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Heparin cofactor II is degraded by heparan sulfate and dextran sulfate

Akio Saito

Division of Basic Medicine, Faculty of Medicine, Kinki University, 377-2 Ohno-Higashi, Osaka-Sayama, Osaka 589-8511, Japan

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ABSTRACT

Heparan sulfate normally binds to heparin cofactor II and modulates the coagulation pathway by inhibiting thrombin. However, when human heparin cofactor II was incubated with heparan sulfate, heparin cofactor II became degraded. Other glycosaminoglycans were tested, including hyaluronic acid, chondroitin sulfates, dermatan sulfate, and heparin, but only dextran sulfate also degraded heparin cofactor II. Pretreatment of heparan sulfate with heparinase reduced its heparin cofactor II-degrading activity. Heparan sulfate and dextran sulfate diminished the thrombin inhibitory activity of heparin cofactor II. Other serpins, including antithrombin III and pigment epithelium-derived factor, were also degraded by heparan sulfate. This is the first evidence of acidic polysaccharides exhibiting protein-degrading activity without the aid of other proteins.

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1. Introduction

Heparan sulfate (HS) is a linear polysaccharide composed of N-acetylated or N-sulfonated glucosamine units and uronic acids; it is present in all animal tissues and occurs as a proteoglycan [1,2]. HS binds to diverse proteins and regulates a wide variety of biological activities such as developmental processes, angiogenesis and blood coagulation [3–5]. Dextran sulfate is a semi-synthetic, sulfated polysaccharide composed of major alpha 1–6 linkages and a minor alpha 1–3 linkage of sulfated glucose; the latter constitutes a branched structure [6]. Dextran sulfate is used to selectively to precipitate lipoproteins and accelerate the hybridization of labeled probes in Southern hybridization [7,8]. Because of its heparin-like activity, dextran sulfate is used as an anticoagulant reagent as well as to reduce triacylglycerol in plasma. During the course of experiments exploring the interactions of glycosaminoglycans (GAGs) with plasma proteins, it was found that HS degraded heparin cofactor II (HCII). Other GAGs, including hyaluronic acid, chondroitin sulfate A and E, dermatan sulfate and heparin, did not degrade HCII. However, dextran sulfate also exhibited HCII-degrading activity. HS also degraded other plasma serpins, such as antithrombin III (ATIII) and pigment epithelium-derived factor (PEDF).

2. Materials and methods

2.1. Materials

The following GAGs were from Seikagaku Corporation (Tokyo, Japan): HS from bovine kidney; chondroitin sulfate A from whale cartilage; chondroitin sulfate E from squid cartilage; dermatan sulfate (chondroitin sulfate B) from hog skin; and hyaluronic acid from human umbilical cord. HS samples from porcine mucosa were from Iduron Ltd. (Manchester, UK) or Sigma–Aldrich (St. Louis, MO, USA). Dextran sulfate (MW 500,000) and dextran (MW 200,000) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Human alpha-thrombin, human ATIII and sheep anti-human HCII were from Haematologic Technologies Inc. (Essex Junction, VT, USA). Rabbit anti-human ATIII was from Biogenesis Ltd. (Poole, UK). Donkey anti-sheep IgG-alkaline phosphatase, heparinase II from *Flavobacterium heparinum* and Amidosulfobetain-14 (ASB-14) were from Sigma–Aldrich. Goat anti-rabbit IgG-alkaline phosphatase was from Cell Signaling Technology (Danvers, MA, USA). Human HCII and Immobilon P were from EMD Millipore Corporation (Bedford, MA, USA). PEDF and protein C inhibitor were purified from human plasma as described previously [9,10]. The silver staining kit was from Atto Corporation (Tokyo, Japan). Chromogenic substrate S-2366 (L-pyroglutamyl-L-prolyl-L-

Abbreviations: HS, heparan sulfate; HCII, heparin cofactor II; ATIII, antithrombin III; PEDF, pigment epithelium-derived factor; GAG, glycosaminoglycan; ASB-14, amidosulfobetain-14; TB 8.5, 20 mM Tris-HCl buffer pH 8.5.

E-mail address: asaito@med.kindai.ac.jp.

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arginine-*p*-nitroanilide) was purchased from Chromogenix Instrumentation Laboratory SpA (Milan, Italy).

2.2. Incubation of HCII with GAGs and dextran sulfate

To 5 ng of HCII, in 10 μL of 20 mM Tris-HCl pH 8.5 buffer (TB 8.5) containing 0.02% of ASB-14, was added various amount of GAGs and dextran sulfate, and the mixture then incubated at 55 °C. ASB-14, a twitterionic detergent, did not affect the HCII-degrading activity of HS, but enhanced the intensity of Western blot bands more than 10-fold. Three different HS samples were employed; however, HS from bovine kidney was generally used, except in Fig. 2 as specified.

2.3. Western blotting

HCII was separated by SDS-PAGE (10% gel) under reducing conditions and blotted to a PVDF membrane using a semi-dry blotting apparatus. The membrane was blocked with 3% BSA in 5 mL of TBS for 15 min, treated with anti-HCII (1/5000) in 5 mL TBS for 3 h at room temperature, washed three times with 0.5% Tween 20 in 25 mL TBS, and then allowed to react with alkaline phosphatase-conjugated secondary antibody (1/10,000) in 5 mL TBS for 1.5 h. Bands on the membrane were visualized with NBT/BCIP (Sigma). For ATIII, Western blots were treated with anti-ATIII and blots performed as above.

