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Interactions of low-molecular-weight semi-synthetic sulfated heparins with human leukocyte elastase and human Cathepsin G

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ABSTRACT

Semi-synthetic low-molecular-weight heparin samples (LMWHs), having homogeneous degree of polymerization and saccharide backbone, but differing in the number and location of sulfate groups, were investigated in their ability to interfere with the pharmacologically relevant targets human leukocyte elastase (EL) and human Cathepsin G (CatG). Spectroscopic studies were performed for a quantitative evaluation of the enzyme-inhibitor dissociation constant, K_i, and of the IC_{50} values for the inhibition of cleavage of target peptide sequences. Both proteases are inhibited by the tested polysaccharides through a mixed hyperbolic binding process. A non-linear relationship was found between degree of sulfation and binding affinity or enzyme inhibition properties, showing a composite correlation between heparin charge density and interference with EL/CatG activity.

1. Introduction

Heparin is a component of the linear polyanionic polysaccharides family called glycosaminoglycans (GAG). Heparin is widely used in clinics as an anti-coagulant and in its low-molecular-weight form (LMWH), as an anti-thrombotic. An important characteristic of GAGs is represented by sulfation at different positions along the polymer chain [1] that favours an interaction with a number of biologically relevant proteins [2]. In particular, heparin pharmacological use as an anti-coagulant is connected to its ability to bind to antithrombin III (AT) and to induce an allosteric modification in the serpine, thereby, greatly enhancing its inhibition of thrombin and Factor Xa [3]. This process is due to the formation of a stable covalent complex between the two protein (serpine and protease) components [4]. Interestingly, antithrombin III bound to heparin is inactivated by the endopeptidase elastase (EL), a serine proteinase stored in the azurophil granules of polymorphonuclear leucocytes [5]. In fact, EL is able to bind heparin in competition with AT, thus modulating anti-coagulant, anti-thrombotic and anti-atherosclerotic drug properties. On the other hand, heparin and other GAGs can reversibly inhibit EL activity [6,7] through a direct binding or by increasing the inhibition rate of specific endogenous inhibitors like mucus proteinase inhibitor [8]. A similar behaviour is observed in the presence of Cathepsin G, a protease closely related to EL in terms of structure and functions [9].

The pharmacological relevance of peptidase interference is well documented in post-operative patients where neutrophil

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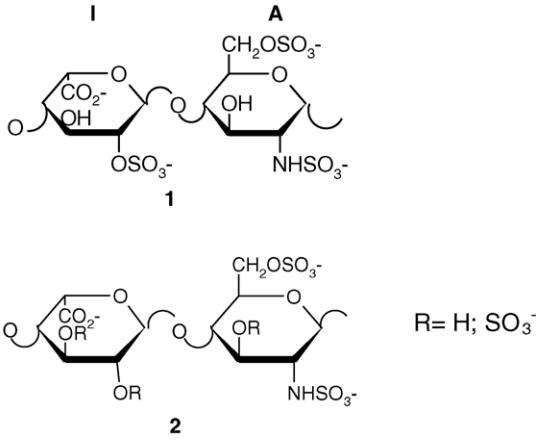


Fig. 1 – Predominant repeating disaccharide units: in heparin (1) and in chemically modified heparins (2). Iduronic acid (I) and glucosamine (A).

activation occurs [10]. Additionally, free heparin has been found in the alveolar walls, just where proteinases attack the extracellular matrix during the chronic lung inflammation associated to emphysema [11].

The GAG-proteins interactions are influenced by carbohydrate backbone, saccharidic composition, degree of sulfation, sulfation patterns and molecular weight. For cationic proteins like EL and CatG, binding is reported to be largely stabilized by electrostatic interactions as it involves positively charged amino acids of the protein and anionic sites of the polysaccharide [12,13]. Nevertheless, other non-ionic interactions can occur to modulate the CatG–polysaccharide interaction process [14].

Given the biological and pharmacological relevance of competitive interactions between GAGs and different target proteins, to assess possible differential effects of sulfate substitution, we investigated quantitatively heparin binding to and interference with EL and CatG using a number of LMW-heparin preparations obtained by solvolytic desulfation under controlled experimental conditions [15,16] of a chemically supersulfated heparin [17] (compounds S0–S3 in Fig. 1 and Table 1). This approach permits to obtain LMW-heparins with systematically different sulfation patterns, difficult to obtain with other methods. It is worth noting that heteronuclear single quantum coherence (HSQC-NMR) spectroscopy [18] confirmed that the test derivatives are essentially equivalent in terms of chain length and saccharide composition, but differ in the amount and distribution of sulfate groups.

