Marine chondroitin sulfate of defined molecular weight by enzymatic depolymerization

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Abstract

Chondroitin sulfate (CS) is a sulfated glycosaminoglycan with diverse biological activities, which are influenced by molecular weight (Mw) and sulfation pattern. In the present work, we take advantage of the characteristic high Mw of fish CS (51-70 kDa) to obtain lower Mw fragments with hyaluronidase and chondroitinase ABC. With this aim, we present a pseudo-mechanistic model capable of reproducing the decrease in Mw of CS from five different fish

species over 24 h at four enzyme to substrate ratios. The fitting parameters of the model for each species allow to establish conditions of reaction to produce CS of the desired Mw. Furthermore, the main features of the sulfation pattern of fish CS remain in the depolymerized fragments, highlighting the feasibility of the proposed approach.

Keywords: Chondroitin sulfate, glycosaminoglycan, hyaluronidase, chondroitinase, enzymatic depolymerization, depolymerization kinetics.

1. Introduction

Chondroitin sulfate (CS) is a sulfated polysaccharide of the glycosaminoglycan family widely distributed at the cell surface and in the extracellular matrix of most animal tissues, where it participates in fundamental cellular events such as cell communication, differentiation and growth (Yamada & Sugahara, 2008). These biological properties make CS interesting for several applications such as cartilage regeneration, either as a nutraceutical or incorporated into tissue engineering scaffolds, nerve regeneration, and as an anticoagulant, anti-inflammatory and anti-metastatic agent (Valcarcel, Novoa-Carballal, Pérez-Martín, Reis, & Vázquez, 2017).

Bioactivity of CS depends largely on its capacity to interact with proteins, which is determined by CS chemical properties and related structure (Benito-Arenas et al., 2018; Djerbal, Lortat-Jacob, & Kwok, 2017; Miller & Hsieh-Wilson, 2015). CS consists of a sequence of glucuronic acid (GlcA) and N-acetyl galactosamine (GalNAc) linked by alternating β -(1 $\rightarrow$ 4) and β -(1 $\rightarrow$ 3) glycosidic bonds. Sulfation can occur at different positions in both rings, giving rise to distinct units sequentially arranged in polymeric chains of variable length. As a result, considerable

heterogeneity exists in CS in terms of charge density, sulfation pattern and molecular weight (Kjellén & Lindahl, 2018; Valcarcel et al., 2017).

Marine CS differs from terrestrial counterparts both in terms of sulfation and molecular weight. The latter contains a majority of 4-sulfated GalNAc units (CS-A), while in marine animals 6-sulfated GalNAc (CS-C) generally prevails, accompanied by a significant proportion of disulfated disaccharides (López-Álvarez et al., 2019). Furthermore, diversity in sulfation is larger in the marine environment, discovering in some cases unique units and structures, e.g. CS-K (GlcA3S-GalNAc4S) in octopus with neuritogenic activity (Higashi et al., 2015) and fucosylated CS in sea cucumbers with anticoagulant activity (Wu et al., 2012).

Beyond sulfation, molecular weight also appears as an important parameter of CS bioactivity. Low molecular weight CS has shown potential for attenuation of osteoarthritis by inhibiting the complement system, especially depolymerized shark CS (1.5 kDa) (Li et al., 2016). Also, the permeability of CS seems to increase as molecular weight decreases, as shown for dietary supplements tested in an *in vitro* intestinal model (Adebowale, Cox, Liang, & Eddington, 2000). However, low molecular weight CS clears from the blood more rapidly than intact CS, as indicated by the positive correlation found between molecular weight (6-50 kDa) and the plasma half-life of CS intravenously injected to mice (Sakai et al., 2002). As a biomaterial, the cellular uptake and transfection efficiency of complexes of CS with chitosan and plasmid DNA for gene delivery have shown to depend on CS molecular weight, with CS of 22 kDa showing the best results (Hagiwara, Nakata, Koyama, & Sato, 2012). Therefore, CS molecular weight presents as an essential parameter to control depending on the application.

Furthermore, while acting on sulfation is challenging and is mostly determined by the source of CS, molecular weight can be more easily modified, adding an extra layer to natural sulfation diversity to tune CS properties. In this context, the characteristic high molecular weight of marine CS offers wider possibilities for modification than terrestrial counterparts. Furthermore, marine CS can be sustainably sourced from chondrichthyan fish such as sharks, rays and skates. Some species in this class are commercially fished, and as a result, substantial quantities of cartilaginous by-products (heads, central skeletons and fins) and fish discards are available as raw materials. Valorization of this biomass to obtain CS can be achieved by sustainable processes, along with other valuable materials, integrated into a marine biorefinery framework (Antelo, de Hijas-Liste, Franco-Uría, Alonso, & Pérez-Martín, 2015; Vázquez et al., 2013).

Reduction of CS molecular weight implies breakage of the glycosidic bond, which can be accomplished by chemical treatment with acids, alkali, oxidants and radicals, but usually undesired structural changes occur, such as desulfation (Li et al., 2016; Moseley, Waddington, Evans, Halliwell, & Embery, 1995). Furthermore, these processes are not environmentally-friendly, as they require hazardous reagents and high temperature (commonly 60 °C), sometimes aided by microwave radiation. Milder conditions of reaction can be achieved by enzymatic depolymerization. A number of chondroitinases and hyaluronidases are capable of breaking CS chains, with distinct mechanisms of action and affinity for the substrate (Wenshuang Wang, 2017).

Chondroitinase ABC, in fact a mixture of endo- and exo-lyases, is commonly used in CS depolymerization, as it acts on glycosidic bonds irrespective of their sulfation pattern producing unsaturated polysaccharides (Hamai et al., 1997). Hyaluronidases degrade CS with lower activity, but on the other hand, maintain the chemical structure of the non-reducing end of the

polysaccharide (Stern & Jedrzejewski, 2006). Previous works have described the enzymatic activity and rate constants of both groups of enzymes on CS (Hamai et al., 1997; Honda, Kaneiwa, Mizumoto, Sugahara, & Yamada, 2012), but very few have determined the actual variation of molecular weight over time (de Souza et al., 2018; Silva, Novoa-Carballal, Reis, & Pashkuleva, 2015). Furthermore, the materials used originate either from terrestrial sources or from unidentified shark species, being the intra-species variability of marine fish practically unexplored.

Even these last studies provide limited information for the design of experiments to produce CS of the desired molecular weight, because of scarce sampling time points and fixed enzyme to substrate ratios. In the present work, we aim to fill this gap by providing an experimental model to establish the conditions of reaction to depolymerize CS of marine origin with chondroitinase ABC (CASE) and hyaluronidase type I (HASE). In the case of CASE, this is the first time that depolymerization kinetics is described in terms of actual molecular weight variation, as previous kinetic studies relied on UV measurements (Shamsi et al., 2016; Thurston, 1975). Inclusion of several enzyme to substrate ratios for each enzyme provide flexibility in the planning of the reaction (i.e. higher ratio or longer reaction time), and depolymerization of CS from five fish species with different initial molecular weights and sulfation patterns allows evaluation of variability in model parameters.

2. Materials and methods

2.1. Depolymerization of CS

Chondroitin sulfate from three shark species (*Prionace glauca*, *Scyliorhinus canicula*, and *Galeus melastomus*), ray (*Raja clavata*) and chimaera (*Chimaera monstrosa*) were used in the present study. These materials were previously isolated and characterized, and details about the isolation process and CS structural features are described elsewhere (Novoa-Carballal et al., 2017; Vázquez et al., 2019; Vázquez et al., 2018). CS samples were depolymerized with chondroitinase ABC (CASE) from *Proteus vulgaris* (EC 4.2.2.4., 0.86 U/mg, Prod. No. C2905, Sigma-Aldrich.) and hyaluronidase (HASE) from bovine testes type I-S (EC 3.2.1.35., 587 U/mg, Prod. No. H3506, Sigma-Aldrich). CS from each species was dissolved at 1g/L in 50 mM TRIS-HCl / 150 mM NaAcO at pH 8, optimal pH for CASE (Hamai et al., 1997), and 5 mM NaH₂PO₄ / 150 mM NaCl at pH 4 for HASE to minimize transglycosylation activity (Hofinger, Bernhardt, & Buschauer, 2007). Reactions were carried out in duplicate at 37°C under magnetic stirring by adding the appropriate amounts of both enzymes to tubes containing 10 mL of CS solutions to reach enzyme to substrate ratios of 0.002, 0.004, 0.009 and 0.014 units of CASE and 2.5, 7.5, 12.5 and 20.0 units of HASE per mg of CS. At various times, 0.7 mL aliquots were taken, and reactions stopped by heating at 70°C for 25 min. After centrifugation at 13306g for 30 min, 100 µL of supernatant was filtered through 0.2 µm PES membranes and stored at -20°C until analysis.

