Exposure to Common Food Additive Carrageenan Leads to Reduced Sulfatase Activity and Increase in Sulfated Glycosaminoglycans in Human Epithelial Cells

Bo Yang[a]

Sumit Bhattacharyya[b,c]

Robert Linhardt[a]

Joanne Tobacman[b,c]

[a]Department of Chemistry and Chemical Biology, Rensselaer Polytechnic Institute, Troy, NY

[b]Department of Medicine, University of Illinois at Chicago, Chicago IL

[c]Jesse Brown VA Medical Center, Chicago, IL

Address correspondence to:

Joanne K. Tobacman, MD
Department of Medicine
University of Illinois at Chicago
840 S. Wood St.
CSN 440, M/C 718
Chicago, IL 60612
Telephone: 312-569-7826
Fax 312-413-8283
E-mail: jkt@uic.edu

Abstract

The commonly used food additive carrageenan, including lambda (λ), kappa (κ) and iota (ι) forms, is composed of galactose disaccharides linked in alpha-1,3 and beta-1,4 glycosidic bonds with up to three sulfate groups per disaccharide residue. Carrageenan closely resembles the endogenous galactose or N-acetylgalactosamine-containing glycosaminoglycans (GAGs), chondroitin sulfate (CS), dermatan sulfate (DS), and keratan sulfate. However, these GAGs have beta-1,3 and beta-1,4 glycosidic bonds, in contrast to the unusual alpha-1,3 glycosidic bond in carrageenan. Since sulfatase activity is inhibited by sulfate, and carrageenan is so highly sulfated, we tested the effect of carrageenan exposure on sulfatase activity in human intestinal and mammary epithelial cell lines and found that carrageenan exposure significantly reduced the activity of sulfatases, including N-acetylgalactosamine-4-sulfatase, galactose-6-sulfatase, iduronate sulfatase, steroid sulfatase, arylsulfatase A, SULF-1,2, and heparan sulfamidase. Consistent with the inhibition of sulfatase activity, following exposure to carrageenan, GAG content increased significantly and showed marked differences in disaccharide composition. Specific changes in CS disaccharides included increases in di-sulfated disaccharide components of CS-D (2S6S) and CS-E, (4S6S), with declines in CS-A (4S) and CS-C (6S). Specific changes in heparin-heparan sulfate disaccharides included increases in 6S disaccharides, as well as increases in NS and 2S6S disaccharides. Study results suggest that carrageenan inhibition of sulfatase activity leads to re-distribution of the cellular GAG composition with increase in di-sulfated CS and with potential consequences for cell structure and function.

Key Words

sulfatase, carrageenan, glycosaminoglycan, disaccharide, heparin, chondroitin

1. Introduction

The common food additive, carrageenan, is consumed in the average diet in sufficient quantities to have biological effects. In contrast to the glycoside digoxin, which is generally prescribed in doses of 0.25 mg daily, average daily ingestion of carrageenan in the typical diet is estimated to be 250 mg/day [1,2]. Individuals who consume several carrageenan-containing foods may ingest several grams of carrageenan per day [3,4]. Carrageenan is found in a wide range of processed foods, including ice cream, whipped cream, infant formula, deli meats, sour cream, puddings, soymilk, yogurt, and dietary supplements. Carrageenan is also used in pharmaceuticals as an excipient, and in room air fresheners, cosmetics, and pet foods, due to its ability to improve the texture and solubility of ingredients. The Joint Expert Committee (Food and Agriculture Organization of the UN and the WHO) on Food Additives has recommended that carrageenan be excluded from infant formula and that current intake of carrageenan in the diet be re-evaluated [5].

Three major types of carrageenan are used in food products. These include lambda (λ)-carrageenan which is the most highly sulfated, with sulfate groups at C2 and C2, C6; kappa (κ)-carrageenan with sulfate groups at C4 of the alpha linked galactose and C3-C6 epimerization of the beta-linked galactose; and iota (ι)-carrageenan that resembles κ-carrageenan, but with the addition of a second sulfate group at C2 of the β-1,4- linked galactose (**Fig.1**). λ-carrageenan is the most soluble and has the lowest gel-forming ability of the carrageenans, while κ- and ι-carrageenans readily form gels in association with potassium or calcium ions, respectively [6,7].

In thousands of experiments, carrageenans have been used to induce inflammation, since inflammation is a predictable effect of exposure to carrageenan in animal and cell-based models. For the most part, these experiments were designed to test the effectiveness of anti-inflammatory agents or to study the mediators of inflammation [8]. In recent experiments, we have identified specific mechanisms by which carrageenan causes inflammation, and have demonstrated activation of an innate immune pathway of inflammation and a reactive-oxygen

species (ROS)-mediated pathway [9-12]. The innate immune pathway is mediated by toll-like receptor (TLR)-4 and B-cell leukemia lymphoma (BCL)10, leading to increased Interleukin (IL)-8 secretion by both canonical and non-canonical pathways of NF-κB activation. The ROS-mediated pathway of inflammation does not involve TLR4-BCL10, but requires Hsp27 and IκB-kinase (IKK)β, leading to increased phosphorylation of IκBα, and thereby enabling the nuclear translocation of NF-κB. Enzymes that hydrolyze the galactosidic bonds of carrageenan affect the extent of activation of the innate immune pathway, with κ-carrageenan-induced inflammation reduced by exposure to α-1→3,6-galactosidase and increased by κ-carrageenase [13]. Carrageenan-induced ROS-mediated inflammation was reduced following exposure of κ-carrageenan to recombinant human arylsulfatase B, (ARSB), suggesting that the presence of sulfate groups on carrageenan contributes to ROS activation [14].

In the current study, we assess the effect of carrageenan exposure on sulfatase activity of human epithelial cells in culture and determine changes in GAG content and disaccharide composition and present these findings to better understand how carrageenan exposure impacts upon the endogenous GAGs.

2. Methods and Materials

2.1 Cell culture

Several intestinal epithelial cell lines were grown in cell culture, including NCM460 (INCELL, San Antonio, TX) [15], T84 (ATCC), CaCO2 (ATCC), rat ileal epithelial cells (ATCC), mouse intestinal epithelial cells, and primary human colonocytes obtained at the time of colonic surgery, in accord with an IRB-approved protocol at the University of Illinois at Chicago. Mammary cell lines included: MCF-7 (ATCC), MCF-10A (ATCC), T47D (ATCC), HCC1937 (ATCC), and primary myoepithelial cells and primary epithelial cells, obtained from reduction mammoplasty in accord with an IRB-approved protocol. The methods for culture of these cells were reported previously and were those that were recommended [16,17]. Cells were grown under established conditions, including 37°C, 5% CO_2, and 95% humidity, with media changes at 2-3 day intervals.

2.3 Measurement of activity of sulfatases

Activity of several sulfatases was measured in NCM460 and MCF-7 cell homogenates at 80-90% confluence after 4-6 days in culture, as indicated in Tables 1 and 2 and in Results 3.1 and 3.2. Cells were exposed to λ-, κ-, or ι-carrageenan (1 ug/mL) (**Fig. 1**), and activity was compared to unexposed cells grown simultaneously under the same conditions. Methods used for assay of N-acetylgalactosamine-4-sulfatase (Arylsulfatase B, ARSB), galactose-6-sulfatase (GALNS), steroid sulfatase (STS), iduronate-2-sulfatase (IDS), and arylsulfatase A (ARSA) were published previously [16]. Cell homogenates were prepared and assays were performed using at least triplicate biological samples with technical replicates of each measurement.

ARSB activity assay was performed by a fluorometric assay with 20 µl of cell homogenate, 80 µl of assay buffer (0.05 M Na acetate buffer, pH 5.6), and 100 µl of substrate (5 mM 4-MUS in assay buffer) in wells of a microplate. The microplate was incubated for 30 minutes at 37˚C. The reaction was stopped by adding 150 µl of stop buffer (Glycine-Carbonate buffer, pH 10.7), and fluorescence was measured at 360 nm (excitation) and 465 nm (emission)

(FLUOstar, BMG Labtech, Inc., Gary, NC). ARSB activity units are nmol/mg protein/hour, and were derived from a standard curve prepared with known quantities of 4-methylumbilleferyl at pH 5.6.