Due to the physiological presence of GAG having different sulfation degree, these heparin derivatives represent a good model for exploiting the biological role of GAG-protein interaction.

2. Materials and methods

The test heparins were prepared by chemical sulfation and controlled depolymerization (S0) of a pig mucosal heparin with sulfuric acid and chlorosulfonic acid mixture leading to a supersulfated LMWH [17], followed by solvolytic desulfation (Me_2SO–MeOH 10%, v/v at 65 °C) using different reaction times (4, 8, 16 h, respectively). Following N-resulfation yielded the desired LMWHs with different degrees of sulfation (Table 1) [15]. Stock solutions (1 mg/ml) were made in water and diluted to the working concentration in the desired buffer. A conventional LMWH (sample N) was prepared by controlled depolymerization of pig mucosal heparin in nitrous acid. Briefly, 4.0 g of heparin were dissolved in 65 ml H_2O at 4 °C. $NaNO_2$ (75 mg) was added and the pH adjusted to 2.0 with 0.1 M HCl. The solution was stirred at 4 °C for 20 min. The pH was, then, brought to 7.0. Solid $NaBH_4$ (1.0 g) was added in several portions under stirring. After 2–3 h, the pH was adjusted to 4.0 with HCl, and the solution was neutralized with NaOH. The product, isolated by ethanolic precipitation, exhibited essentially the same molecular weight (5000 Da evaluated by GPC) as the chemically modified LMWH preparations of the present study.

Human leukocyte elastase, purified by affinity chromatography, was purchased from Elastin Products (Owensville, USA). The protein purity was checked by gel electrophoresis (SDS-PAGE). EL was stored in 70 mM sodium acetate buffer, pH 4.5, containing 0.05% (v/v) Triton X100. Its molar extinction at 280 nm was 20,100 l/(mol cm).

Table 1 – Molecular mass and characterization of sulfation patterns of the test heparins. Sulfation degree (SD) and determination of variously substituted monosaccharide components were evaluated by NMR spectroscopy. The semi-synthetic samples are labeled S0–S3 and the conventional low-molecular-weight heparin is N

Sample	Mw[a] (kDa)	D[b]	SD[c]	Glucosamine sulfate groups (mol%)			Iduronic acid residues (%)			
				6-SO_3^-	3-SO_3^-	N-SO_3^-	Non-sulfated	2,3-O-sulfated	2-O-sulfated	3-O-sulfated
N	5.0	1.2	2.7	84	5	84	10	0	85	0
S0	6.3	1.3	4.8	100	100	85	0	100	0	0
S1	5.4	1.2	3.3	52	86	85	0	<5	>95	0
S2	5.3	1.2	3.0	36	80	85	0	<5	>95	0
S3	5.3	1.2	2.3	22	56	85	37	<5	54	9

[a] Average ponderal molecular mass determined by size exclusion chromatography with a triple detector array.
[b] Molecular mass polydispersity.
[c] Sulfation degree obtained by NMR evaluation of sulfate residues.

Cathepsin G was purchased from Europa Bioproducts and dissolved before use in sodium acetate 50 mM pH 5.5 and NaCl 150 mM.

2.1. NMR experimental procedure

Proton and HSQC-NMR spectra were obtained at 500 MHz with a Bruker Avance 500 spectrometer equipped with a 5-mm TXI probe. Chemical shift values were measured downfield from trimethylsilyl propionate sodium salt (TSP) as standard at 40 °C. Samples (10 mg) were previously submitted to a double lyophilization from D_2O and finally dissolved in 0.6 ml of deuterium oxide (99.996 at.% D_2O). Mono-dimensional ^1H spectra were obtained with presaturation of residual HDO, 128 scans, and a recycle delay of 10 s. Proton resonances were assigned with the use of conventional double-quantum-filtered COSY and TOCSY spectra. Data were acquired using 16 scans per series of 1 K × 512 W data points with zero filling in F1 and a shifted squared cosine function was applied prior to Fourier transformation. Two-dimensional gradient enhanced HSQC-NMR spectra were recorded with carbon decoupling during acquisition with 512 increments of 64 scans each. The polarization transfer delay ($D = 1/[2 \times 1JC–H]$) was set with a 1JC–H coupling values of 139–150–170 Hz for heparin samples. The matrix size 1 K × 512 was zero filled to 4 K × 2 K by application of a squared cosine function prior to Fourier transformation. Integration of cross-peaks was made using standard Bruker XWINNMR 3.5 software.