2.2. Determination of molecular weight

Filtered samples were injected onto a GPC system (Agilent1260) equipped with quaternary pump (G1311B), injector (G1329B), column oven (G1316A), refractive index (G1362A) and dual-angle static light scattering (G7800A) detectors. Sample separation was performed with a set of four Suprema columns (PSS, Mainz, Germany): precolumn 5 µm, 8x50 mm; 30Å 5 µm, 8x300 mm; 100Å 5 µm, 8x300 mm; and ultrahigh 10 µm, 8x300 mm. A 100 µL sample volume

was injected onto the system and eluted at a flow rate of 1 mL/min with 0.1 M NaN_3 and 0.01 M NaH_2PO_4 at pH 6.6. Column oven and light scattering detector were kept at 30°C and refractive index detector at 40°C. Both detectors were calibrated with a polyethylene oxide standard (PSS, Mainz, Germany) of 106 kDa (M_w) and polydispersity index (PDI) 1.05, dissolved in the mobile phase solution at 1 g/L. Absolute molecular weights were determined combining refractive index and dual angle light scattering signals using refractive index increments (dn/dc) of 0.110.

2.3. CS ultrafiltration and disaccharide composition

Depolymerized CS samples of molecular weight around 30 kDa and 10 kDa were subjected to ultrafiltration through 3 kDa membranes (Amicon Ultracel centrifugal filters, Millipore) as specified by the manufacturer, to separate the polymeric material from short oligosaccharides and disaccharides.

Disaccharide composition of CS in both eluate and filtrate fractions was determined by strong anion exchange (SAX) chromatography after extensive enzymatic digestion with CASE at 0.2 U mg^{-1} of CS. The reaction was carried out in 0.05 M Tris-HCl / 0.15 M sodium acetate buffer at pH 8 for depolymerization experiments with CASE, adjusting pH to 8 for experiments carried out with HASE. After 4 days at 37°C, the enzyme was inactivated by heating at 70°C for 25 min, followed by centrifugation at 12857 g. Supernatants were collected and filtered through 0.2 μm polyethersulfone (PES) syringe filters. Unsaturated disaccharide standards were purchased from Grampenz (Aberdeen, UK) and dissolved in water. Samples and standards were manually injected onto an HPLC system (Agilent 1200) consisting of binary pump (G1312A), column oven (G1316A) and UV-visible detector (G1314B). Separation was carried out with a Waters Spherisorb SAX column (5 μm , 4.6x250mm, Prod. No. PSS832715) fitted with a guard cartridge

(Waters Spherisorb, 5 μ m, 4.6x10 mm) based on a previously reported method (Volpi, 2000).

Elution was performed in isocratic mode from 0 to 5 min with 50 mM NaCl at pH 4. A linear

gradient was applied from 5 to 20 min starting with 50 mM NaCl at pH 4 and ending with 76%

50 mM NaCl at pH 4 and 24% 1.2M NaCl at pH 4. A sample volume of 20 μ l was injected onto

the system with a flow rate of 1.5 ml/min. Detection was made at 232 nm. An external

calibration curve was built with each standard to calculate the amount of disaccharide units in the

sample and reported as percentage in weight.

2.4. Depolymerization modelling and estimation

Depolymerization kinetics follows the next expression:

$$\frac{dM_n}{dt} = -k(M_n - M_n^\infty)^n \quad (1)$$

where M_n is the molecular weight of CS (as number average molecular weight, in kDa), n is the

kinetic order (dimensionless) and k the rate of decay (in h^{-1}) and the molecular weight after

depolymerization for 24 h is M_n^∞ (in kDa). Note that the model combines in one kinetic equation

the action of several different chemical reactions. Therefore the decay is not necessarily

exponential ($n=1$) or a second-order decay ($n=2$) (de Souza et al., 2018). The enzyme to substrate

ratio (E) affects both the kinetic rate and the molecular weight after depolymerization for 24 h.

Good correlation of experimental data was observed for the following expressions:

$$k(E) = a + bE \quad (2)$$

$$M_n^\infty(E) = M_n^0 e^{-\mu E} \quad (3)$$

The relationship is linear for the kinetic rate and decays exponentially with the enzyme to substrate ratio (in U/mg) for the molecular weight reached after 24 h of depolymerization. M_n^0 denotes the initial molecular weight (in kDa).

There are four parameters (n , a , b and μ) with different units (dimensionless, h^{-1} , $\text{mg h}^{-1} \text{U}^{-1}$ and mg U^{-1} , respectively) that are unknown a priori but can be estimated from depolymerization data with, at least, two levels of enzyme. For the estimation of the parameters, we minimized the residual sum of squares with the AMIGO2 toolbox (Balsa-Canto, Henriques, Gábor, & Banga, 2016). For the implementation, we used an estimation protocol previously described (Vilas, Arias-Méndez, García, Alonso, & Balsa-Canto, 2018). A cross-validation study was carried out to assess model validity (Elsner & Schmertmann, 1994; García & Cabo, 2018).

3. Results and discussion

3.1. Molecular weight distribution profiles

The evolution of molecular weight distributions of CS isolated from *P. glauca* (PGLA) depicted in Fig. 1 serves to illustrate the dynamics of depolymerization with CASE and HASE. Elution at higher retention volumes as reaction time increases indicates a reduction in molecular weight. In CASE, the area of the CS signal diminishes as the reaction progresses, along with a growing peak that appears close to the end of the column. Injection of an unsaturated disaccharide standard of $\Delta\text{UA-GalNAc6S}$ (557 Da), the most common disaccharide unit in fish CS, eluted approximately at the same elution volume as the new peak. Therefore, it is reasonable to assume that this signal corresponds to unsaturated disaccharides being released from CS. CASE is actually a mixture of two enzymes, an endolyase (EC 4.2.2.20) capable of randomly cleaving the glycosidic bond between N-acetylgalactosamine and glucuronic acid, and an exolyase (EC

4.2.2.21) with the same substrate specificity as the endolyase but only removing disaccharide residues from the non-reducing ends of CS, either native or products of EC 4.2.2.21 (Hamai et al., 1997). For depolymerisation by HASE the disaccharide produced would overlap with the solvent signal (27.5 min) and therefore cannot be quantified. However, no reduction in the area of the CS signal occurs regardless of the depolymerization stage, indicating no disaccharide release. This agrees with the mode of action of HASE used in the present study, an endolytic hydrolase capable of cleaving the β 1,4 glycosidic bond between N-acetylgalactosamine and glucuronic acid up to tetrasaccharides (Stern & Jedrzejewski, 2006). It is noteworthy that while a single distribution appears for HASE, in CASE a second distribution arises at lower elution volumes as the reaction progresses and remains stable after 2-3 h of reaction (at the maximum enzyme to substrate ratio tested). M_n of this signal could only be estimated after 2 h of reaction at 3 kDa, because the intensity of the light scattering signal was too weak at longer reaction times. This might correspond to a fraction resistant to hydrolysis, accounting for around 8% of the initial CS material based on peak areas. Previously, CASE has been reported to only produce 70% hydrolysis after 2.5 h. Even extensive digestion with lyases ABC and C for seven days could only convert 80–85% of the initial polymer to disaccharides (Pomin et al., 2012).