Substrates for determinations of activity of galactose-6-sulfatase (GALNS) and iduronate-2-sulfatase (IDS) were obtained (Moscerdam Substrates, Rotterdam, The Netherlands), and the assays were performed in accord with previously published protocols [18,19]. GALNS assay was performed with 5 µl cell homogenate made in ddH_2O by sonication with metal tip combined with 5 µl 0.2% heat-inactivated BSA (or 10 µl of 0.2% heat-inactivated BSA for blank) and 20 µl of substrate [10 mM 4-methylumbilliferyl-ß-D-galactoside-6-sulfateNH_4 (MU-ßGal-6S)] in substrate buffer [0.1 M sodium acetate / 0.1M acetic acid at pH 4.3 with 0.1 M NaCl, 5 mM Pb-acetate (1.9 mg/ml) and 0.02% Na-azide] in wells of a microtiter plate. The plate was sealed, and incubated for 17 hours at 37˚C. Next, 5 µl 0.9 M Na-Phosphate buffer at pH 4.3 with 0.02% Na-azide was added, as well as 10 µl of 10 U ß-D-Galactoside galactohydrolase (Sigma) / ml 0.2% heat-inactivated BSA. Reactants were incubated for 2 hours at 37˚C, and then 200 µl of stop buffer [0.5 M $NaHCO_3$ / 0.5 M Na_2CO_3 at pH 10.7 with 0.025% Triton-X-100] was added. Fluorescence readings were taken at 360 nm and 465 nm. GALNS activity is expressed as nmol/mg protein/hour.

For IDS activity, 10 µl of cell homogenate made in water by sonification with a metal tip [or 10 µl 0.2% heat-inactivated bovine serum albumin (BSA) for the blank] was combined with 20 µl of substrate in substrate buffer (1.25 mM 4-MUS in 0.1 M Na-acetate / 0.1 M acetic acid at pH 5.0 with 10 mM Pb-acetate). Cell homogenate, substrate, and substrate buffer were incubated 37˚C x 4 hours. After 4 hours, 40 µl of 0.4 M Na-Phosphate / 0.2 M citrate buffer at pH 4.5 and 0.02% Na-azide were added, as well as 10 µl of LEBT (lysosomal enzymes purified from bovine testis), followed by incubation for 24 hours at 37˚C. Next, 200 µl of stop buffer consisting of 0.5 M $NaHCO_3$ / Na_2CO_3 at pH 10.7 with 0.025% Triton-X-100 was added, and readings were taken at 360 nm and 465 nm. IDS activity is expressed as nmol/mg protein/hour.

ARSA activity was determined by a spectrophotometric technique using *p*-nitrocatechol sulfate (NCS) as substrate, and based on the retention of ARSA activity at low temperature, in contrast to ARSB activity [20,21]. Pre-chilled reagents were used, and cells were homogenized in ddH_2O by sonication. Cell homogenates were centrifuged for 10 minutes at 5000g at 4˚ C, and the supernatant was used for the enzyme activity assay. Cell extracts were incubated in a total volume of 200 µl 0.5M Na acetate buffer at pH 5.0 with 200 µl of 10mM *p*-NCS in an ice-bath. After 17 hours, the reaction was stopped by adding 600 µl of 1M NaOH, and the reaction mixture was centrifuged for 5 minutes to remove precipitated material. The optical density of the clear supernatant was measured at 516 nm in a Beckman spectrophotometer. ARSA activity is reported as nmol/mg protein/hour.

STS activity assay was performed using 20 µl of cell homogenate, 80 µl of assay buffer, and 100 µl of substrate [16,22]. The substrate was 0.5 mM 4-methylumbilleferyl-sulfate (4-MUS) in substrate buffer consisting of 0.5 M Tris-C Buffer at pH 7.5. The reaction mixture was incubated for 4 hours at 37˚C, then 100 µl of stop buffer (1 M Tris-Cl buffer at pH 10.4) was added. Fluorescence was measured at 360 nm and 465 nm (FLUOstar, BMG Labtech Inc., Cary, NC). STS activity units are nmol/mg protein/hour, and were calculated by a standard curve prepared with known quantities of 4-methylumbilleferyl at pH 7.5.

Measurement of heparan sulfamidase activity was performed following an established assay procedure [23], using the substrate methylumbilleferyl-α-D-N-sulphoglucosaminide.Na (MU-αGlcNS), the substrate buffer consisting of Na-barbital in Na-acetate prepared with 0.7% NaCl at pH 6.5 with 0.02% (w/v) Na-azide, and the protease inhibitor 4-(2-aminoethyl)-benzenesulfonylfluoride.HCL (Roche, Indianapolis, ID). α-Glucosidase from *Bacillus stearothermophilus* (Sigma Chemical Company, St. Louis, MO) 10 U / ml BSA-0.2%, double concentrated McIlvains phosphate/citrate (Pi/Ci) buffer, and bicarbonate stop buffer at pH 10.7 with 0.025% Triton X-100 were required. Cell homogenates (30 µg protein) were prepared in water by sonification with a metal tip. Cell preparations were incubated with 10 µl heat

denatured BSA-0.2%, 10 µl MU-αGlcNS and 10 µl of the protease inhibitor for 17 h at 47°C. Then the reaction was stopped with 6 µl Pi/Ci buffer and mixed with 10 µl α-glucosidase and incubated for 24 h at 37°C. The reaction was terminated by addition of 200 µl of the stop buffer, and the fluorescence of the MU read. Activity was expressed as nmol/mg protein/hour.

SULF-1 and SULF-2 are endosulfatases that act extracellularly to remove sulfate groups from heparin and heparin sulfate. Activity was measured in the spent media of the NCM460 cells following exposure to λ-carrageenan or unexposed control following an established procedure [24]. Concentrated spent media were mixed with 10 mM 4-MUS, 10 mM lead acetate, and 50 mM HEPES at pH 8.0 yielding a total volume of 30 µl. The mixture was incubated for 2 h at 37°C. 100 µl of 0.5 M Na_2CO_3/$NaHCO_3$ was used to stop the reaction, and fluorescence was measured, the OD was correlated with known concentrations of 4-MU at pH 8.0, and the activity expressed as nmol/mg protein/h.

2.3 Cell-based ELISA and cDNA microarray of sulfatases

2.3.1 Cell-based ELISA is designed as a microplate-based Western blot (Active Motif, Carlsbad, CA), and was used to detect the protein content of ARSB, ARSA, GALNS, and STS in NCM460 and T84 cells after exposure to λ-carrageenan for 48 hours [25]. Activity at 48 hours was determined by the procedures described above and were within the standard deviation of the activity at 4 days that is reported in Table 1 for these enzymes. Antibodies used for detection were ARSB rabbit polyclonal antibody (1:250; Open Biosystems, Huntsville, AL), GALNS rabbit polyclonal antibody (1:250; Open Biosystems, Huntsville, AL), STS mouse monoclonal (2 µg/ml, Abcam, Cambridge, MA) and ARSA mouse monoclonal (4 µg/ml; R&D, St. Paul, MN). Rabbit or mouse IgG was used for control experiments. For the cell-based ELISAs, NCM460 or T84 cells were seeded at densities of 2500, 5000, and 10,000 cells per well in a 96-well plate with n=5 for each of the determinations. After 48 hours, the media were aspirated, the cells were washed with PBS, and then fixed x 20 minutes at room temperature with 100 µl of formaldehyde 4% in PBS. The formaldehyde was removed and cells were washed with wash