2.2. Molecular weight determination

The average molecular weights (Mw), in Da, and polydispersion (D) were measured by GPC–HPLC on a Viscotex instrument equipped with VE1121 pump, Rheodyne valve 100 μl and TDA (Triple Detector Array) 302 equipped with R.I., viscosimeter and 90° light scattering systems. Two 300 mm× 7.8 mm TSK GMPWXL Viscotek columns were used, with 0.1 M $NaNO_3$ as eluent (flow 0.6 ml/min). Samples were dissolved in the eluent solution at the concentration of 15 mg/ml. [19].

2.3. Protease activity

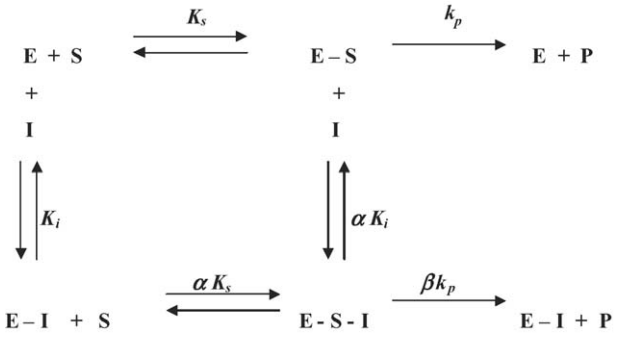
The enzymatic activity of EL and CatG was evaluated from the extent of hydrolysis of the peptide substrate MeO-Suc-Ala-Ala-Pro-Val-*p*-NO_2-anilide and Suc-Ala-Ala-Pro-Phe-pNA (Calbiochem), respectively. The release of *p*-NO_2-aniline was determined in 50 mM sodium hydrogen phosphate, pH 7.4, containing 0.1% (w/v) PEG 8000. The concentration of the aniline products as a function of time of incubation, was calculated spectrophotometrically with a Perkin Elmer Lambda 20 spectrophotometer, using a molar extinction of 8800 $M^{-1}\,cm^{-1}$ at 410 nm.

The experimentally determined K_M are 70 μM and 7.1 mM for EL and CatG, respectively.

To monitor inhibition of protease activity by LMWHs, solutions of proteases and substrate (25 nM and 60 μM for EL and 100 nM and 350 μM for CatG, respectively) were incubated in the presence of increasing polysaccharide concentrations. IC_{50} is the heparin concentration required to reduce the initial rate of substrate hydrolysis by 50%.

2.4. Data analysis

The kinetic data were elaborated in terms of the following general inhibitor-binding scheme [20]:



where E is the enzyme, S the substrate, I the inhibitor and P the product. E–S, E–I and E–S–I are the complexes formed between the various species. K_i and K_s are the equilibrium constants for E binding to I and to S. The parameter k_p represents the kinetic rate constant for the formation of P from ES. The parameter α is related to the competitive character of the inhibition process and the parameter β to reaction completeness.

The initial rate of substrate hydrolysis was evaluated from the initial slope of a plot absorption at 410 nm versus time. The initial rate in the presence of the inhibitor, v_i, is related to the initial rate in the absence of inhibitor (v_0) according to the following equation valid for a hyperbolic binding process [21,22]:

$$v_i = \frac{v_0}{2}(1-\beta)\left(\left(\frac{K_i + [I]}{[E]} - 1\right)^2 + 4\frac{K_i}{[E]}\right)^{0.5} + \left(\frac{1+\beta}{1-\beta} - \frac{K_i + [I]}{[E]}\right)$$

[I] and [E] represent the total heparin and protease concentrations, respectively. The parameter β is the ratio of the hydrolysis rate in the presence/absence of saturating concentrations of inhibitor and K_i the dissociation constant between heparin and EL.

3. Results

3.1. Preparation and characterization of low-molecular-weight heparins

Sulfation with sulfuric acid–chlorosulfonic acid of unfractionated heparin yields to the exhaustively sulfated LMWH preparation S0 [17]. Graded solvolytic desulfation of this compound, followed by restoring of the original N-sulfation at the glucosamine residues, yielded samples (S1–S3), with an average sulfation degree similar to that of the original heparin. Moreover, the distribution of sulfate groups in the various positions of the uronic acid and glucosamine residues (sulfation pattern) are different from those characterizing heparin samples. In fact, S0–S3 are significantly richer in 3-*O*-sulfated glucosamine residues [15]. The molecular mass (Mw) and the molecular mass distribution (D) of the various LMWHs were essentially the same (Table 1).