3.2. Disaccharide composition of depolymerized fragments

Enzymatic depolymerization may alter the sulfation pattern of CS fragments, as both CASE and HASE have shown enhanced activity toward disaccharide units with specific sulfation (Hamai et al., 1997; Pomin et al., 2012). To test the extent of this effect on CS products, digests of similar molecular weights (20-30 kDa and 5-10 kDa) from each species treated with both enzymes were subjected to ultrafiltration through 3kDa membranes, which retained polymeric material form eluted disaccharides and oligosaccharides. Disaccharide composition of the retentate can then be

Figure 1. GPC eluograms (refractive index detector signals) of chondroitin sulfate (CS) from *P. glauca* depolymerized with hyaluronidase (20 U/mg CS) and chondroitinase ABC (0.14 U/mg CS) at different reaction times. Molecular weights expressed as M_n (number average molecular weight in kDa).* Light scattering signals were too weak to determine M_n . CS disaccharide: Δ UA-GalNAc6S of 557 Da. Refractive index signal (RI) expressed as arbitrary units (AU).

determined by quantification of CS unsaturated disaccharides by SAX-HPLC released after exhaustive enzymatic digestion with chondroitinase ABC. The results are presented in Table 1, showing changes in disaccharide composition as depolymerization progresses. Within each species, there is no distinct pattern for the disaccharides produced with increasing time, in line with a previous study that found divergent changes in bovine, porcine and shark CS of 2-2.6 kDa obtained after CASE digestion (Li et al., 2016). As an exception, the percentage of D-units decreases during CASE digestion in all the species tested, save for *G. melastomus*. While this

could be interpreted as a preference of CASE for D-units, it most likely reflects an important endo- action, as hexasaccharides isolated from depolymerised shark CS have shown enrichment in CS-D (-endo), whereas a lower proportion of these units appears in disaccharides stripped off the polymer (-exo) (Pomin et al., 2012). Furthermore, the -endo form of CASE seems to preferentially attack 4S GalNAc with activity one order of magnitude greater than the -exo form. The temperature of the reaction may also play a role here, as maximum -endo activity takes place at 37°C, used in the present study, while optimum -endo action occurs at 40°C (Hamai et al., 1997). This difference represents an advantage for generating low-molecular weight CS, as a lower amount of the initial polymer is lost as disaccharides.

Other studies have also found preference of CASE for 4-sulfated GalNAc, as well as HASE, after fractionation of enzymatic depolymerised CS. This has been linked to a decrease in 4-sulfation for CS retained on 10 kDa membranes, with the opposite occurring in the eluted fraction after both CASE and HASE digestion (Silva et al., 2015), or increases in CS-4S for CS treated with HASE and fractionated between 5 and 10 kDa (de Souza et al., 2018). As above mentioned, the data presented here does not suggest a high proportion of CS-4S, and this varies significantly among the species assessed. Beyond the source of variation in disaccharide composition, in all of the CS fragments analyzed the main sulfation pattern features of fish CS remain, i.e. prevalent sulfation at position 6 of the GalNAc ring and a substantial amount of disulfated D-units. Therefore, reductions in molecular weight of up to 90% can be accomplished with both CASE and HASE without drastic changes in the disaccharide profile of low molecular weight CS fragments.

Species		Initial	CHONDROITINASE		HYALURONIDASE	
			20 min	1 h	1 h	24 h
<i>R. clavata</i>	M_n	51.2 kDa (1.12)	23.9 kDa (1.28)	10.1 kDa (1.53)	27.8 kDa (1.28)	6.4 kDa (1.33)
	0	12.6±0.1	13.3±0.1	16.0±0.8	13.2±0.1	13.6±0.9
	C	50.0±0.1	50.5±0.0	50.3±0.6	50.1±0.5	47.0±0.1
	A	21.2±0.1	21.1±0.2	18.6±0.4	20.2±0.1	22.1±0.1
	D	15.5±0.2	14.2±0.2	14.2±0.0	15.4±0.3	16.1±0.0
	E	0.3±0.1	0.4±0.0	0.4±0.3	0.5±0.0	0.6±0.0
	B	0.4±0.1	0.5±0.0	0.6±0.1	0.5±0.0	0.6±0.0
<i>C. monstrosa</i>	M_n	62.5 kDa (1.19)	24.8 kDa (1.37)	8.9 kDa (1.60)	27.2 kDa (1.38)	6.0 kDa (1.43)
	0	9.6±0.5	14.8±0.8	18.5±4.0	10.2±0.2	11.0±2.7
	C	54.5±0.1	53.7±0.4	51.7±0.4	55.0±0.4	53.2±0.4
	A	16.3±0.7	13.6±0.2	12.1±1.3	12.9±0.3	17.8±1.3
	D	17.8±0.1	16.1±0.3	16.1±0.5	20.5±0.1	16.3±0.7
	E	0.8±0.0	0.9±0.0	0.8±0.1	0.4±0.0	0.7±0.0
	B	0.9±0.0	0.9±0.0	0.8±0.0	1.0±0.0	0.9±0.0
<i>P. glauca</i>	M_n	57.5 kDa (1.16)	25.2 kDa (1.28)	9.4 kDa (1.37)	25.6 kDa (1.20)	7.7 kDa (1.26)
	0	14.9±0.4	17.3±1.2	14.7±2.6	14.2±6.4	21.2±7.0
	C	68.5±0.6	67.3±0.4	69.3±4.6	67.5±2.6	61.6±2.6
	A	7.5±0.1	7.3±0.4	7.6±0.8	8.6±0.8	7.1±0.6
	D	8.9±0.3	8.0±0.2	8.2±1.3	9.5±1.6	9.9±0.2
	E	0.1±0.0	0.1±0.1	0.1±0.0	0.1±0.0	0.1±0.0
	B	0.1±0.0	0.1±0.1	0.1±0.0	0.1±0.0	0.2±0.1
<i>S. canicula</i>	M_n	70 kDa (1.12)	26.7 kDa (1.22)	10.1 kDa (1.59)	28.3 kDa (1.27)	8.0 kDa (1.32)
	0	15.4±0.1	14.0±0.5	13.0±0.8	9.1±1.5	11.4±0.8
	C	61.2±1.3	63.1±0.5	64.7±1.3	66.2±3.3	63.8±0.3
	A	10.2±0.0	10.4±0.2	10.1±0.5	12.4±0.5	12.7±1.2
	D	12.5±0.8	11.8±0.3	11.5±0.3	11.5±1.5	11.3±0.1
	E	0.3±0.0	0.3±0.0	0.4±0.2	0.5±0.2	0.4±0.0
	B	0.4±0.0	0.4±0.0	0.3±0.1	0.4±0.2	0.4±0.0
<i>G. melastomus</i>	M_n	63.2 kDa (1.16)	36.4 kDa (1.32)	15.3 kDa (1.57)	29.2 kDa (1.35)	10.5 kDa (1.46)
	0	12.6±0.3	13.8±0.0	17.4±1.4	20.9±1.4	17.8±0.7
	C	54.6±0.1	52.4±0.4	50.5±1.4	50.4±0.6	50.4±1.7
	A	15.9±1.2	16.1±0.1	14.8±0.1	9.6±0.4	13.9±0.2
	D	15.7±0.1	16.3±0.4	16.3±1.7	18.5±0.2	16.9±1.7
	E	0.6±0.0	0.7±0.0	0.3±0.0	0.2±0.1	0.3±0.0
	B	0.6±0.0	0.6±0.0	0.6±0.3	0.5±0.0	0.6±0.3

Table 1. CS composition as mean % of each disaccharide unit ± standard deviation (n=2) by SAX-HPLC and number average molecular weight (kDa) by GPC of native CS and retentates of selected digests after 3 kDa ultrafiltration. M_n : number average molecular weight; 0: GlcA-GalNAc 0S; A: GlcA-GalNAc 4S; B: GlcA 2S-GalNAc 4S; C: GlcA-GalNAc 6S; D: GlcA 2S-GalNAc 6S; E: GlcA -GalNAc 4,6S. Digests were depolymerised with 0.009 U CASE/mg CS and 20 U HASE/mg CS for the times specified in the table. Values in brackets correspond to the polydispersity index.

3.3. Depolymerization kinetics

In all of the experiments, reduction in molecular weight was more intense at the beginning of the reaction, varying very slightly after 5-8 h (Fig. 2). In experiments with CASE above 0.002-0.004 U/mg CS, reactions ended earlier as discussed in the previous section. However, we considered in all cases depolymerization after 24 h as the final point of the reactions, even though some could theoretically progress to lower molecular weight oligosaccharides and disaccharides (Hamai et al., 1997; Pomin et al., 2012).