buffer (0.1% Triton X-100 in PBS) 200 µl three times, wash buffer was aspirated, and cells were incubated with quenching buffer (1% H_2O_2 in wash buffer) 100 µl for 20 minutes at room temperature. Then quenching buffer was removed and cells were washed three times with wash buffer 200 µl. Wash buffer was aspirated and cells were incubated with blocking buffer (PBS with 5% non-fat milk/FBS) 100 µl to each well for 1 hour at room temperature. Blocking buffer was aspirated, and primary antibody to either ARSB, ARSA, GALNS, or STS was added (diluted in PBS with 0.1% heat-inactivated BSA), and the plate the sealed and incubated for 2 hours at room temperature. Primary antibody was aspirated, and cells were washed with 200 µl of wash buffer three times, and the secondary antibody (100 µl) was added to each well, the plate was sealed and incubated for 1 hour at room temperature. The secondary antibody was aspirated and cells were washed with wash buffer (200 µl) three times, then 100 µl of developing solution was added to each well, and incubated at room temperature x 15 minutes and protected from light. Stop solution (2N sulfuric acid; 100 µl) was added to each well, then absorbance was read at 450 nm. Cells were washed with 200 µl of wash buffer x 3, excess liquid was removed from the wells, and the plates were air-dried at room temperature for 5 minutes. Crystal violet solution 100 µl was added to each well, and the plates were incubated for 30 minutes at room temperature, washed with wash buffer three times, and 100 µl SDS solution was added to each well, and the plate was incubated on a shaker for 1 hour at room temperature and final absorbance was read at 595 nm. The linearity of the determinations was confirmed by comparisons of the intensity at three different cell concentrations.

2.3.2 cDNA microarrays were performed on NCM460 cells after exposure to λ-carrageenan (1µg/ml) for 4 days using the Affymetrix Human Gene Chip U133 plus 2.0 system (Santa Clara, CA) [26]. Total RNA was extracted using the RNeasy minikit (Qiagen) from replicate biological samples of carrageenan-treated and control samples. Each chip contained 11 probeset pairs (22 probes total) for each gene with 54,613 transcripts present on the chip. Pair-wise

comparisons of control vs. carrageenan-treated mean fluorescent intensity values were performed, and a total of 790 transcripts had uncorrected P-values less than 0.001.

2.4 Materials and reagents for disaccharide analysis

Actinase E was from Kaken Biochemicals (Tokyo, Japan). Chondroitin lyases ABC and ACII and heparin lyases 1, 2 and 3 were from Seikagaku Corporation (Tokyo, Japan). Urea, CHAPS were from Sigma Chemical Company (St. Louis, MO, USA). Vivapure MINI Q H spin columns were from Viva science (Edgewood, NJ, USA). Microcon Centrifugal Filter Units YM-3 was from Millipore Company (Bedford, MA, USA). Unsaturated disaccharide standards of CS/DS (ΔDi-0S: ΔUA-GalNAc, ΔDi-4S: ΔUA-GalNAc4S, ΔDi-6S: ΔUAGalNAc6S, ΔDi-2S: ΔUA2S-GalNAc, ΔDi-diSB: ΔUA2S-GalNAc4S, ΔDi-diSD: ΔUA2S-GalNAc6S, ΔDi-diSE: ΔUA-GalNAc4S6S, ΔDi-triS: ΔUA2SGalNAc4S6S) and unsaturated disaccharides standards of HP/HS (ΔDi-0S: ΔUA-GlcNAc, ΔDi-NS: ΔUA-GlcNS, ΔDi-6S: ΔUA-GlcNAc6S, ΔDi-2S: ΔUA2S-GlcNAc, ΔDi-2SNS: ΔUA2S-GlcNS, ΔDi-NS6S: ΔUA-GlcNS6S, ΔDi-2S6S: ΔUA2S-GlcNAc6S, ΔDi-triS: ΔUA2S-GlcNS6S) were obtained from Seikagaku Corporation (Japan). All other chemicals were of regent grade.

The molecular weights (MW) of the CS disaccharides are: Di-0S (UA-GalNAc) 379; Di-4S(UA-GalNAc4S) 459; Di-6S(UAGalNAc6S) 459; Di-2S(UA2S-GalNAc) 459;Di-diSB(UA2S-GalNAc4S) 539; Di-diSD(UA2S-GalNAc6S) 539; Di-diSE(UA-GalNAc4S6S) 539; and Di-triS(UA2SGalNAc4S6S) 637. The MW of the HS disaccharides are: Di-0S(UA-GlcNAc) 379; Di-NS(UA-GlcNS) 417; Di-6S(UA-GlcNAc6S) 458; Di-2S(UA2S-GlcNAc) 458; Di-2SNS(UA2S-GlcNS) 497; Di-NS6S(UA-GlcNS6S) 497; Di-2S6S(UA2S-GlcNAc6S) 537; and Di-triS(UA2S-GlcNS6S) 576. The molecular weights of the carrageenan disaccharides are different than those of the GAGs disaccharides. The carrageenan-derived disaccharides consist of 3,6-anhydro-galactose(A-Unit) and galactose(G-Unit) or two galactose (G-unit). The MW of the carrageenan-derived disaccharides are: kappa-carra-disaccharide (A-G4S) 404; iota-carra-disaccharide (A2S-G4S) 484; and lambda-carra-disaccharide (G4S-G2S6S) 582.

2.5 Isolation and purification of GAGs from cells

The cell samples (in 1 mL water) were individually subjected to proteolysis at 55°C with 10% of actinase E (20 mg/mL) for 20 h. After proteolysis, dry urea and dry CHAPS were added to each sample (2 wt% in CHAPS and 8 M in urea). Particulates were removed from the resulting solutions by passing each through a syringe filter containing a 0.22 μm membrane. A Vivapure MINI Q H spin column was prepared by equilibrating with 200 μL of 8 M urea containing 2% CHAPS (pH 8.3). The clarified filtered samples were loaded onto and run through the Vivapure MINI Q H spin columns under centrifugal force (2,000×g). The columns were first washed with 200 μL of 8 M urea containing 2% CHAPS at pH 8.3. The columns were then washed five-times with 200 μL of 200 mM NaCl. GAGs were released from the spin column by washing three-times with 50 μL of 16% NaCl. GAGs were desalted with a Microcon Centrifugal Filter Units YM-3 (3,000 MWCO) spin column. The GAGs were freeze-dried for future use.

2.6 Quantification of glycosaminoglycans

The isolated glycosaminoglycans (GAGs) were subjected to carbazole assay to quantify the amount of GAGs in each sample using heparan sulfate as standard. A standard curve of the heparan sulfate gave the equation y =0.1065x+0.0498, R^2=0.9986. The amount of GAGs was determined in samples of 10^7 cells treated with carrageenan for 4 days. The carbazole assay measures the hexuronic containing GAGs, including unsulfated GAGs (e.g. hyaluronan) and disaccharides, but not keratan sulfate or its digests.

The total sulfated GAGs, including chondroitin-4-sulfate (C4S), chondroitin-6-sulfate, keratan sulfate, dermatan sulfate, heparan sulfate, and heparin, but not including disaccharides, were measured in cell lysates by sulfated GAG assay (Blyscan™, Biocolor Ltd, Newtownabbey, Northern Ireland). The cationic dye 1,9-dimethylmethylene blue reacts with the sulfated GAG, producing an insoluble dye-GAG complex, and the sGAG content is determined by the amount of dye recovered from the test sample following exposure to Blyscan Dissociation Reagent. The ratio of the GAG dye-binding capability to the C4S sulfation level is reported as 1.0 for C4S from

bovine trachea [27]. Absorbance maximum of 1,9-dimethylmethylene blue is 656 nm. Concentration by Blyscan assay is expressed as μg / mg protein of cell lysate. C4S was determined by this assay following immunoprecipitation of cell lysates from treated and control cells using the C4S (4D1) antibody (Abnova) to detect native C4S, as previously described [28].

2.7 Disaccharide composition analysis using LC-MS

GAG samples (5 μg) were incubated with the chondroitinase ABC (10 m-units) and chondroitinase ACII (5 m-units) at 37°C for 10 h [29,30]. The enzymatic products were recovered by centrifugal filtration (YM-30, 30,000 MWCO, Millipore, Bedford, MA). CS/DS disaccharides passed through the filter, were freeze-dried and ready for LC-MS analysis. Next, the heparinases I, II and III (5 mU each) were add into the remainder and incubated at 37°C for 10 h. The products were again recovered by centrifugal filtration and the heparin/HS disaccharides were similarly collected and freeze-dried and ready for LC–MS analysis.