As shown in previous studies [15,16] on similar chemically modified heparin derivatives, severe signal overlap in mono-

dimensional NMR spectra did not permit quantification of sulfate substitution at positions 2 and 3 of iduronic acid residues. Recently, integration of heteronuclear single quantum coherence (HSQC-NMR) spectra [18] was validated for determination of compositional analysis of heparins and applied to chemically modified glycosaminoglycans samples. This method was used for the characterization of the LMW-heparin derivatives used in this work. Assignments of the principal signals were confirmed by COSY and TOCSY experiments and the ratio between the volumes of the HSQC-NMR signals were measured. Fig. 2 shows the anomeric region of the HSQC-NMR spectra of samples S0–S3, with signals assignments. Analytical data of the reference LMWH (N) and of chemically modified LMW-heparins S0–S3 are summarized in Table 1 as the relative molar percentages of 6-OSO_3, 3-OSO_3 and NSO_3 groups for glucosamine residues and of non-sulfated, 2,3-OSO_3, 2-OSO_3, and 3-OSO_3 groups for iduronic acid residues. Signals corresponding to other minor components, such as those belonging to non-sulfated glucuronic acid residues, can be detected by HSQC-NMR spectra in samples S1–S3, but are not accurately quantified. In Table 1, only the contents of iduronic acid residues are indicated together with the average sulfation degrees (SD), calculated by addition of the molar fraction of sulfate groups per disaccharidic unit. This could be overestimated to about 7–10%.

It can be observed that, in glucosamine residues, position 6 is more sensitive to desulfation in comparison to position 3. The oversulfated derivative S0 showed a large extent of 2,3-disulfation on the iduronic acid. However, during the desulfation step the 3-OSO_3 groups are more easily released than the 2-OSO_3 groups and the final products S1 and S2 are 2-*O*-sulfated to an extent comparable to non-chemically modified LMW-heparin N. N and S3 have similar charge density (SD) but different degree of sulfation at positions 6 and 3 of glucosamine residues. S1 and S2 have similar charge density and statistical sulfate distribution, S2 has about 15% less of 6SO_3 glucosamine.

3.2. *Inhibition of elastase activity*

To evaluate the kinetics of EL enzymatic activity in the presence/absence of heparins, we performed spectrophotometric measurements. We determined the extent of cleavage of an appropriate chromogenic peptide substrate from the production of the 4-nitroaniline chromophore.

Several types of measurements were performed using different substrate and heparins concentrations. An example is reported in Fig. 3, where inhibition of protease activity is occurring upon addition of increasing amounts of heparin S3.

