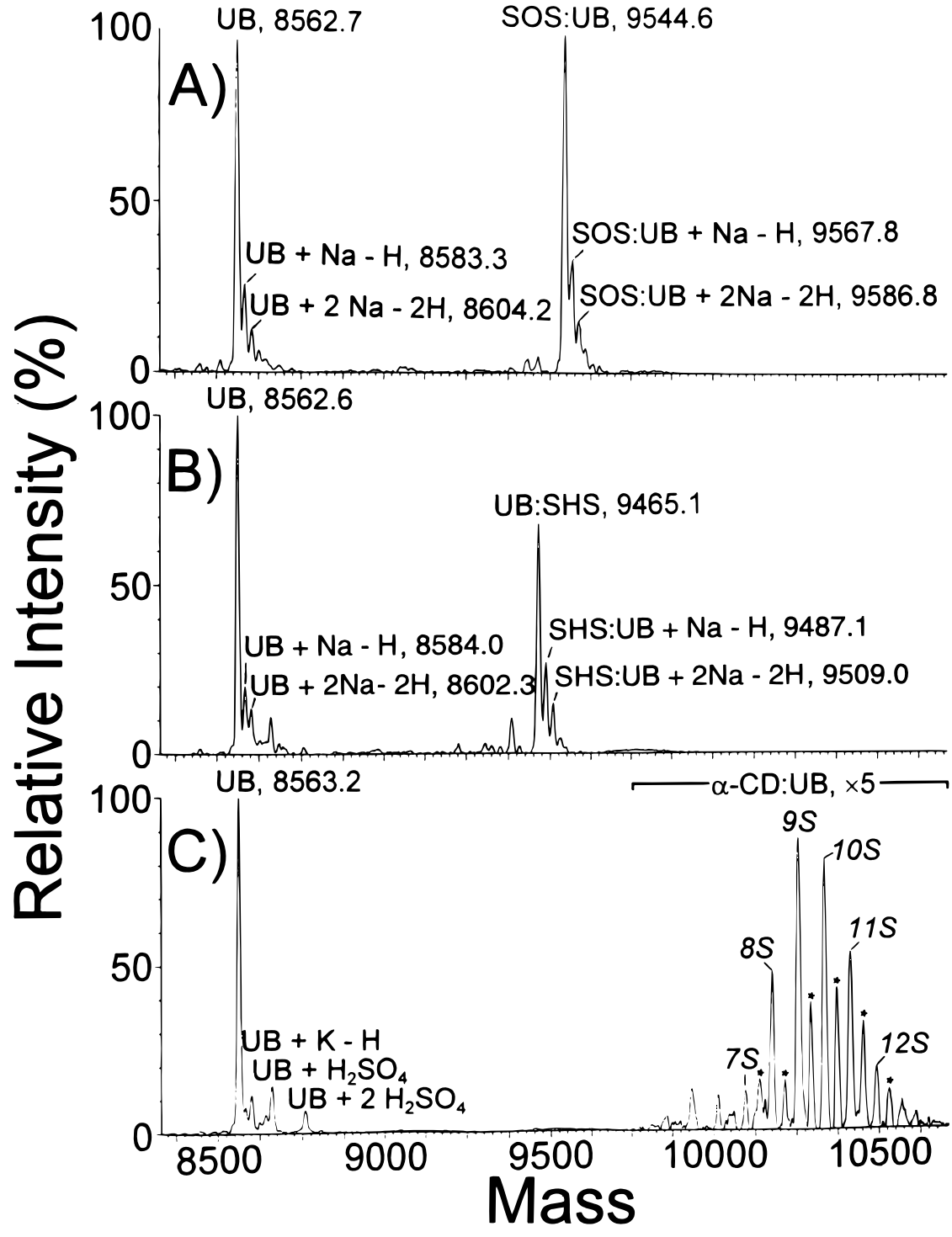

Figure 6. Transformed negative ion ESI mass spectra of SOS, SHS and α-CD complexes with ubiquitin (UB). The average molecular masses for SOS, SHS, α-CD and bovine UB are 982.8, 902.7, 972 + (80 × *n*) (*n* = number of sulfate groups) and 8565, respectively. Numbers followed by an S indicate the number of sulfate groups. See Table 5 for the complete mass assignments. (A) UB and 1 : 1 SOS–UB complex; (B) 1 : 1 SHS–UB complex; and (C) 1 : 1 α-CD–UB complex. The peaks marked with asterisks correspond to potassium adducts (K–H). The nozzle–skimmer voltage was −20 V, the solvent was 1 : 1 (v/v) water–acetonitrile and the source temperature was 80 °C.

Ion pairing of polysulfated oligosaccharides and polyamines

Two polyamines, spermidine (SPD) ($H_2N(CH_2)_4NH(CH_2)_3NH_2$) and spermine (SPM) ($H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$), were used to prepare ion-paired complexes with SOS. These polyamines were chosen because they are known to complex readily with acidic peptides[32] and polynucleotides.[33] When mixtures of SOS and polyamines were prepared