MS analyses were performed on an Agilent 1100 LC/MSD instrument (Agilent Technologies, Inc. Wilmington, DE) equipped with an ion trap, binary pump followed by microflow, and a UV detector. For CS/DS disaccharide analysis, the column used was a 1.7 μm Acquity UPLC BEH C18 column (2.1 × 150 mm, Waters, Milford, MA, USA). Solutions A and B for UPLC were 0 and 75% acetonitrile, respectively, containing the same concentration of 15 mM hexylamine as an ion-pairing reagent and 100 mM hexafluoroisopropanol as an organic modifier. The column temperature was maintained at 45 °C. For disaccharide analysis, solution A for 10 min was followed by a linear gradient from 10 to 40 min of 0 to 50% solution B at the flow rate of 100 μL/min. The electrospray interface was set in positive ionization mode with the skimmer potential 40.0 V, capillary exit 40.0 V, and a source of temperature of 350 °C to obtain maximum abundance of the ions in a full-scan spectra (350–2000 Da, 10 full scans/s). Nitrogen was used as a drying gas (8 L/min) and a nebulizing gas (40 psi).

For HS/HP disaccharide analysis, eluent A was water/acetonitrile (85:15) v/v, and eluent B was water/acetonitrile (35:65) v/v. Both eluents contained 12 mM tributylamine (TrBA) and 38

mM NH_4OAc with pH adjusted to 6.5 with HOAc. The column temperature was maintained at 45 °C. Solution A for 10 min, followed by a linear gradient from 10 to 40 min of 0 to 50% solution B at the flow rate of 100 µL/min, was used for disaccharide analysis. The electrospray interface was set in negative ionization mode with the skimmer potential -40.0 V, capillary exit -40.0 V, and a source of temperature of 350 °C to obtain maximum abundance of the ions in a full-scan spectra (350–1500 Da, 10 full scans/s). Nitrogen was used as a drying gas (8 L/min) and a nebulizing gas (40 psi)

2.8 Statistical Analysis

Sulfatase activity data are the mean ± Standard Deviation (S. D.) of at least three independent biological samples with two technical replicates of each determination. Statistical significance was determined by one-way ANOVA followed by a post-hoc Tukey–Kramer test for multiple comparisons, unless stated otherwise in the Results or Figure Legends, using InStat software (GraphPad, San Diego, CA). Asterisks represent significant differences compared to control, with * for $p \leq 0.05$, ** for $p \leq 0.01$, and *** for $p \leq 0.001$.

3. Results

3.1 Sulfatase activity in colonic epithelial cells following carrageenan

Table 1 presents the sulfatase activity data in colonic epithelial cells following exposure to λ-carrageenan for 4 days (**Table 1**). In all circumstances, exposure to carrageenan lowered the activity of the sulfatase enzymes significantly ($p<0.001$ or $p<0.01$, 1-way ANOVA with Tukey-Kramer post-test). Declines in activity occurred in ARSB, ARSA, GALNS, IDS, and STS in NCM460, T84, and CaCO2 cell lines, and in primary human colonocytes. The malignant, colonic epithelial cell line T84, derived from a metastatic lesion, had significantly lower baseline ARSB, ARSA, GALNS, and STS activity than the other human colonic cell lines, and declined further carrageenan exposure. IDS activity in the T84 cells was similar to the activity in the NCM460 cells and primary colonocytes and greater than in the CaCO2 cells, in contrast to the ARSB activity which was highest in the CaCO2 cells and least in the T84 cells. All of the activity measurements declined following carrageenan exposure.

In the NCM460 cell line, which was derived from normal colonic epithelium, the effects of exposure to kappa and iota carrageenan were compared to effects of lambda-carrageenan (1 μg/ml x 4 d) (**Table 1**). λ-carrageenan had the greatest inhibitory effect on activity of the panel of sulfatase enzymes, and ι-carrageenan showed the least inhibition. Similarly, in the T84 cells, the inhibitory effect of λ-carrageenan on ARSB activity was greatest, with activity declining from 60.0 ± 3.8 to 32.8 ± 1.4 nmol/mg protein/h ($p<0.001$), and the effect of i-carrageenan was least, declining to 80% of baseline (48.0 ± 1.6 nmol/mg protein/h; $p<0.001$). The differences in structure of λ- κ- and ι-carrageenans are depicted in **Figure 1**.

In addition to the effects on the sulfatases that remove O-sulfo groups at the non-reducing end of GAGs, carrageenan exposure (1 μg/ml x 96 h) to NCM460 cells inhibited the activity of the endosulfatase SULF-1,2 (12.4 ± 0.6 to 6.6 ± 0.6 nmol/mg protein/h; $p<0.001$), which removes the O-sulfo group from internal sites in GAG chains, and of heparan sulfamidase (4.6 ± 0.4 to 3.0 ± 0.2 nmol/mg protein/h; $p<0.001$).

In contrast to marked declines in activity assays, no significant differences in the protein content of ARSB, ARSA, GALNS, or STS in the NCM460 or T84 cells were detected by the cell-based ELISA assays following exposure to λ-carrageenan x 48 h. Similarly, expression of sulfatases was not significantly modified in a cDNA microarray to be significantly changed.

3.2 Sulfatase activity in human mammary cells following carrageenan

Consistent with the observed effects of carrageenan exposure on sulfatase activity in the human colonic epithelial cells, sulfatase activity declined significantly in several human mammary cell lines, including MCF-7, MCF10A, T47D, HCC1937, as well as primary myoepithelial and epithelial cells (**Table 2**) ($p<0.001$) following exposure to λ-carrageenan (1 μg/ml x 6 days). ARSB, ARSA, STS, GALNS, and IDS all declined following exposure to λ-carrageenan ($p<0.001$). As reported previously, baseline activity of ARSB, ARSA, STS, and GALNS was higher in the normal, primary epithelial and myoepithelial cells than in the malignant, metastatic cell lines (MCF-7 and T47D), and sulfatase activity in the MCF10A cell line was reduced for ARSB, ARSA, and GALNS, but STS and IDS activity was similar to the activity in the primary mammary cells [16]. Carrageenan exposure produced significant reductions in activity of each sulfatase in the different cell types ($p<0.001$ or $p<0.01$). As in the colonic cells, exposure to λ-carrageenan produced greater declines than κ- or ι-carrageenan in ARSB activity in the MCF-7 and MCF-10A cells, although all declines were highly significant ($p<0.001$).

3.3 GAG content following carrageenan in NCM460 cells

The GAGs detected by carbazole assay are the uronic-acid containing disaccharides and glycosaminoglycans, including hyaluronan (HA), a nonsulfated GAG, but excluding keratan sulfate, a sulfated GAG not containing an uronic acid residue. NCM460 cells exposed to kappa, iota or lambda carrageenan for 4 days and unexposed cell control were isolated by a three-step procedure involving protease digestion, strong anion-exchange chromatography on a spin column, followed by salt release. Under the conditions used in the strong anion exchange spin

column step, unsulfated HA is poorly recovered. The GAG samples were subjected to carbazole assay to quantify the amount of GAG in each sample of 10^7 cells (~1 mg protein/10^6 cells). The GAG content from cells exposed to carrageenans was markedly higher than GAGs from the unexposed cell control (**Table 3A**) by carbazole assay.

The total sulfated GAG and the C4S content, determined by 1,9-dimethylmethylene blue assay, also demonstrated significant increases following carrageenan exposure (**Table 3B**). The differences between the two assays reflect differences in specific GAGs and degradation products, and suggest that the effects of ι-carrageenan exposure on the accumulation of unsulfated GAGs are greater than the impact of either λ- or κ-carrageenan.