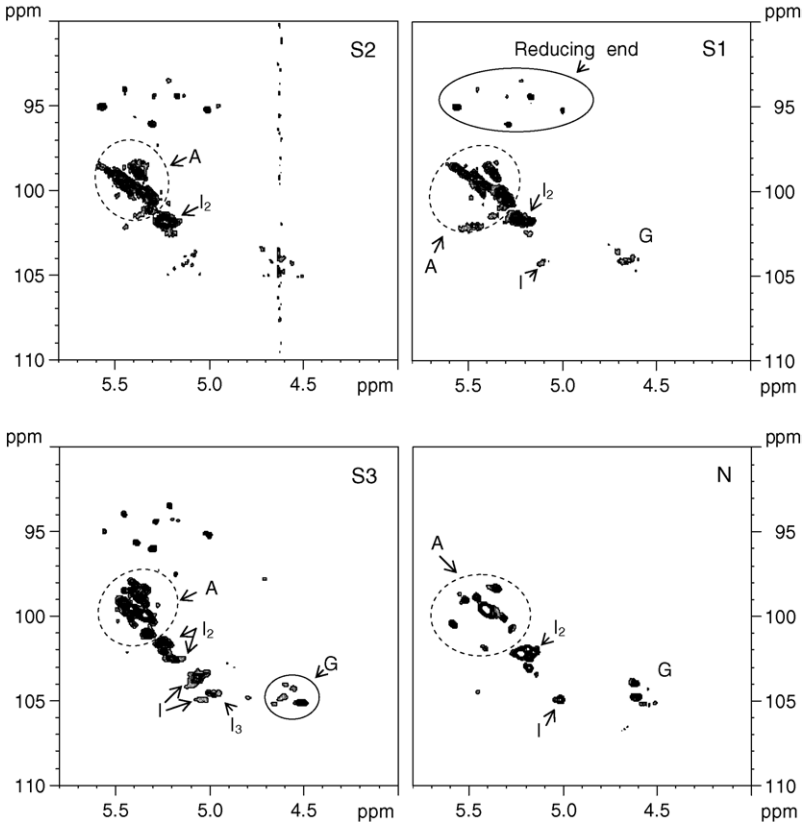


Fig. 2 – Partial HSQC-NMR spectra of LMW-heparin (N) and chemically modified heparins (S1–S3) containing the signals used for quantification of the uronic acid residues: anomeric signals of iduronic acid (I), iduronic acid 2-*O*-sulfated (I_2) iduronic acid 3-*O*-sulfated (I_3), glucuronic acid (G). Dashed circles A outlines the anomeric protons of glucosamine residues, not used for quantification purposes.

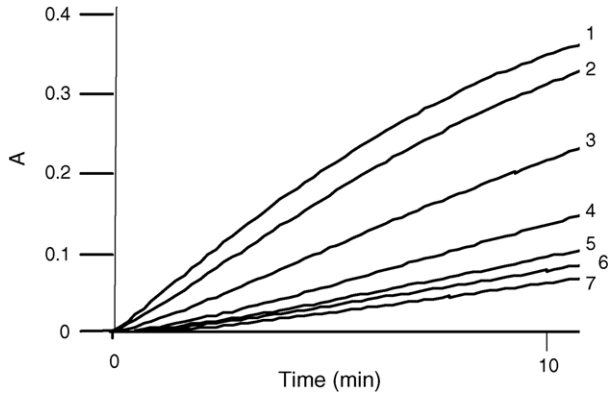


Fig. 3 – Time-dependent production of p-NO_2-aniline resulting from hydrolysis (50 mM phosphate buffer, pH 7.4 at 25 °C) of the substrate MeO-Suc-Ala-Ala-Pro-Val-p-NO_2-anilide (60 μM) catalyzed by human elastase (25 nM) in the presence of the following heparin S3 concentrations: curve 1, 0 nM; curve 2, 8 nM; curve 3, 25 nM; curve 4, 35 nM; curve 5, 100 nM; curve 6, 250 nM; curve 7, 500 nM.

Heparin binds EL and behaves as a hyperbolic non-competitive inhibitor (the reciprocal of the inhibition rate is an hyperbolic function of the inhibitor concentration) [7,23].

The catalytic inhibition properties determined for all test heparins confirmed the same mode of interaction. A representative example is presented in Fig. 4 where a progressive reduction in the initial rate of substrate hydrolysis produced by the protease occurs upon increasing polysaccharide concentration. These experimental data were analyzed as reported [19] and the evaluated K_i values are reported in Table 2. The parameter α was set equal to 1, since in our experimental conditions (phosphate buffer) the inhibitory mechanism is non-competitive [24]. The experimentally calculated β parameter was 0.11 for all test GAGs, in agreement with literature data [12].

To avoid mass effects due to different sulfation degree of the test samples, we analyzed the binding process evaluating the polysaccharide concentration in terms of disaccharide units.

All K_i values are in the nM range: the lowest values are found for heparins S0 and S3. Derivatives S1 and S2 behave similarly to the natural product N.

A correlation of K_i with SD (Fig. 5) showed a non-linear relationship between these two parameters. In fact, the most favourable interactions are observed either at very low (sample S3) or at very high (sample S0) SD.