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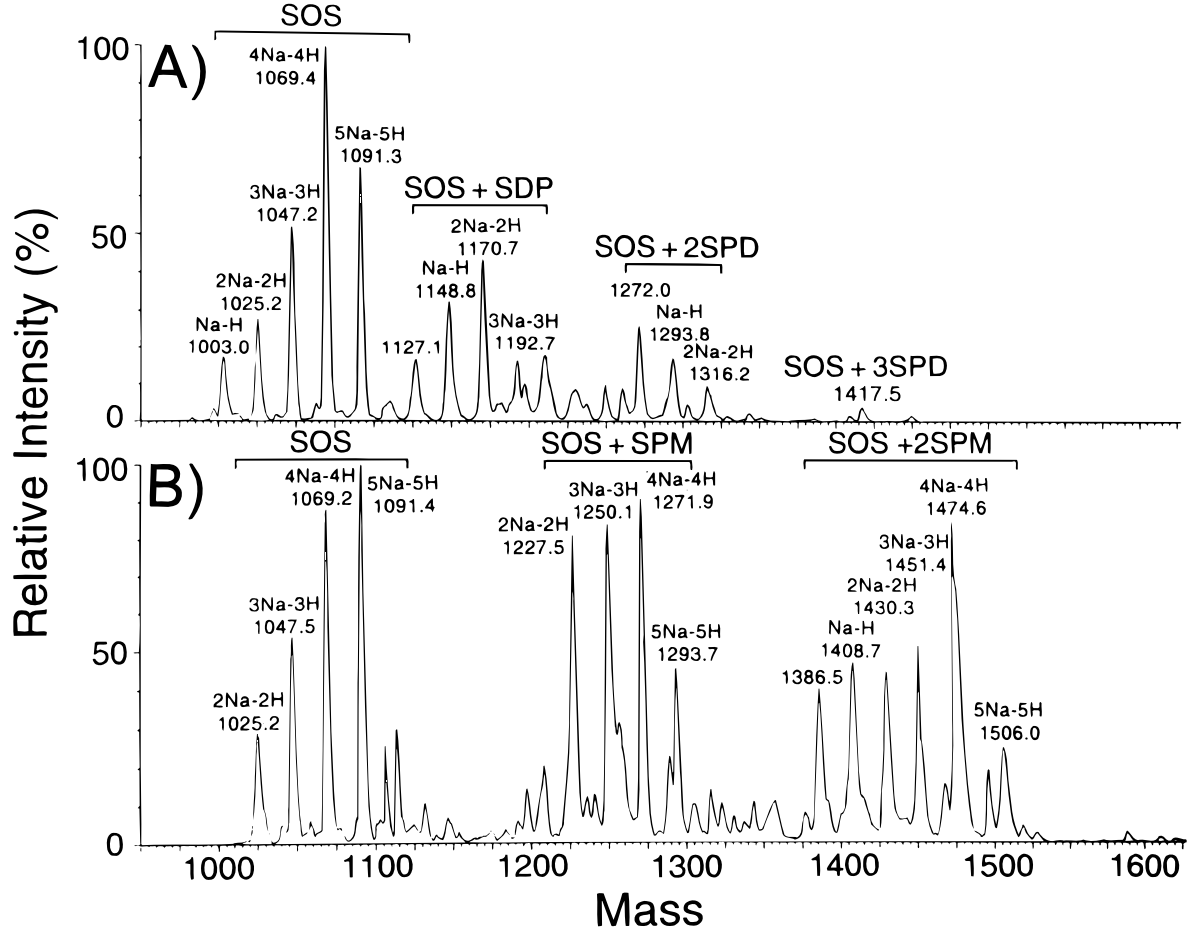

Figure 7. Transformed negative ion ESI mass spectra of SOS ion-paired complexes with polyamines. The average molecular masses of SOS, sperimidine (SPD) and spermine (SPM) are 982.8, 145.3 and 202.3, respectively. (A) 1 : 0, 1 : 2 and 1 : 2 SOS–SPD complexes are observed as differentially sodiated species; (B) 1 : 0, 1 : 1 and 1 : 2 SOS–SPD complexes are observed as multiply sodiated species. The nozzle–skimmer voltage was −12 V, the solvent was 1 : 1 (v/v) water–acetonitrile and the source temperature was 80 °C.

with molar ratios of 1 : 20, the transformed negative ion ESI mass spectra [Fig. 7(A) and (B)] exhibited multiply sodiated peaks corresponding to 2 : 1 and 1 : 1 polyamine–SOS complexes in addition to uncomplexed SOS. No fragmentation (loss of SO_3) was observed in these spectra. However, when mixtures of SOS and polyamines were prepared with molar ratios of 1 : 5, multiply sodiated peaks appeared corresponding to a 1 : 1 polyamine–SOS complex and uncomplexed SOS, each showing a loss of SO_3. The presence of multiply sodiated species and the loss of SO_3 as a function of decreasing polyamine molar ratio complicates the use of polyamines for the analysis of SOS purity.

CONCLUSIONS

Ion-pair complexes can be readily formed in negative ion ESI-MS between acidic polysulfated oligosaccharides and basic peptides and proteins for quantitating the distributions of the sulfate groups. The optimization of the experimental conditions, particularly the careful adjustment of the nozzle–skimmer voltage, could control the loss of SO_3 groups. The major limitation in the technique is that the formation of the complex is highly dependent upon the nature of the chemical structures of the sulfated oligosaccharide and the complexing peptide or protein and the number of charges present in them. To form stable complexes, the choice of best peptides or proteins for complexation for a given polysulfate should be determined empirically. These non-covalent complexes are due to non-specific ion pairing between the polyanionic and polycationic species. This work may also represent an important new approach for the study of non-covalent interactions between basic peptides and proteins and sulfated oligosaccharides and may account for non-covalent complexes reported in the literature. The methods described using ESI-MS may also be applicable to polyphosphate (DNA)–peptide complexes recently studied by MALDI/MS.[34,35]

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