Enzymatic depolymerization is the consequence of a set of multiple reaction steps, and depolymerization kinetics change with sulfation and molecular weight (Sugahara, Nadanaka, Takeda, & Kojima, 1996). Simulation using kinetic mechanistic models is complex and requires either large models with a large set of unknown parameters or distributed models, sometimes difficult to be estimated from the available information (see for example a mechanistic model for enzymatic hydrolysis of cellulose (Griggs, Stickel, & Lischeske, 2012)).

In this work, we propose a pseudo-mechanistic model encoding the essence of multiple reaction steps in one simple equation. In the proposed model, the critical parameter is the estimation of the kinetic order, that may be >1 and non integer due to the approximation of using one simple overall kinetic equation.

As a first approach, we considered a first-order reaction to reproduce the depolymerization data for both enzymes ($n=1$ in equation (1)), but data fit was poor (Table 2). Depolymerization with CASE fitted to second order kinetics. In the case of enzymatic action with HASE, four out of five species fitted to a rate order of three, but was variable between species. Consequently,

Figure 2. Evolution of number average molecular weight (M_n) of CS during depolymerization reactions with chondroitinase and hyaluronidase at four enzyme to substrate ratios for each fish species tested. Model solution represented by solid lines; scatter data: average M_n (kDa) at each sampling time; error bars: standard deviation (n=2).

the reaction rate was left free in the model, resulting in reaction rates between 1.7 and 2.1 for CASE and between 1.9 and 3.4 for HASE depending on the species (Table 3). In the first case,

all reaction rates are close to two, but for HASE only *P. glauca* shows $n=2$, the same order recently used to model depolymerization of CS from bovine trachea (de Souza et al., 2018). Interestingly, the other four fish species display intermediates rates around 2.5 and 3.5, which illustrates the importance of CS origin, even down to the species level.

	$n=1$	$n=2$	$n=3$
HASE – <i>R. clavata</i>	654	317	190
HASE – <i>C. monstrosa</i>	778	317	315
HASE – <i>P. glauca</i>	1165	433	161
HASE – <i>S. canicula</i>	723	201	185
HASE – <i>G. melastomus</i>	551	343	388
CASE – <i>R. clavata</i>	380	167	385
CASE – <i>C. monstrosa</i>	417	154	635
CASE – <i>P. glauca</i>	621	273	396
CASE – <i>S. canicula</i>	722	220	631
CASE – <i>G. melastomus</i>	641	455	663

Table 2. Residual sum of squares (RSS) for depolymerization reaction rates (n) 1 to 3. HASE: hyaluronidase; CASE: chondroitinase.

The model with free reaction rates proves capable of reproducing the experimental data within the enzyme to substrate ratios and timeframes defined, as shown in Fig. 2. Adjusted correlation coefficients ranged between 0.966 and 0.994, demonstrating the goodness of fit of the model. We assumed that the enzyme to substrate ratio (E) affected the kinetic rate (k) and the molecular weight after depolymerization for 24 h (M_n^∞). We initially tried a Michaelis-Menten adjustment for k , but the simpler linear equation (2) gave a better fit. Dependence of M_n^∞ with E was modelled with a negative exponential equation (3), as linear or second order polynomials resulted in poorer data-fit. The estimated values for the four adjustment parameters required are shown in Table 3.

	RSS	R_{adj}^2	b	a	μ	n	R^2	$R^2(CV)$
HASE – <i>R. clavata</i>	175	0.985	0.104	0.509	0.210	3.477	0.986	0.977
HASE – <i>C. monstrosa</i>	275	0.987	0.775	1.61×10^{-3}	0.230	2.509	0.987	0.965
HASE – <i>P. glauca</i>	138	0.992	6.15×10^{-3}	0.863	0.274	3.393	0.992	0.981
HASE – <i>S. canicula</i>	147	0.994	4.05×10^{-7}	3.17×10^{-3}	0.184	2.517	0.994	0.991
HASE – <i>G. melastomus</i>	343	0.977	4.52×10^{-7}	0.032	0.126	1.946	0.979	0.953
CASE – <i>R. clavata</i>	162	0.984	76.509	0.043	620.38	1.842	0.985	0.962
CASE – <i>C. monstrosa</i>	117	0.991	23.525	0.029	836.90	1.696	0.992	0.986
CASE – <i>P. glauca</i>	272	0.975	53.081	0.016	673.25	2.077	0.977	0.937
CASE – <i>S. canicula</i>	212	0.988	82.314	0.064	537.94	1.864	0.989	0.979
CASE – <i>G. melastomus</i>	438	0.966	52.879	0.073	418.82	1.726	0.969	0.909

Table 3. Fitting parameters. RSS: residual sum of squares. R_{adj}^2 : adjusted determination coefficient. R^2 : correlation coefficient (all data); $R^2(CV)$ correlation coefficient (cross-validation).

Model predictive capabilities were assessed by cross-validation. For each species tested, model parameters were estimated as above described using only three E/S levels, setting aside the data of the fourth E/S level. The resulting model was then applied to this remaining level to predict molecular weights at every testing time. This procedure was iteratively repeated until all data sets were validated. Cross-validation correlation coefficients of estimated versus experimental values were calculated for each species and enzymes tested ($R^2(CV)$ in Table 3). As can be expected, these are slightly worse than the correlation coefficients (not adjusted for parameters) using the whole data set (R^2 in Table 3). However, the cross-validation model proves capable of adequately fitting the data, as shown by correlation coefficients ranging between 0.909 and 0.991. Hence, it is possible to predict reasonably well M_n values within the specified experimental range. Accuracy will be higher towards the centre of the range, as more significant differences between predicted and experimental values are generally seen at extreme E/S ratios (Fig. 3, Table S1). In particular, low E/S presents the poorest R^2 in cross-validation experiments in comparison to higher enzymatic levels; therefore, more uncertainty is expected close to these lower bounds. This behaviour consistently appears for both enzymes, but it is remarkable in

HASE, reaching minimum values in *R. clavata* and *G. melastomus* (Table 4). The rest of HASE coefficients range from 0.849 to 0.994, which indicate the validity of the model, however it is not possible to ascertain model accuracy in the mentioned species around the lower E/S level.

Figure 3. Prediction of M_n with cross-validation models (solid lines) compared to experimental data (scatter) during depolymerization reactions of CS with chondroitinase and hyaluronidase at four enzyme to substrate ratios for each fish species tested. Scatter data as average M_n (kDa) at each sampling time; error bars: standard deviation ($n=2$).

	HASE				CASE			
	2.5	7.5	12.5	20.0	2.0	4.0	9.0	14.0
<i>R. clavata</i>	0.211	0.924	0.980	0.986	0.885	0.961	0.995	0.986
<i>C. monstrosa</i>	0.886	0.992	0.966	0.910	0.977	0.982	0.993	0.996
<i>P. glauca</i>	0.849	0.967	0.986	0.973	0.855	0.956	0.981	0.933
<i>S. canicula</i>	0.930	0.984	0.994	0.989	0.961	0.958	0.991	0.991
<i>G. melastomus</i>	0.499	0.967	0.972	0.929	0.807	0.851	0.992	0.976

Table 4. Correlation coefficients (R^2) for each cross-validation experiment. Numerical column headers are the enzyme to substrate ratios (U /mg for HASE; mU/mg for CASE)

Bearing this in mind, the model proposed permits the determination of what conditions are required to provide a desired molecular weight. Fig. 4 depicts the possible combinations of E and reaction time as a function of M_n . As can be seen, a given M_n can be reached by increased t at low E or vice versa. Reaction times at chosen E can be calculated integrating differential equation (1) and replacing the relationships (2) and (3). The integrated form of equation (1) reads:

$$\frac{1}{(M_n - M_n^\infty)^{n-1}} = \frac{1}{(M_n^0 - M_n^\infty)^{n-1}} + (n-1)kt \quad (4)$$

Where

$$t = \left(\frac{1}{(n-1)k} \right) \left(\frac{1}{(M_n - M_n^\infty)^{n-1}} - \frac{1}{(M_n^0 - M_n^\infty)^{n-1}} \right)$$

Substituting relationships (2) and (3):

$$t = \left(\frac{1}{(n-1)(\alpha + bE)} \right) \left(\frac{1}{(M_n - M_n^0 e^{-\mu E})^{n-1}} - \frac{1}{(M_n^0 - M_n^0 e^{-\mu E})^{n-1}} \right) \quad (5)$$

This equation gives the explicit expression to calculate the required time to achieve a particular M_n for a given enzymatic level (E). It should be noted that the enzymatic level can also be

Figure 4. Variation in molecular weight (M_n ; kDa) of CS with reaction time (t; hours) and enzyme to substrate ratios (E; enzyme U/mg CS).

designed using equation (4) for a given M_w and time of reaction. However, the equation has not an explicit solution, and numerical methods have to be used.