3.4 Disaccharide composition analysis

3.4.1 CS/DS-derived disaccharides

It is noteworthy that the control cells (not exposed to carrageenans) have ~10-fold lower CS/DS levels than the samples exposed to carrageenans, as indicated by the noisy baseline and low intensity peaks observed in the control analysis. For the CS/DS disaccharide analysis, three main types of disaccharides (ΔDi-0S, ΔDi-6S and ΔDi-4S) were detected in all samples (**Fig. 2A; Table 4**). Similar percentages of ΔDi-6S and ΔDi-4S were present in all samples: control (22.7%: 72.8%); κ-carrageenan (20.0%: 70.3%); ι-carrageenan (20.1%: 71.2%); and λ-carrageenan (21.2%: 71.1%). In the zoom-in figure (**Fig. 2B**), small amounts of di-sulfated disaccharides (ΔDi-diSD and ΔDi-diSE) were only detected in the GAG samples from cells exposed to carrageenan. In contrast, these disaccharides were not present in the unexposed control cell sample.

3.4.2 HP/HS-derived disaccharides

A new UPLC/MS method was used to analyze the HP/HS disaccharide composition of GAGs from the NCM460 cells. This method is particularly useful in the analysis of small amounts of biological samples or samples having very low amounts of GAG. For the HS/HP disaccharide analysis, marked differences between carrageenan-exposed samples and

unexposed control were found (**Fig. 3**). First, the cultured cells (not exposed to carrageenans) had 10-100 fold lower HS/HP levels than the samples exposed to carrageenan, as indicted by the noisy baseline and low intensity peaks. ΔDi-TriS and ΔDi-0S were the only assignable peaks in the untreated sample. In contrast, three other types of disaccharides (ΔDi-6S, ΔDi-2S6S, and ΔDi-NS) were detected in all of the carrageenan-exposed samples. The disaccharide composition (**Table 5**) again demonstrates a remarkable similarity in disaccharide composition upon carrageenan exposure and a major difference from the control untreated cells.

4. Discussion

The commonly used food additive carrageenan resembles the naturally occurring sulfated GAGs, since it is composed of repeating sulfated disaccharide units. However, the disaccharide linkages in carrageenan differ from those in the endogenous human GAGs, due to the presence of the unusual α-D-Gal(1→3)D-Gal bond. In contrast, chondroitin, dermatan, and keratan sulfates have β-1 →3 and β-1→4 linkages and heparin and heparan sulfate have β-1→4 and α-1→4 linkages between their sugar moieties. The α-D-Gal(1→3)D-Gal is recognized by anti-Gal antibodies [31] and appears to stimulate innate immune responses involving TLR4, BCL10, and NF-κB [9-12]. Since carrageenan is so highly sulfated and resembles the endogenous GAG, the studies in this report were performed to determine the impact of carrageenan on activity of several sulfatase enzymes and the impact on cellular GAG composition.

Study findings indicate marked decline in activity of several sulfatase enzymes, including ARSB, ARSA, GALNS, STS, IDS, heparan sulfamidase, and SULF-1,2 following exposure to carrageenan. The assays were performed using standard methods that involve exogenous substrates and non-physiological conditions, and assays compared activity following carrageenan exposure vs. control under similar growth conditions. The assays have been developed to be sulfatase-specific and employ different temperature, pH, substrates, buffers, and inhibitors. Measurements consistently demonstrated that exposure of human colonic epithelial cells and mammary cells to carrageenan reduced the sulfatase activity. In contrast, cDNA microarray and cell-based ELISA did not demonstrate significant differences in sulfatase expression following carrageenan, suggesting a direct effect on enzyme function or on enzyme activation. The precise mechanism by which carrageenan inhibits enzyme activity is not yet determined, but may be attributable to an increase in sulfate, since sulfate is reported to inhibit the activity of the sulfatases [32,33]. Alternatively, carrageenan might directly interact with the sulfatase enzymes, such as indicated by κ-carrageenan mimicry of C4S and dermatan sulfate

[34]. Carrageenans may interfere with the endogenous substrate-enzyme reaction and product dissociation, and thereby inhibit the measurable enzyme activity in the sulfatase assays that utilize the exogenous substrates.

Since degradation of chondroitin-4-sulfate requires removal of the sulfate at the non-reducing end by ARSB (N-acetylgalactosamine-4-sulfatase), silencing ARSB activity by siRNA increased the abundance of cellular GAGs, in experiments reported in bronchial, mammary, renal, and colonic epithelial cells [16,28,35-37]. In association with the reduced ARSB activity, cellular sulfated GAG and C4S were increased, secretion of vital molecules, including IL-8 and bradykinin, was reduced, and cellular sequestration of IL-8 and kininogen was increased, indicating the impact of sulfatase activity and GAG sulfation upon vital cell functions [35,37]. Silencing and overexpression of GALNS and ARSB produced corresponding changes in mRNA and protein expression of syndecan-1 and decorin in MCF-7 cells, demonstrating that changes in proteoglycans followed changes in GAG sulfation [28]. Also, since chondroitin-4-sulfation is critical for plasmodial attachment, carrageenan, by effects on ARSB, and, thereby, on chondroitin sulfation, may have an impact on malarial infectivity [36].

In addition to the inhibitory effects on activity of sulfatase enzymes, and consistent with these effects, carrageenan exposure leads to marked increase in total cellular sulfated GAG content. This effect is attributable in part to impaired degradation of the GAGs when hydrolysis of sulfate groups is inhibited. Consistent with the inhibition of sulfatase enzyme activity and the overall increase in the GAG abundance, disaccharide analysis confirmed that the carrageenan exposure provokes profound changes in the composition of these cellular GAGs.

The CS/DS-derived disaccharides post-carrageenan included chondroitin sulfate D (ΔDi-diS_D) and chondroitin sulfate E (ΔDi-diS_E) which were not originally present. Galectin-3, -7, and -9 binding to sulfated GAGs has been linked to the extent of sulfation, suggesting that the effects of carrageenan on sulfatase activity may impact upon galectin binding [38]. Since galectins are linked to multiple critical cellular events, the carrageenan-induced changes in GAGs may lead to

profound changes in cell functions and cell regulation through transcriptional effects, as well as altered GAG interactions [39-41]. Further analysis of the impact of carrageenan exposure may provide new insights into how GAG sulfation and sulfatases influence cell fate. The implications for human disease may be profound, since carrageenan is consumed in significant quantity in the human diet.

5. Conclusions

Exposure to the common food additive significantly reduced the activity of multiple sulfatase enzymes in human colonic and mammary epithelial cells. These changes in sulfatase activity were accompanied by marked increase in cellular GAGs. Disaccharide analysis demonstrated that CSD- and CSE-derived disaccharides were present following carrageenan exposure, but not in the untreated control. Analysis of HP/HS-derived disaccharides demonstrated marked changes in the specific GAG composition following carrageenan, including the presence of ΔDi-6S, ΔDi-2S6S, and ΔDi-NS that were not originally present. These determinations indicate that exposure to the common food additive carrageenan has profound effects on sulfatase activity and GAG abundance and composition that may affect vital cell processes.

Acknowledgement

The authors acknowledge the contributions of Dr. Anuj Jain to performance of the cell-based ELISA experiments.