Table 2 – Values for the dissociation constants (K_i) of the semisynthetic heparin–elastase complex in 50 mM phosphate buffer, pH 7.4, 25 °C

Heparin	β	K_i (nM)
S0	0.11	30 ± 10
S1	0.11	150 ± 40
S2	0.11	100 ± 20
S3	0.11	70 ± 10
N	0.11	120 ± 10

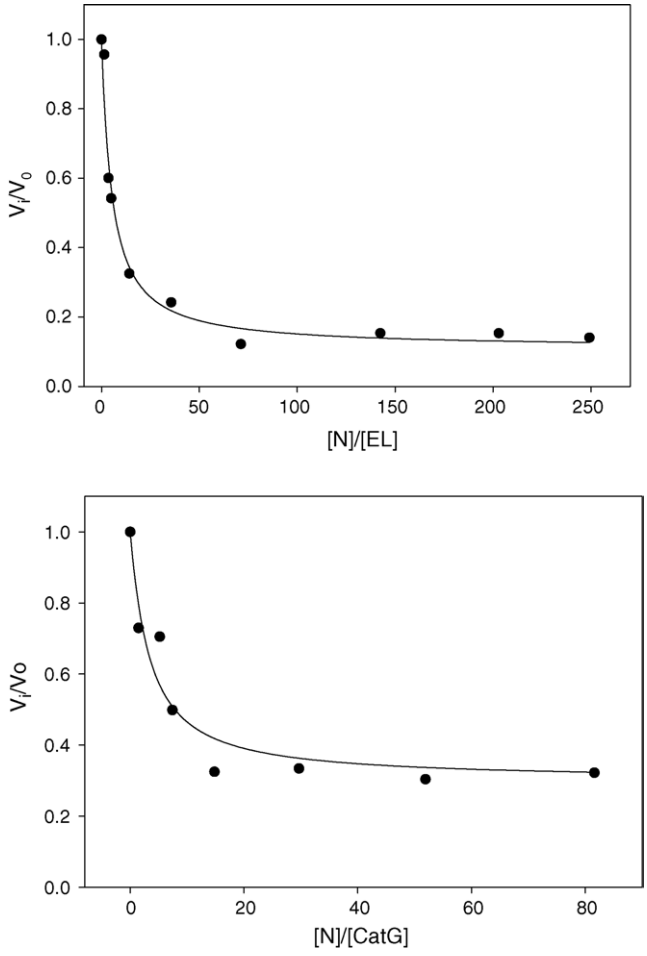


Fig. 4 – Ratio of initial hydrolysis rate in the presence (v_i) and in the absence (v_0) of heparin N as a function of the ratio heparin concentration/protease concentration. Experimental conditions as in Fig. 3. Reported lines were obtained by fitting the experimental data on the equation given in the data analysis section.

In addition to the binding properties, the IC_{50} values for each heparin were estimated and a similar trend was observed.

3.3. *Inhibition of Cathepsin G activity*

The enzymatic properties of CatG in the presence/absence of test heparins have been evaluated according to the protocols followed for EL. The results in terms of β and K_i are summarized in Table 3. The major difference between the two proteases rests in the markedly dissimilar K_m values observed with the selected substrate. In particular, the experimentally determined K_m for CatG is in the millimolar range. Thus, higher enzyme concentrations were used in comparison to EL to properly monitor enzyme cleavage activity. Nevertheless, the two proteases share a common binding mechanism for heparin interaction. Hence, complex formation parameters were evaluated according to the hyperbolic binding model. In our experimental conditions, a non-competitive inhibition mechanism was confirmed, with

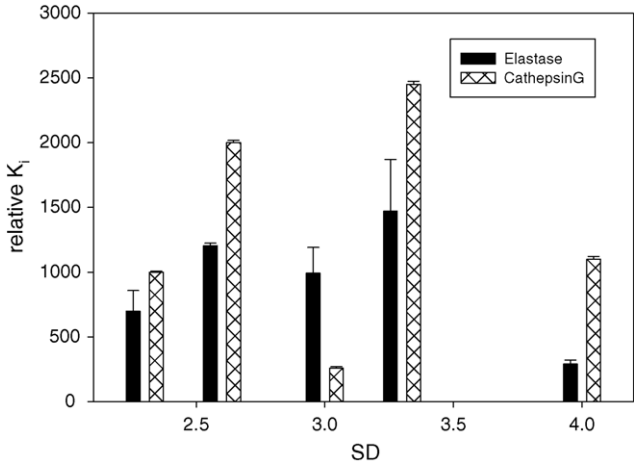


Fig. 5 – Plot of relative K_i versus sulfation degree (SD) for the tested heparin samples. Black and white bars refer to EL and CatG respectively. Bars represents K_i (nM) × 10 for EL and K_i (nM) for CatG.

an α factor close to 1 [23]. In contrast to EL, the experimentally calculated β parameter was found to depend upon the nature of the heparin sample tested, S1/N and S3 showing the lowest and highest values, respectively.