When choosing specific reaction conditions, it must be considered that shortening reaction times by increasing E results in a more pronounced decrease of M_n . Stopping the reaction under these conditions may compromise M_n accuracy, as small variations in time can result in substantial M_n changes. This is particularly relevant, as inactivation of the enzymes by heating requires some time, especially if the reaction is scaled-up. Concerning enzyme selection, several aspects require attention: i) CASE is considerably more expensive than HASE, therefore minimizing the amount of enzyme used in the reaction may be important in the first case; ii) The $-exo$ action of CASE strips off disaccharides from the CS chain, leading to mass loss in the depolymerized products. As seen in Fig. 1, the proportion of disaccharides released is low until M_n of 20-25 kDa is reached, as $-endo$ activity predominates, but increases dramatically from these values downwards. Therefore, CASE may be suitable to produce mid-sized polymers, but the yield would be meager for small CS fragments; iii) HASE preserves the chemical structure of undepolymerized CS, CASE on the other hand yields reaction products carrying unsaturation at the non-reducing end. Such structural changes may alter CS bioactivity and must be evaluated based on the intended application.

4. Conclusions

We have described the depolymerization of marine chondroitin sulfate with hyaluronidase and chondroitinase through a pseudo-mechanistic model. CS from five fish species was tested at four enzyme to substrate ratios. Good fits are obtained for reaction orders between 1.7 and 2.1 for CASE and between 1.9 and 3.4 for HASE depending on the species. The predictive capabilities

of the model were assessed by cross-validation, resulting in correlation coefficients with experimental data greater than 0.9. The proposed equations allow to define combinations of enzyme amount and reaction time to obtain CS of the desired molecular weight. Furthermore, while sulfation pattern changes as the reaction progresses, the main features of marine CS remain in the reaction products tested, i.e. prevalent 6S sulfation and a substantial amount of disulfated disaccharides.

Conflict of interest

The authors declare no conflict of interest.

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Species	time	CASE				HASE			
		E/S	Mn (rep 1)	Mn (rep 2)	Mn (est.)	E/S	Mn (rep 1)	Mn (rep 2)	Mn (est.)
R. clavata	0.00	2	61.02	59.12	51.65	2.5	61.02	59.12	50.77
	0.08		57.75	59.15	48.82		57.75	59.15	50.54
	0.17		58.05	56.84	46.38		58.05	56.84	50.31
	0.33		52.92	56.61	42.44		52.92	56.61	49.88
	0.50		57.76	57.56	39.39		57.76	57.56	49.48
	0.67		55.37	54.38	36.97		55.37	54.38	49.11
	0.83		55.21	52.16	35.01		55.21	52.16	48.76
	1.00		51.69	51.22	33.39		51.69	51.22	48.43
	2.00		48.28	49.12	27.65		48.28	49.12	46.78
	3.00		48.07	45.80	24.95		48.07	45.80	45.53
	4.00		47.57	42.85	23.41		47.57	42.85	44.54
	6.00		47.47	42.18	21.76		47.47	42.18	43.73
	8.00		44.36	42.07	20.91		44.36	42.07	41.97
	11.00		45.53	41.96	20.22		45.53	41.96	40.77
	14.00		-	41.34	19.83		-	41.34	39.20
	24.00		44.21	41.27	19.26		44.21	41.27	38.04
	0.00	4	61.02	59.12	51.65	7.5	61.02	59.12	50.77
	0.08		52.87	50.94	44.73		52.87	50.94	47.47
	0.17		48.78	46.58	39.44		48.78	46.58	44.98
	0.33		41.28	42.87	31.94		41.28	42.87	41.40
	0.50		38.51	42.74	26.90		38.51	42.74	38.91
	0.67		38.29	39.52	23.30		38.29	39.52	37.05
	0.83		37.70	38.48	20.62		37.70	38.48	35.59
	1.00		34.90	35.39	18.54		34.90	35.39	34.40
	2.00		29.14	30.96	12.04		29.14	30.96	30.12
	3.00		26.74	25.20	9.38		26.74	25.20	27.92
	4.00		22.86	23.54	7.96		22.86	23.54	26.51
	6.00		22.51	19.51	6.51		22.51	19.51	25.52
	8.00		21.29	16.37	5.78		21.29	16.37	23.68
	11.00		16.16	14.18	5.19		16.16	14.18	22.62
	14.00		-	13.06	4.87		-	13.06	21.39
	24.00		16.12	12.14	4.39		16.12	12.14	20.56
	0.00	9	61.02	59.12	51.65	12.5	61.02	59.12	50.77
	0.08		55.74	46.75	40.81		55.74	46.75	42.85
	0.17		46.92	41.14	33.61		46.92	41.14	38.25
	0.33		38.06	40.43	24.67		38.06	40.43	32.82
	0.50		35.56	34.81	19.38		35.56	34.81	29.55
	0.67		32.09	31.59	15.90		32.09	31.59	27.30
	0.83		33.20	28.89	13.44		33.20	28.89	25.61
	1.00		29.05	26.70	11.62		29.05	26.70	24.28
	2.00		21.41	24.23	6.33		21.41	24.23	19.73
	3.00		20.84	17.74	4.31		20.84	17.74	17.46
	4.00		19.11	15.94	3.26		19.11	15.94	16.02
	6.00		15.60	14.48	2.19		15.60	14.48	14.99
	8.00		12.01	12.75	1.66		12.01	12.75	13.08
	11.00		13.03	12.44	1.22		13.03	12.44	11.96
	14.00		-	11.98	0.98		-	11.98	10.63
	24.00		11.30	11.74	0.62		11.30	11.74	9.72
0.00	14	61.02	59.12	51.65	20	61.02	59.12	50.77	
0.08		48.28	46.08	38.93		48.28	46.08	40.18	
0.17		40.32	38.95	30.97		40.32	38.95	34.87	