References

1. J. West, K. A. Miller, California's Living Marine Resources: A status report. Agarophytes and carrageenophytes, California Department of Fish and Game, 2001.
2. "algae." *Encyclopædia Britannica.* Encyclopædia Britannica Online. Encyclopædia Britannica, 2010. Web. 22 Jan 2010 <http://search.eb.com/eb.article-31714.
3. Food and Drug Administration. Processed Eucheuma Seaweed. Working paper; 1992.
4. Z. C. Shah, F. G. Huffman, Current availability and consumption of carrageenan-containing Foods, Ecol. Food Nutr. 42 (2003) 1-15.
5. Joint FAO/WHO Expert Committee on Food Additives. Sixty-eighth meeting, 2007. Summary and conclusions. http://www.Jecfa68 meeting.pdf
6. W. M. Marrs, The stability of carrageenan to processing, In: Williams, PA and Phillips, GO, editors, Gums and Stabilizers for the Food Industry, Cambridge, England: The Royal Society of Chemistry (1998), pp. 345-357.
7. International Agency for Research on Cancer, I*ARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans,* Volume 31, Lyon, France: IARC (1983) 79-94.
8. J. K. Tobacman, Review of harmful gastrointestinal effects of carrageenan in animal experiments, Environ. Health Perspect.109 (2001) 983-994.
9. S. Bhattaycharyya, A. Borthakur, P. K. Dudeja, J. K. Tobacman, Carrageenan induces interleukin-8 production through distinct Bcl10 pathway in normal human colonic epithelial cells, Am. J. Physiol. Gastrointest. Liver Physiol. 292 (2007) G829-838.
10. S. Bhattacharyya, P. K. Dudeja, J. K. Tobacman, Carrageenan-induced NFκB activation depends on distinct pathways mediated by reactive oxygen species and Hsp27 or by Bcl10, Biochim. Biophys. Acta. 1780 (2008) 973-982.

11. S. Bhattacharyya, R. Gill, M. L. Chen, F. Zhang, R. J. Linhardt, P. K. Dudeja, J. K. Tobacman, Toll-like receptor 4 mediates induction of Bcl10-NFκB-IL-8 inflammatory pathway by carrageenan in human intestinal epithelial cells, J. Biol. Chem. 283 (2008) 10550-10558.
12. S. Bhattacharyya, A. Borthakur, R. Gill, M. L. Chen, P. K. Dudeja, J. K. Tobacman, BCL10 is required for NF-κB nuclear translocation by both canonical and non-canonical pathways and for NIK phosphorylation, J. Biol. Chem. 285 (2010) 522-530.
13. S. Bhattacharyya, H. Liu, Z. Zhang, M. Jam, P. K. Dudeja, G. Michel, R. J. Linhardt, J. K. Tobacman, Carrageenan-induced innate immune response is modified by enzymes that hydrolyze distinct galactosidic bonds, J. Nutr. Biochem. 21 (2010) 906-13.
14. S. Bhattacharyya, J. K. Tobacman, Molecular signature of kappa-carrageenan mimics chondroitin-4-sulfate and dermatan sulfate and enables interaction with arylsulfatase B. J. Nutr. Biochem, (2011) [Epub ahead of print]
15. M. P. Moyer, L. A. Manzano, R. L. Merriman, J. S. Stauffer, L. R. Tanzer, NCM460, a normal human colon mucosal epithelial cell line, In Vitro Cell. Dev. Biol. Anim. 32 (1996) 315-317.
16. S. Bhattacharyya, J. K. Tobacman, Steroid sulfatase, arylsulfatases A and B, galactose 6-sulfatase, and iduronate sulfatase in mammary cells and effects of sulfated and non-sulfated estrogens on sulfatase activity, J. Steroid Biochem. Mol. Biol. 103 (2007) 20-34.
17. S. Bhattacharyya, J. K. Tobacman, Arylsulfatase B regulates colonic epithelial cell migration by effects on MMP9 expression and RhoA activation, Clin. Exp. Metastasis 26 (2009) 535-545.
18. O. P. van Diggelen, H. Zhao, W. J. Kleijer, H.C. Janse, B. J. Poorthuis, J. van Pelt, J. P. Kamerling, H. Galjaard, A fluorimetric enzyme assay for the diagnosis of Morquio disease type A, Clin Chim Acta 187 (1990) 131-140.
19. Y. V. Voznyi, J. L. M. Keulemans, O. P. van Diggelen, A fluorogenic assay for the diagnosis of Hunter disease, J Inh Metab Dis 24 (2001), 675-680.

20. M. Lee-Vaupel, E. Conzelmann, A simple chromogenic assay for arylsulphatase A, Clin Chim Acta 164 (1987) 171-180.

21. J. W. Rip, B. A. Gordon, A simple spectrophotometric enzyme assay with absolute specificity for arylsulfatase A, Clin Biochem 31 (1998) 29-31.

22 B. Wolff B, A. Billich, W. Brunowsky, G. Herzig, I. Lindley, P. Nussbaumer, E. Pursch, C. Rabeck, G. Winkler G, Microtiter plate cellular assay for human steroid sulfatase with fluorescence readout, Anal Biochem 318 (2003) 276-284.

23. E. A. Karpova, Y. V. Voznyi, J. L. M. Keuleans, A. T. Hoogeveen, B. Winchester, I. Tsvetkova, O. P. van Diggelen, A fluorogenic assay for the diagnosis of Sanfilippo disease type A (MPS III A), J. Inher. Metab. Dis. 19 (1996) 278-285.

24. K. Uchimura, M. Morimoto-Tomita, S. D. Rosen, Measuring the Activities of the Sulfs: Two Novel Heparin/Heparan Sulfate Endosulfatases, *Methods in Enzymology*, Volume 416, (2006) pp. 243-253.

25. S. Bhattacharyya, P. K. Dudeja, J. K. Tobacman, Carrageenan-induced NFkappaB activation depends on distinct pathways mediated by reactive oxygen species and Hsp27 or by Bcl10, Biochim Biophys Acta. 1780(2008), 973-82.

26. S. Bhattacharyya, A. Borthakur, P. D. Dudeja, J. K. Tobacman, Carrageenan induces cell cycle arrest in human intestinal epithelial cells in vitro, J. Nutr. 138 (2008) 469-475.

27. Blyscan. Sulfated glycosaminoglycan assay. www. biocolor.co.uk

28. S. Bhattacharyya, K. Kotlo, S. Shukla, R. S. Danziger, Tobacman JK, Distinct effects of N-acetylgalactosamine-4-sulfatase and galactose-6-sulfatase expression on chondroitin sulfate, J. Biol. Chem. 283 (2008) 9523-9530.

29. M. J. Hernáiz, R. J. Linhardt, Degradation of chondroitin sulfate and dermatan sulfate with chondroitin lysases, Methods Mol. Biol. 171 (2001) 363-371.

30. K. Solakyildirim, Z. Zhang, R. J. Linhardt, Ultraperformance liquid chromatography with electrospray ionization ion trap mass spectrometry for chondroitinal disaccharide analysis, Anal. Biochem. 397 (2010) 24-32.

31. U. Galili, The alpha-gal epitope and the anti-Gal antibody in xenotransplantation and in cancer immunotherapy, Immunol. Cell Biol. 83 (2005) 674-686.

32. B. Wojczyk, Lysosomal arylsulfatases A and B from horse blood leukocytes: purification and physico-chemical properties, Biol. Cell 57 (1986) 147-152.

33. G. J. Rao, M. E. Christe, Inhibition of rabbit liver arylsulfatase B by phosphate esters, Biochim. Biophys. Acta 788 (1984) 58-61.

34. S. Bhattacharyya, J. K. Tobacman, Molecular signature of kappa-carrageenan mimics chondroitin-4-sulfate and dermatan sulfate and enables interaction with arylsulfatase B. J Nutr Biochem. 2011 Nov 11. [Epub ahead of print]

35. S. Bhattacharyya, K. Solakyildirim, Z. Zhang, R. J. Linhardt, J. K. Tobacman, Cell-bound IL-8 increases in bronchial epithelial cells following Arylsulfatase B silencing, Am. J. Respir. Cell. Mol. Biol. 42 (2010) 51-61.

36. S. Bhattacharyya, K. Solakyildirim, Z. Zhang, R. J. Linhardt, J. K. Tobacman, Chloroquine reduces Arylsulfatase B activity and increases chondroitin 4-sulfate: Implications for mechanisms of action and resistance, Malaria J. 1 (2009) 303.

37. S. Bhattacharyya, K. Kotlo, R. S. Danziger, J. K. Tobacman, Arylsulfatase B regulates interaction of chondroitin-4-sulfate and kininogen in renal epithelial cells, Biochim. Biophys. Acta. 1802 (2010) 472-477.

38. J. Iwaki, T. Minamisawa, H. Tateno, J. Kominami, K. Suzuk, et al, Desulfated galactosaminoglycans are potential ligands for galectins: evidence from frontal affinity chromatography, Biochem. Biophys. Res. Commun. 373 (2008) 206-212.