K_i values are not linearly related to the SD (Fig. 5) and they follow the ranking order S2 < S3 = S0 < N ≈ S1. Finally, K_i are linearly related to the calculated IC_{50}.

4. Discussion

We have used a number of semi-synthetic LMW-heparin samples obtained by desulfation of a single LMW-supersulfated compound (S0) to investigate heparin binding to EL and CatG. This approach is quite useful to properly assess the effects of sulfation on protein recognition without interference from other variables, such as molecular weight, molecular weight distribution and saccharide sequence.

Obviously, the knowledge of monosaccharidic composition and sulfation pattern of natural and chemically modified GAGs is important for deriving structure–activity relationships. Here, we solved this problem using proton-carbon correlation 2D-NMR experiments. In fact, it was recently demonstrated that heteronuclear single quantum coherence (HSQC-NMR) spectroscopy can be used for the quantitative analysis of both major and minor monosaccharide constituents of heparins and other GAGs [18]. On this basis, an accurate analysis devoted to unveil the fine details of GAG-target recognition process could be performed with the test samples.

As far as heparin–EL interactions are concerned, it is worth noting that the residual activity of the enzyme saturated with the inhibitor is essentially the same irrespective of the heparin used. This suggests a common mechanism of enzyme inhibition. However, a modulation in binding affinity is observed upon changing SD, the best binders being the over-sulfated S0 and the under-sulfated S3. While it is easily understandable why, in the presence of strong electrostatic interactions between EL and GAG, complex formation and subsequent modulation of the catalytic activity are very efficient [12,13], the reasons for the prominent effects seen in the presence of the low-sulfated sample S3 are not immediately evident. As a possible explanation, other non-charged interactions (e.g. hydrogen bonding) are likely affecting complex formation, thus, overcoming the loss of electrostatic binding free energy on decreasing SD. In addition, the low SD renders sample S3 more flexible as it does not experience the strong electrostatic repulsions characteristic of highly sulfated heparins. This could favour complex formation through a better ''induced fit'' when the GAG approaches the protein.

This conclusion is in agreement with previously reported data on CatG and suggests that the saccharide–proteases interaction is not strictly dependent on charge density [14].

Similar results were obtained with CatG, consistent with the close structural similarity of the two test proteases. However, two main differences emerged. First of all, the test heparins induced the formation of a protein–polysaccharide complex exhibiting variable residual activity in the presence of CatG but not of EL. The β parameter was always at least three-fold higher for CatG than for EL. These findings suggest the formation of CatG–heparin complexes distinct from those involving EL. Indeed, comparison of the charge distribution of the two protease surfaces showed a marked difference in the region surrounding the channel leading to the catalytic site [25,26]. Thus, the charged polysaccharide can likely bind different sites on the protein surface producing differential effects at the active sites in terms of conformation or access availability.

Second, with CatG a substantially different inhibitory effect is produced by samples S1 and S2, in spite of their comparable SD value. The main difference between these two polysaccharides rests on the extent of glucosamine sulfation at position 6 (52% for S1 versus 36% for S2). Interestingly, the level of sulfation at the above position is known to be crucial for polysaccharide recognition of different proteins like ATIII or fibroblast growth factors [27,28]. This property likely rests on the establishment of specific ionic bonds between the two counterparts [29,30]. Hence, the different charge distribution presented by EL and CatG can explain the remarkable differences in S1/S2 binding process in spite of a similar overall charge density.

In conclusion, our results demonstrate that independently of their sulfation degree, all tested LMWHs act on both EL and CatG by a mixed hyperbolic inhibition mechanism. Additionally, it is not just sufficient to provide a high degree of sulfation (charge density) to stimulate an effective biological interaction

Table 3 – Values for the residual activity (β) of the semisynthetic heparin–Catepsin G complex in 50 mM phosphate buffer, pH 7.4, 25 °C

Heparin	β	K_i (nM)
S0	0.38 ± 0.03	820 ± 20
S1	0.30 ± 0.02	2130 ± 30
S2	0.38 ± 0.03	30 ± 10
S3	0.45 ± 0.01	810 ± 10
N	0.30 ± 0.04	1720 ± 20

(and response) by GAGs. Indeed, it is necessary to attain a delicate balance among a number of factors including, besides extent and location of sulfate groups, non-charged interactions, chain conformation and structural flexibility. The development of a chemistry that allows to change the extent of sulfation at selected positions of a polysaccharide chain will surely help in addressing this interesting issue.

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