Species	time	CASE			HASE				
		E/S	Mn (rep 1)	Mn (rep 2)	Mn (est.)	E/S	Mn (rep 1)	Mn (rep 2)	Mn (est.)
<i>R. clavata</i>	0.33	14	31.98	32.78	21.64	20	31.98	32.78	29.08
	0.50		31.58	30.41	16.42		31.58	30.41	25.78
	0.67		24.64	26.57	13.12		24.64	26.57	23.55
	0.83		24.37	25.93	10.86		24.37	25.93	21.90
	1.00		22.81	22.72	9.22		22.81	22.72	20.62
	2.00		16.32	18.31	4.66		16.32	18.31	16.29
	3.00		13.86	12.99	3.02		13.86	12.99	14.15
	4.00		13.82	12.36	2.20		13.82	12.36	12.79
	6.00		11.34	10.85	1.39		11.34	10.85	11.83
	8.00		8.39	8.49	1.00		8.39	8.49	10.03
	11.00		7.90	9.01	0.69		7.90	9.01	8.97
	14.00		-	7.12	0.52		-	7.12	7.71
	24.00		8.55	6.88	0.28		8.55	6.88	6.84
<i>C. monstrosa</i>	0.00	2	61.02	59.12	60.63	2.5	61.02	59.12	64.36
	0.08		57.75	59.15	55.46		57.75	59.15	63.86
	0.17		58.05	56.84	51.20		58.05	56.84	63.39
	0.33		52.92	56.61	44.61		52.92	56.61	62.50
	0.50		57.76	57.56	39.78		57.76	57.56	61.68
	0.67		55.37	54.38	36.10		55.37	54.38	60.92
	0.83		55.21	52.16	33.23		55.21	52.16	60.21
	1.00		51.69	51.22	30.92		51.69	51.22	59.56
	2.00		48.28	49.12	23.25		48.28	49.12	56.39
	3.00		48.07	45.80	19.93		48.07	45.80	54.11
	4.00		47.57	42.85	18.13		47.57	42.85	52.37
	6.00		47.47	42.18	16.27		47.47	42.18	50.99
	8.00		44.36	42.07	15.34		44.36	42.07	48.13
	11.00		45.53	41.96	14.61		45.53	41.96	46.31
	14.00		-	41.34	14.21		-	41.34	44.06
	24.00		44.21	41.27	13.64		44.21	41.27	42.51
	0.00	4	61.02	59.12	60.63	7.5	61.02	59.12	64.36
	0.08		52.87	50.94	52.36		52.87	50.94	60.96
	0.17		48.78	46.58	45.82		48.78	46.58	58.06
	0.33		41.28	42.87	36.25		41.28	42.87	53.34
	0.50		38.51	42.74	29.67		38.51	42.74	49.65
	0.67		38.29	39.52	24.92		38.29	39.52	46.67
	0.83		37.70	38.48	21.36		37.70	38.48	44.20
	1.00		34.90	35.39	18.63		34.90	35.39	42.12
	2.00		29.14	30.96	10.31		29.14	30.96	34.15
	3.00		26.74	25.20	7.15		26.74	25.20	29.85
	4.00		22.86	23.54	5.58		22.86	23.54	27.09
	6.00		22.51	19.51	4.10		22.51	19.51	25.14
	8.00		21.29	16.37	3.43		21.29	16.37	21.61
	11.00		16.16	14.18	2.94		16.16	14.18	19.64
	14.00		-	13.06	2.69		-	13.06	17.45
	24.00		16.12	12.14	2.37		16.12	12.14	16.06
	0.00	9	61.02	59.12	60.63	12.5	61.02	59.12	64.36
	0.08		55.74	46.75	43.87		55.74	46.75	57.87
	0.17		46.92	41.14	33.74		46.92	41.14	52.92
	0.33		38.06	40.43	22.35		38.06	40.43	45.76
	0.50		35.56	34.81	16.27		35.56	34.81	40.78
	0.67		32.09	31.59	12.57		32.09	31.59	37.08

Species	time	CASE			HASE				
		E/S	Mn (rep 1)	Mn (rep 2)	Mn (est.)	E/S	Mn (rep 1)	Mn (rep 2)	Mn (est.)
<i>C. monstrosa</i>	0.83	9	33.20	28.89	10.10	12.5	33.20	28.89	34.20
	1.00		29.05	26.70	8.37		29.05	26.70	31.88
	2.00		21.41	24.23	3.82		21.41	24.23	23.81
	3.00		20.84	17.74	2.32		20.84	17.74	19.85
	4.00		19.11	15.94	1.62		19.11	15.94	17.43
	6.00		15.60	14.48	0.96		15.60	14.48	15.77
	8.00		12.01	12.75	0.66		12.01	12.75	12.84
	11.00		13.03	12.44	0.44		13.03	12.44	11.25
	14.00		-	11.98	0.33		-	11.98	9.50
	24.00		11.30	11.74	0.17		11.30	11.74	8.40
	0.00	14	61.02	59.12	60.63	20	61.02	59.12	64.36
	0.08		48.28	46.08	38.97		48.28	46.08	59.02
	0.17		40.32	38.95	27.69		40.32	38.95	54.67
	0.33		31.98	32.78	16.56		31.98	32.78	47.96
	0.50		31.58	30.41	11.29		31.58	30.41	42.99
	0.67		24.64	26.57	8.32		24.64	26.57	39.14
	0.83		24.37	25.93	6.45		24.37	25.93	36.05
	1.00		22.81	22.72	5.19		22.81	22.72	33.51
	2.00		16.32	18.31	2.13		16.32	18.31	24.30
	3.00		13.86	12.99	1.22		13.86	12.99	19.61
	4.00		13.82	12.36	0.81		13.82	12.36	16.71
	6.00		11.34	10.85	0.45		11.34	10.85	14.70
	8.00		8.39	8.49	0.30		8.39	8.49	11.15
	11.00		7.90	9.01	0.19		7.90	9.01	9.22
	14.00		-	7.12	0.13		-	7.12	7.10
	24.00		8.55	6.88	0.06		8.55	6.88	5.79
	0.00	2	61.02	59.12	54.86	2.5	61.02	59.12	60.07
	0.08		57.75	59.15	51.73		57.75	59.15	59.50
	0.17		58.05	56.84	49.11		58.05	56.84	58.96
	0.33		52.92	56.61	44.93		52.92	56.61	57.97
	0.50		57.76	57.56	41.76		57.76	57.56	57.07
	0.67		55.37	54.38	39.26		55.37	54.38	56.25
	0.83		55.21	52.16	37.25		55.21	52.16	55.50
	1.00		51.69	51.22	35.58		51.69	51.22	54.80
	2.00		48.28	49.12	29.57		48.28	49.12	51.53
	3.00		48.07	45.80	26.59		48.07	45.80	49.22
	4.00		47.57	42.85	24.80		47.57	42.85	47.48
	6.00		47.47	42.18	22.75		47.47	42.18	46.10
	8.00		44.36	42.07	21.59		44.36	42.07	43.20
	11.00		45.53	41.96	20.56		45.53	41.96	41.31
	14.00		-	41.34	19.94		-	41.34	38.89
	24.00		44.21	41.27	18.91		44.21	41.27	37.14
	0.00	4	61.02	59.12	54.86	7.5	61.02	59.12	60.07
	0.08		52.87	50.94	45.17		52.87	50.94	53.93
	0.17		48.78	46.58	38.63		48.78	46.58	49.73
	0.33		41.28	42.87	30.32		41.28	42.87	44.21
	0.50		38.51	42.74	25.25		38.51	42.74	40.63
	0.67		38.29	39.52	21.83		38.29	39.52	38.06
	0.83		37.70	38.48	19.35		37.70	38.48	36.10
	1.00		34.90	35.39	17.48		34.90	35.39	34.54
	2.00		29.14	30.96	11.73		29.14	30.96	29.13
<i>P. glauca</i>	0.00	2	61.02	59.12	54.86	2.5	61.02	59.12	60.07
	0.08		57.75	59.15	51.73		57.75	59.15	59.50
	0.17		58.05	56.84	49.11		58.05	56.84	58.96
	0.33		52.92	56.61	44.93		52.92	56.61	57.97
	0.50		57.76	57.56	41.76		57.76	57.56	57.07
	0.67		55.37	54.38	39.26		55.37	54.38	56.25
	0.83		55.21	52.16	37.25		55.21	52.16	55.50
	1.00		51.69	51.22	35.58		51.69	51.22	54.80
	2.00		48.28	49.12	29.57		48.28	49.12	51.53
	3.00		48.07	45.80	26.59		48.07	45.80	49.22
	4.00		47.57	42.85	24.80		47.57	42.85	47.48
	6.00		47.47	42.18	22.75		47.47	42.18	46.10
	8.00		44.36	42.07	21.59		44.36	42.07	43.20
	11.00		45.53	41.96	20.56		45.53	41.96	41.31
	14.00		-	41.34	19.94		-	41.34	38.89
	24.00		44.21	41.27	18.91		44.21	41.27	37.14
	0.00	4	61.02	59.12	54.86	7.5	61.02	59.12	60.07
	0.08		52.87	50.94	45.17		52.87	50.94	53.93
	0.17		48.78	46.58	38.63		48.78	46.58	49.73
	0.33		41.28	42.87	30.32		41.28	42.87	44.21
	0.50		38.51	42.74	25.25		38.51	42.74	40.63
	0.67		38.29	39.52	21.83		38.29	39.52	38.06
	0.83		37.70	38.48	19.35		37.70	38.48	36.10
	1.00		34.90	35.39	17.48		34.90	35.39	34.54
	2.00		29.14	30.96	11.73		29.14	30.96	29.13
	0.00	2	61.02	59.12	54.86	2.5	61.02	59.12	60.07
	0.08		57.75	59.15	51.73		57.75	59.15	59.50
	0.17		58.05	56.84	49.11		58.05	56.84	58.96
	0.33		52.92	56.61	44.93		52.92	56.61	57.97
	0.50		57.76	57.56	41.76		57.76	57.56	57.07
	0.67		55.37	54.38	39.26		55.37	54.38	56.25
	0.83		55.21	52.16	37.25		55.21	52.16	55.50
	1.00		51.69	51.22	35.58		51.69	51.22	54.80
	2.00		48.28	49.12	29.57		48.28	49.12	51.53
	3.00		48.07	45.80	26.59		48.07	45.80	49.22
	4.00		47.57	42.85	24.80		47.57	42.85	47.48
	6.00		47.47	42.18	22.75		47.47	42.18	46.10
	8.00		44.36	42.07	21.59		44.36	42.07	43.20
	11.00		45.53	41.96	20.56		45.53	41.96	41.31
	14.00		-	41.34	19.94		-	41.34	38.89
	24.00		44.21	41.27	18.91		44.21	41.27	37.14
	0.00	4	61.02	59.12	54.86	7.5	61.02	59.12	60.07
	0.08		52.87	50.94	45.17		52.87	50.94	53.93
	0.17		48.78	46.58	38.63		48.78	46.58	49.73
	0.33		41.28	42.87	30.32		41.28	42.87	44.21
	0.50		38.51	42.74	25.25		38.51	42.74	40.63
	0.67		38.29	39.52	21.83		38.29	39.52	38.06
	0.83		37.70	38.48	19.35		37.70	38.48	36.10
	1.00		34.90	35.39	17.48		34.90	35.39	34.54
	2.00		29.14	30.96	11.73		29.14	30.96	29.13