39. http://www.functionalglycomics.org/CFGparadigms/index.php/Galectin-3

40. N.C. Henderson, T. Sethi,The regulation of inflammation by galectin-3, Immunol. Rev. 230 (2009) 160-171.
41. S. Nakahara, V. Hogan, H. Inohara, A. Raz, Importin-mediated nuclear translocation of galectin-3, J. Biol. Chem. 281 (2006) 39649-39659.

Figure Legends

Figure 1. Structures of kappa(κ)-, iota(ι)-, and lambda(λ)-carrageenan.

The disaccharides of kappa-, iota-, and lambda-carrageenan are depicted, showing the β-1→4 and the α-1→3 bonds.

Figure 2. CS/DS disaccharide composition analysis by LC–MS.

A. Extracted ion chromatography (EIC) of CS/DS disaccharides at normal magnification. (A)= CS/DS disaccharide standards; (B) untreated GAG sample from NCM460 cells; (C) KCG-treated; (D) ICG-treated; and (E) LCG-treated. [GAG=glycosaminoglycan; CS/DS=chondroitin sulfate/dermatan sulfate; KCG=kappa carrageenan; ICG = iota-carrageenan; LCG=lambda-carrageenan]

B. Zoom in figure of samples identified above.

Figure 3. HS/heparin disaccharide composition analysis by LC–MS.

Extracted ion chromatography (EIC) of HS/HP disaccharide standards; (B), (C), (D) and (E) EIC of HS/HP disaccharides from the GAG samples CN, KCG, ICG, and LCG, respectively. [GAG=glycosaminoglycan; CS/DS=chondroitin sulfate/dermatan sulfate; KCG=kappa carrageenan; ICG = iota-carrageenan; LCG=lambda-carrageenan]

Table 1. Sulfatase activity in intestinal epithelial cells following exposure to λ-carrageenan x 4 days

A

CELL TYPE	TREATMENT	ARSB[a]	ARSA[b]	STS[c]	GALNS[d]	IDS[e]
		nmol/mg protein/h ± (S.D.)	nmol/mg protein/h ± (S.D.)	nmol/mg protein/h ± (S.D.)	nmol/mg protein/h ± (S.D.)	nmol/mg protein/h ± (S.D.)
NCM460	control	125.5 (1.1)	107.6 (8.8)	26.8 (0.5)	8.6 (0.3)	11.1 (0.2)
	λ-carrageenan	75.2 (4.6)***	66.9 (5.9)**	13.9 (0.2)***	5.2(0.002)***	8.4 (0.4)***
	κ-carrageenan	83.7 (1.5)***	75.6 (7.0)**	16.2 (0.9)***	6.9 (0.5)**	8.6 (0.4)***
	ι-carrageenan	90.3 (3.8)***	79.4 (6.9)**	16.7 (2.4)***	7.2 (2.5)**	8.6 (0.6)***
CaCO2	control	143.4 (10.3)	97.9 (3.7)	22.9 (0.3)	5.4 (0.2)	5.9 (0.1)
	λ-carrageenan	107.5(4.9)**	61.8 (5.0)***	16.8 (0.3)***	3.5 (0.1)***	3.0 (0.1)***
colonocytes	control	113.6 (2.9)	141.0 (9.0)	28.0 (0.6)	9.3 (0.4)	12.1 (0.4)
	λ-carrageenan	66.4 (4.4)***	107.4(1.8)**	14.7 (0.7)***	4.8 (0.4)***	7.8 (0.4)***
T84	control	60.0 (3.8)	52.5 (4.8)	20.1 (1.2)	6.3 (0.3)	12.7 (0.6)
	λ-carrageenan	32.8 (1.4)***	37.8 (2.8)**	12.5 (0.5)***	4.2 (0.2)***	8.7 (0.1)***

[a]ARSB = arylsulfatase B; [b]ARSA = arylsulfatase A; [c]STS = steroid sulfatase; [d]GALNS = galactose-6-sulfatase; [e]IDS = iduronate sulfatase;
*** for $p<0.001$; ** for $p<0.01$; unpaired t-test, two-tail

Table 2. Sulfatase activity in mammary cells after exposure to λ-carrageenan x 6 days

CELL TYPE	TREATMENT	ARSB[a]	ARSA[b]	STS[c]	GALNS[d]	IDS[e]
		nmol/mg protein/h ± (S.D.)	nmol/mg protein/h ± (S.D.)	nmol/mg protein/h ± (S.D.)	nmol/mg protein/h ± (S.D.)	nmol/mg protein/h ± (S.D.)
MCF-7	control	53.4 (1.8)	85.4 (4.6)	14.2 (0.7)	3.7 (0.3)	13.4 (1.3)
	λ-carrageenan	31.2 (0.9)***	60.1 (1.0)***	6.1 (0.5) ***	2.4 (0.9)**	7.2 (0.2)***
MCF10A	control	74.8 (2.8)	57.9 (1.6)	62.4 (3.6)	4.1 (0.2)	16.3 (0.5)
	λ-carrageenan	22.4 (1.7)***	29.5 (0.9)***	42.7 (4.4)***	1.8 (0.1)***	11.2 (1.0)***
MEC[f]	control	412.4 (18.8)	262.5 (2.1)	81.1 (3.9)	5.6 (0.2)	13.3 (0.2)
	λ-carrageenan	177.8(15.0)***	117.8 (1.7)***	38.7 (0.3)***	2.3 (0.03)***	8.5 (0.2)***
T47D	control	47.4 (0.9)	86.8 (0.2)	14.5 (0.2)	4.6 (0.1)	16.9 (0.2)
	λ-carrageenan	20.4 (0.5)***	62.7 (1.7)***	9.4 (0.2)***	2.8 (0.03)***	8.8 (0.1)***
HCC1937	control	73.5 (3.9)	121.3 (6.3)	25.2 (0.6)	4.4 (0.1)	17.3 (0.03)
	λ-carrageenan	44.9 (1.7)***	96.6 (4.4)**	15.0 (0.2)***	2.4 (0.01)***	10.0 (0.4)***
EC[g]	control	233.3 (10.1)	ND	62.8 (3.3)	8.4 (0.3)	13.1 (0.1)
	λ-carrageenan	162.7 (3.8)***	ND	39.4 (0.9)***	6.2 (0.1)***	7.7 (0.4)***

[a]ARSB = arylsulfatase B; [b]ARSA = arylsulfatase A; [c]STS = steroid sulfatase; [d]GALNS = galactose-6-sulfatase; [e]IDS = iduronate sulfatase
[f]MEC = primary mammary myoepithelial cells; [g]EC = primary mammary epithelial cells
*** for $p<0.001$; ** for $p<0.01$ for difference following carrageenan exposure

Table 3. GAGs following exposure to different types of carrageenan

A

	Control	κ-carrageenan	ι-carrageenan	λ-carrageenan
GAGs (μg)	4.2	135.5	192.3	141.2
Cell Number	10^7	10^7	10^7	10^7

B

	NCM460 cells	**T84 cells**
sGAG μg/mg protein ± (S.D.)		
control	14.6 (0.4)	19.1 (0.9)
λ-carrageenan	20.4 (1.2)***	25.7 (0.3)***
κ-carrageenan	17.9 (0.6)**	23.8 (0.7)**
ι-carrageenan	16.4 (0.6)*	21.9 (1.4)*
C4S μg/mg protein ± (S.D.)		
control	7.4 (0.3)	11.8 (0.4)
λ-carrageenan	11.4 (0.4)***	16.6 (0.2)***
κ-carrageenan	10.1 (0.5)***	15.4 (0.7)***
ι-carrageenan	8.8 (0.6)*	13.5 (0.2)**

The carbazole assay detects uronic acid containing glycosaminoglycans and oligosaccharides, including hyaluronan, chondroitin-4-sulfate, chondroitin-6-sulfate, dermatan sulfate, heparin, heparan sulfate, and disaccharides, but not keratan sulfate or carrageenan. The 1,9-dimethylmethylene blue assay detects sulfated GAG, but does not detect disaccharides, carrageenan, hyaluronan, or other unsulfated oligosaccharides.