Species	time	CASE			HASE				
		E/S	Mn (rep 1)	Mn (rep 2)	Mn (est.)	E/S	Mn (rep 1)	Mn (rep 2)	Mn (est.)
<i>P. glauca</i>	3.00	4	26.74	25.20	9.35	7.5	26.74	25.20	26.43
	4.00		22.86	23.54	8.05		22.86	23.54	24.73
	6.00		22.51	19.51	6.64		22.51	19.51	23.54
	8.00		21.29	16.37	5.89		21.29	16.37	21.34
	11.00		16.16	14.18	5.25		16.16	14.18	20.07
	14.00		-	13.06	4.87		-	13.06	18.60
	24.00		16.12	12.14	4.27		16.12	12.14	17.62
	0.00	9	61.02	59.12	54.86	12.5	61.02	59.12	60.07
	0.08		55.74	46.75	38.87		55.74	46.75	50.16
	0.17		46.92	41.14	30.41		46.92	41.14	44.28
	0.33		38.06	40.43	21.51		38.06	40.43	37.28
	0.50		35.56	34.81	16.81		35.56	34.81	33.07
	0.67		32.09	31.59	13.89		32.09	31.59	30.18
	0.83		33.20	28.89	11.89		33.20	28.89	28.02
	1.00		29.05	26.70	10.42		29.05	26.70	26.33
	2.00		21.41	24.23	6.15		21.41	24.23	20.61
	3.00		20.84	17.74	4.46		20.84	17.74	17.81
	4.00		19.11	15.94	3.54		19.11	15.94	16.05
	6.00		15.60	14.48	2.56		15.60	14.48	14.82
	8.00		12.01	12.75	2.03		12.01	12.75	12.55
	11.00		13.03	12.44	1.57		13.03	12.44	11.25
	14.00		-	11.98	1.30		-	11.98	9.72
	24.00		11.30	11.74	0.86		11.30	11.74	8.69
	0.00	14	61.02	59.12	54.86	20	61.02	59.12	60.07
	0.08		48.28	46.08	38.50		48.28	46.08	49.31
	0.17		40.32	38.95	29.31		40.32	38.95	43.22
	0.33		31.98	32.78	19.47		31.98	32.78	36.15
	0.50		31.58	30.41	14.37		31.58	30.41	31.97
	0.67		24.64	26.57	11.28		24.64	26.57	29.10
	0.83		24.37	25.93	9.23		24.37	25.93	26.97
	1.00		22.81	22.72	7.77		22.81	22.72	25.30
	2.00		16.32	18.31	3.84		16.32	18.31	19.63
	3.00		13.86	12.99	2.48		13.86	12.99	16.84
	4.00		13.82	12.36	1.80		13.82	12.36	15.08
	6.00		11.34	10.85	1.14		11.34	10.85	13.83
	8.00		8.39	8.49	0.82		8.39	8.49	11.53
	11.00		7.90	9.01	0.57		7.90	9.01	10.18
	14.00		-	7.12	0.43		-	7.12	8.59
	24.00		8.55	6.88	0.23		8.55	6.88	7.51
	0.00	2	61.02	59.12	69.38	2.5	61.02	59.12	70.65
	0.08		57.75	59.15	61.41		57.75	59.15	69.86
	0.17		58.05	56.84	55.79		58.05	56.84	69.13
	0.33		52.92	56.61	48.43		52.92	56.61	67.81
	0.50		57.76	57.56	43.87		57.76	57.56	66.66
	0.67		55.37	54.38	40.77		55.37	54.38	65.63
	0.83		55.21	52.16	38.55		55.21	52.16	64.72
	1.00		51.69	51.22	36.88		51.69	51.22	63.90
	2.00		48.28	49.12	31.91		48.28	49.12	60.26
	3.00		48.07	45.80	29.98		48.07	45.80	57.92
	4.00		47.57	42.85	28.97		47.57	42.85	56.26
	6.00		47.47	42.18	27.96		47.47	42.18	55.00
<i>S. canicula</i>	0.00	2	61.02	59.12	69.38	2.5	61.02	59.12	70.65
	0.08		57.75	59.15	61.41		57.75	59.15	69.86
	0.17		58.05	56.84	55.79		58.05	56.84	69.13
	0.33		52.92	56.61	48.43		52.92	56.61	67.81
	0.50		57.76	57.56	43.87		57.76	57.56	66.66
	0.67		55.37	54.38	40.77		55.37	54.38	65.63
	0.83		55.21	52.16	38.55		55.21	52.16	64.72
	1.00		51.69	51.22	36.88		51.69	51.22	63.90
	2.00		48.28	49.12	31.91		48.28	49.12	60.26
	3.00		48.07	45.80	29.98		48.07	45.80	57.92
	4.00		47.57	42.85	28.97		47.57	42.85	56.26
	6.00		47.47	42.18	27.96		47.47	42.18	55.00