Table 4. CS/DS disaccharide composition analysis by LC-MS following exposure to different types of carrageenan

Sample	CS/DS disaccharide composition							
	ΔDi-0S	ΔDi-2S	ΔDi-6S	ΔDi-4S	ΔDi-diS_D	ΔDi-diS_B	ΔDi-diS_E	ΔDi-TriS
untreated	4.5	-	22.7	72.8	-	-	-	-
kappa	9.2	-	20.0	70.3	0.2	-	0.2	-
Iota	8.1	-	20.1	71.2	0.2	-	0.4	-
lambda	6.6	-	21.2	71.1	0.1	-	0.9	-

LC-MS = liquid chromatography-mass spectrometry. CS=chondroitin sulfate; DS=dermatan sulfate; ΔDi=disaccharide; ΔDi-0S = ΔUA-GalNAc; ΔDi-4S = ΔUA-GalNAc4S; ΔDi-6S = ΔUA-GalNAc6S; ΔDi-2S = ΔUA2S-GalNAc, ΔDi-diS_B = ΔUA2S-GalNAc4S; ΔDi-diS_D = ΔUA2S-GalNAc6S, ΔDi-diS_E = ΔUA-GalNAc4S6S, ΔDi-triS = ΔUA2S-GalNAc4S6S

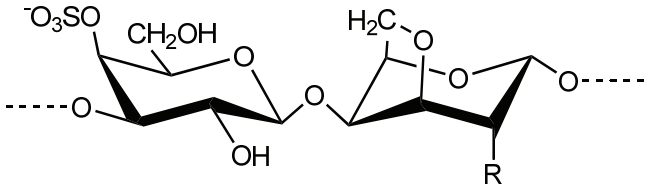
Table 5. HS/HP disaccharide composition analysis by LC-MS

Sample	HS/heparin disaccharide composition							
	ΔDi-0S	ΔDi-NS	ΔDi-6S	ΔDi-2S	ΔDi-NS6S	ΔDi-NS2S	ΔDi-2S6S	ΔDi-TriS
untreated	6.9	-	-	-	-	-	-	93.1
kappa	1.1	1.9	94.8	-	-	-	2.2	-
iota	0.6	0.9	96.9	-	-	-	1.7	-
lambda	2.3	2.1	91.5	-	-	-	4.6	-

LC-MS = liquid chromatography-mass spectrometry. HS=heparan sulfate; HP=heparin; ΔDi=disaccharide; ΔDi-0S = ΔUA-GlcNAc; ΔDi-NS = ΔUA-GlcNS, ΔDi-6S = ΔUA-GlcNAc6S; ΔDi-2S = ΔUA2S-GlcNAc; ΔDi-2SNS = ΔUA2S-GlcNS; ΔDi-NS6S = ΔUA-GlcNS6S; ΔDi-2S6S = ΔUA2S-GlcNAc6S; ΔDi-triS = ΔUA2S-GlcNS6S)

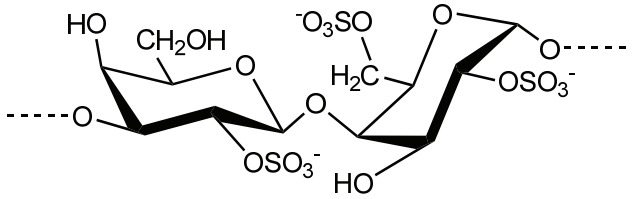
Figure 1. Structure of kappa(κ)-, iota(ι)- and lambda(λ)- carrageenan

->3) β-D-Galactose-4-sulfate (1->4) α-D-3,6-Anhydrogalactose (1->



R = OH, κ-carrageenan; R = OSO_3^-, ι-carrageenan

->3) β-D-Galactose-2-sulfate (1->4) α-D-Galactose-2,6-disulfate (1->



λ-carrageenan

Figure 2. CS/DS disaccharide composition analysis by LC–MS

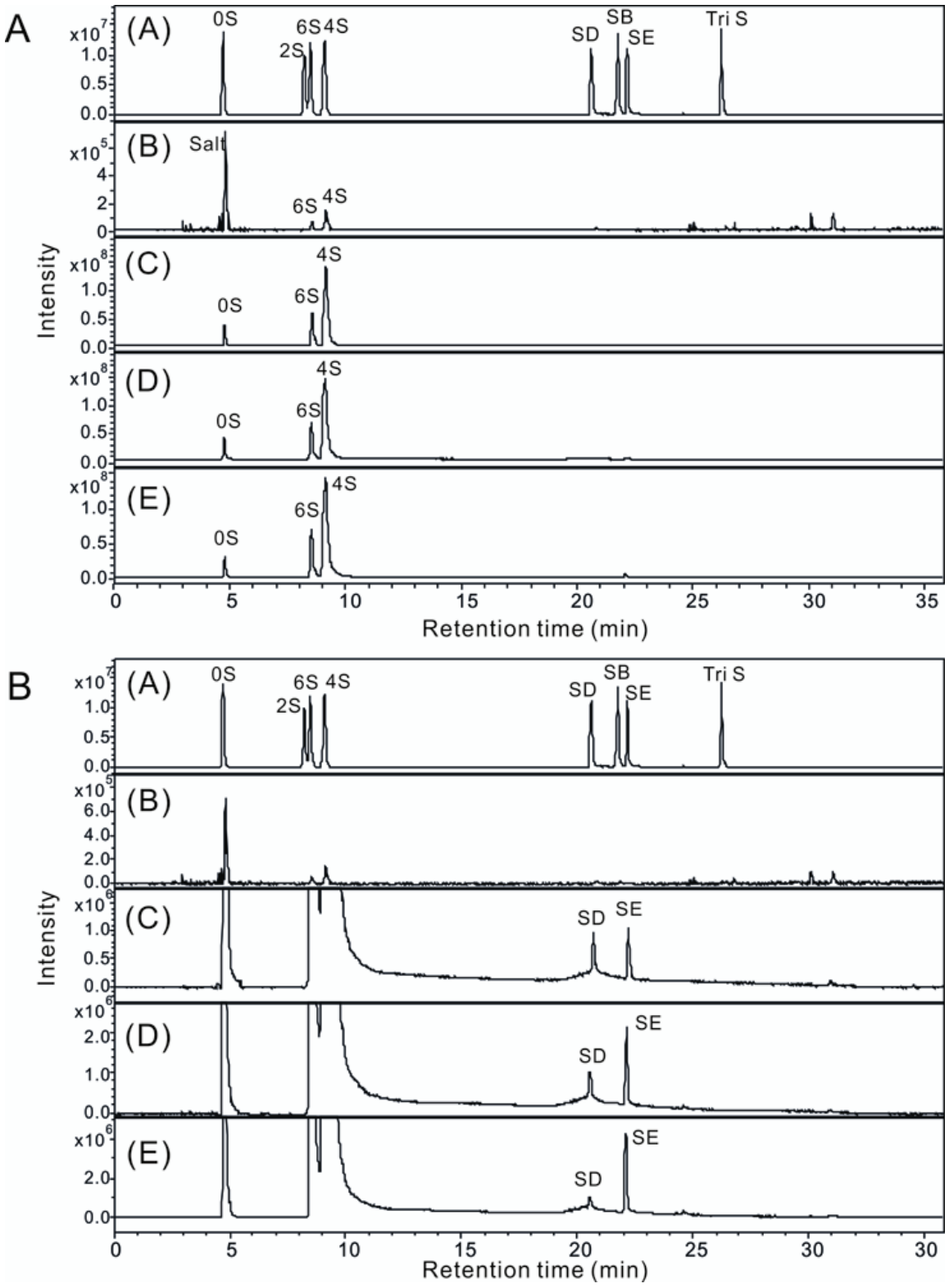


Figure 3. HS/heparin disaccharide composition analysis by LC–MS