Species	time	CASE			HASE				
		E/S	Mn (rep 1)	Mn (rep 2)	Mn (est.)	E/S	Mn (rep 1)	Mn (rep 2)	Mn (est.)
<i>S. canicula</i>	8.00	2	44.36	42.07	27.45	2.5	44.36	42.07	52.54
	11.00		45.53	41.96	27.04		45.53	41.96	51.06
	14.00		-	41.34	26.81		-	41.34	49.30
	24.00		44.21	41.27	26.48		44.21	41.27	48.13
	0.00	4	61.02	59.12	69.38	7.5	61.02	59.12	70.65
	0.08		52.87	50.94	57.24		52.87	50.94	65.58
	0.17		48.78	46.58	48.75		48.78	46.58	61.59
	0.33		41.28	42.87	37.75		41.28	42.87	55.68
	0.50		38.51	42.74	31.03		38.51	42.74	51.46
	0.67		38.29	39.52	26.54		38.29	39.52	48.27
	0.83		37.70	38.48	23.36		37.70	38.48	45.75
	1.00		34.90	35.39	21.00		34.90	35.39	43.72
	2.00		29.14	30.96	14.18		29.14	30.96	36.50
	3.00		26.74	25.20	11.67		26.74	25.20	32.91
	4.00		22.86	23.54	10.40		22.86	23.54	30.70
	6.00		22.51	19.51	9.16		22.51	19.51	29.18
	8.00		21.29	16.37	8.57		21.29	16.37	26.50
	11.00		16.16	14.18	8.11		16.16	14.18	25.04
	14.00		-	13.06	7.86		-	13.06	23.43
	24.00		16.12	12.14	7.52		16.12	12.14	22.43
	0.00	9	61.02	59.12	69.38	12.5	61.02	59.12	70.65
	0.08		55.74	46.75	46.65		55.74	46.75	62.74
	0.17		46.92	41.14	34.84		46.92	41.14	56.90
	0.33		38.06	40.43	22.88		38.06	40.43	48.77
	0.50		35.56	34.81	16.92		35.56	34.81	43.31
	0.67		32.09	31.59	13.38		32.09	31.59	39.34
	0.83		33.20	28.89	11.05		33.20	28.89	36.30
	1.00		29.05	26.70	9.40		29.05	26.70	33.89
	2.00		21.41	24.23	4.99		21.41	24.23	25.70
	3.00		20.84	17.74	3.45		20.84	17.74	21.80
	4.00		19.11	15.94	2.68		19.11	15.94	19.46
	6.00		15.60	14.48	1.92		15.60	14.48	17.86
	8.00		12.01	12.75	1.55		12.01	12.75	15.09
	11.00		13.03	12.44	1.25		13.03	12.44	13.60
	14.00		-	11.98	1.09		-	11.98	11.98
	24.00		11.30	11.74	0.85		11.30	11.74	10.98
	0.00	14	61.02	59.12	69.38	20	61.02	59.12	70.65
	0.08		48.28	46.08	44.89		48.28	46.08	62.16
	0.17		40.32	38.95	32.81		40.32	38.95	55.73
	0.33		31.98	32.78	20.99		31.98	32.78	46.58
	0.50		31.58	30.41	15.25		31.58	30.41	40.33
	0.67		24.64	26.57	11.90		24.64	26.57	35.78
	0.83		24.37	25.93	9.71		24.37	25.93	32.29
	1.00		22.81	22.72	8.17		22.81	22.72	29.52
	2.00		16.32	18.31	4.09		16.32	18.31	20.24
	3.00		13.86	12.99	2.68		13.86	12.99	15.96
	4.00		13.82	12.36	1.98		13.82	12.36	13.44
	6.00		11.34	10.85	1.28		11.34	10.85	11.77
	8.00		8.39	8.49	0.94		8.39	8.49	8.95
	11.00		7.90	9.01	0.67		7.90	9.01	7.51
	14.00		-	7.12	0.52		-	7.12	6.00

Species	time	CASE			HASE				
		E/S	Mn (rep 1)	Mn (rep 2)	Mn (est.)	E/S	Mn (rep 1)	Mn (rep 2)	Mn (est.)
<i>S. canicula</i>	24.00	14	8.55	6.88	0.30	20	8.55	6.88	5.10
<i>G. melastomus</i>	0.00	2	61.02	59.12	64.64	2.5	61.02	59.12	61.76
	0.08		57.75	59.15	61.46		57.75	59.15	61.37
	0.17		58.05	56.84	58.77		58.05	56.84	61.01
	0.33		52.92	56.61	54.53		52.92	56.61	60.33
	0.50		57.76	57.56	51.36		57.76	57.56	59.71
	0.67		55.37	54.38	48.90		55.37	54.38	59.15
	0.83		55.21	52.16	46.97		55.21	52.16	58.63
	1.00		51.69	51.22	45.41		51.69	51.22	58.16
	2.00		48.28	49.12	40.20		48.28	49.12	55.90
	3.00		48.07	45.80	37.98		48.07	45.80	54.32
	4.00		47.57	42.85	36.80		47.57	42.85	53.14
	6.00		47.47	42.18	35.64		47.47	42.18	52.21
	8.00		44.36	42.07	35.08		44.36	42.07	50.30
	11.00		45.53	41.96	34.67		45.53	41.96	49.09
	14.00		-	41.34	34.45		-	41.34	47.60
	24.00		44.21	41.27	34.16		44.21	41.27	46.56
	0.00	4	61.02	59.12	64.64	7.5	61.02	59.12	61.76
	0.08		52.87	50.94	58.21		52.87	50.94	58.96
	0.17		48.78	46.58	52.98		48.78	46.58	56.53
	0.33		41.28	42.87	45.03		41.28	42.87	52.51
	0.50		38.51	42.74	39.30		38.51	42.74	49.35
	0.67		38.29	39.52	34.98		38.29	39.52	46.79
	0.83		37.70	38.48	31.63		37.70	38.48	44.70
	1.00		34.90	35.39	28.95		34.90	35.39	42.96
	2.00		29.14	30.96	20.06		29.14	30.96	36.71
	3.00		26.74	25.20	16.18		26.74	25.20	33.75
	4.00		22.86	23.54	14.04		22.86	23.54	32.08
	6.00		22.51	19.51	11.78		22.51	19.51	31.03
	8.00		21.29	16.37	10.63		21.29	16.37	29.42
	11.00		16.16	14.18	9.69		16.16	14.18	28.71
	14.00		-	13.06	9.17		-	13.06	28.09
	24.00		16.12	12.14	8.39		16.12	12.14	27.79
	0.00	9	61.02	59.12	64.64	12.5	61.02	59.12	61.76
	0.08		55.74	46.75	53.65		55.74	46.75	56.04
	0.17		46.92	41.14	45.55		46.92	41.14	51.66
	0.33		38.06	40.43	34.54		38.06	40.43	45.32
	0.50		35.56	34.81	27.50		35.56	34.81	40.91
	0.67		32.09	31.59	22.68		32.09	31.59	37.62
	0.83		33.20	28.89	19.20		33.20	28.89	35.06
	1.00		29.05	26.70	16.59		29.05	26.70	33.01
	2.00		21.41	24.23	8.99		21.41	24.23	25.82
	3.00		20.84	17.74	6.20		20.84	17.74	22.31
	4.00		19.11	15.94	4.81		19.11	15.94	20.15
	6.00		15.60	14.48	3.49		15.60	14.48	18.68
	8.00		12.01	12.75	2.87		12.01	12.75	16.07
	11.00		13.03	12.44	2.41		13.03	12.44	14.66
	14.00		-	11.98	2.17		-	11.98	13.10
	24.00		11.30	11.74	1.84		11.30	11.74	12.13
	0.00	14	61.02	59.12	64.64	20	61.02	59.12	61.76
	0.08		48.28	46.08	52.86		48.28	46.08	57.11

Species	time	CASE				HASE			
		E/S	Mn (rep 1)	Mn (rep 2)	Mn (est.)	E/S	Mn (rep 1)	Mn (rep 2)	Mn (est.)
<i>G. melastomus</i>	0.17	14	40.32	38.95	44.21	20	40.32	38.95	52.99
	0.33		31.98	32.78	32.54		31.98	32.78	46.02
	0.50		31.58	30.41	25.18		31.58	30.41	40.40
	0.67		24.64	26.57	20.19		24.64	26.57	35.81
	0.83		24.37	25.93	16.64		24.37	25.93	32.04
	1.00		22.81	22.72	14.01		22.81	22.72	28.89
	2.00		16.32	18.31	6.58		16.32	18.31	17.84
	3.00		13.86	12.99	4.00		13.86	12.99	13.05
	4.00		13.82	12.36	2.77		13.82	12.36	10.61
	6.00		11.34	10.85	1.65		11.34	10.85	9.24
	8.00		8.39	8.49	1.15		8.39	8.49	7.49
	11.00		7.90	9.01	0.80		7.90	9.01	6.90
	14.00		-	7.12	0.62		-	7.12	6.52
	24.00		8.55	6.88	0.39		8.55	6.88	6.39

Table S1. Number average molecular weight (Mn, kDa) of chondroitin sulfate (CS) from five fish species depolymerised with chondroitinase ABC (CASE) and hyaluronidase from bovine testes (HASE) over 24 hours, at four enzyme to substrate ratios (E/S). Mn (rep 1) and (rep 2): experimental data; Mn (est.): values estimated by the model; time in h, E/S in U HASE / mg CS or mU CASE / mg CS.