2.4. Inhibition of thrombin by HCII and ATIII

HCII (0.5 μg) or ATIII (0.2 μg) was incubated in the presence and absence of HS (1 μg) or dextran sulfate (5 ng) for 1 h at 55 °C in 10 μL of TB 8.5. The mixture was diluted with 500 μL of TBS containing 50 ng of thrombin and 1 μg of heparin, rested for 5 min, and then incubated with 5 μL of 20 mM S-2366 at room temperature. Absorbances at 405 nm were measured after 10, 20 and 30 min.

3. Results

3.1. Degradation of HCII and ATIII by HS

HCII is a member of the serpin family and modulates the blood coagulation cascade by inhibiting thrombin [11–14]. HCII binds to dermatan sulfate, in addition to HS and heparin, and the inhibition of thrombin is greatly enhanced by these GAGs [15–17]. ATIII is another serpin protein and, with the aid of HS and heparin, inhibits several proteases during coagulation [17–19]. When HCII or ATIII were incubated at 55 °C with various GAGs, including hyaluronic acid, chondroitin sulfate A or E, dermatan sulfate, HS or heparin, only HS completely degraded these proteins as shown in Fig. 1A and B, lane 6.

The incubation temperature used was high compared to that of a conventional enzyme reaction, but both HCII and ATIII were stable at this temperature for 20 h (Fig. 1A and B, lane 1, respectively), as judged by Western blotting. These incubation conditions reduced the possibility of contamination by proteases as most proteases would not withstand such a high temperature. When the reaction temperature was elevated to 75 °C, the HCII or ATIII protein band on the blot became vague.

Because HS can bind to HCII or ATIII, HS interaction may lead to the cleavage of these proteins. It is interesting that heparin did not degrade HCII or ATIII, in spite of heparin's ability to bind to these proteins.

Similar experiments were carried out with other serpin molecules, and it was consequently found that PEDF, a non-inhibitory serpin, was also degraded by HS; however, protein C inhibitor and antitrypsin were not degraded by any GAGs (data not shown).

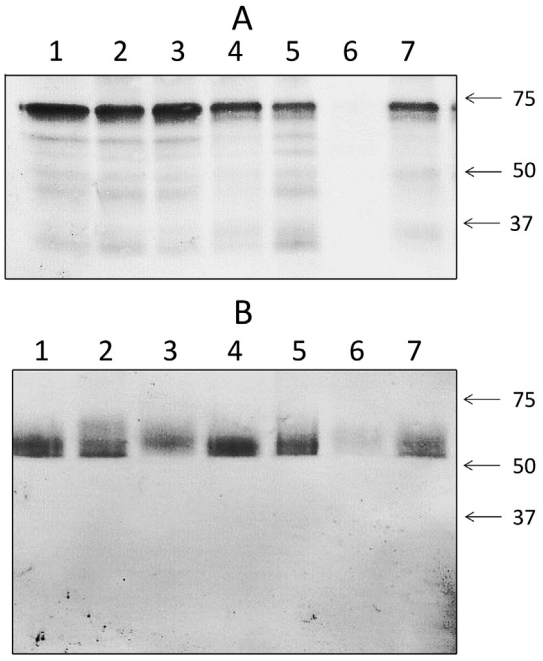

Fig. 1. Degradation of HCII and ATIII by GAGs. Five μg of various GAGs were mixed with 5 ng of HCII (panel A) or 50 ng of ATIII (panel B) in 10 μL of TB 8.5, and incubated at 55 °C for 20 h. ASB-14 (0.02%) was included in HCII samples only. Samples were subjected to 10% SDS-PAGE and Western blotting. Lane 1, HCII or ATIII only; lane 2, hyaluronic acid; lane 3, chondroitin sulfate A; lane 4, dermatan sulfate; lane 5, chondroitin sulfate E; lane 6, HS; lane 7, heparin. Molecular weights (kDa) are shown to the right.

3.2. Dose-dependent degradation of HCII by HS and dextran sulfate

The structure of GAGs is not homologous in general, and HS has pronounced diversity in molecular size and its extent of sulfation [3]. Thus, the degradation of HCII by HS was investigated using HS extracted from different sources and at varying concentrations. HS derived from bovine kidney and two different samples derived from porcine intestinal mucosa were incubated with HCII. Most of the HCII was degraded by 20 μg/mL of bovine kidney HS, but 100 μg/mL of the two porcine HS samples was required before degradation of HCII was noted (Fig. 2A, lanes 3, 6 and 8, respectively). Hyaluronic acid was not effective (Fig. 2A, lanes 9 and 10).

Dextran sulphate degraded HCII at lower concentrations than HS since the effective concentrations of dextran sulfate and HS were 0.1–0.5 (Fig. 2A, lanes 12 and 13) and 20–100 μg/mL (Fig. 2A, lanes 3 and 4), respectively. The dose-dependent profile of HCII degradation by dextran sulfate was interesting, since both the lowest and highest concentrations showed no degradation activity (Fig. 2A, lanes 11, 14 and 15). Different molecular sizes of dextran sulfate, from 5,000 to 500,000, were examined for their HCII degradation abilities; it was found that dextran sulfate of a higher molecular weight was more effective (data not shown). Because dextran was not effective in the degradation of HCII (data not shown), this suggests that sulfation of the dextran molecule could be a prerequisite for its degradation activity.

Since HS and dextran sulfate individually degraded HCII, any potential additive effect of these molecules was investigated. When 5 ng of dextran sulfate was mixed with 50 ng of HS in a 10 μL solution, HCII became degraded (Fig. 2B, lane 3). But 500 ng of dextran sulfate, even in the presence of 50 ng of HS, suppressed the degradation (lane 4 in Fig. 2B). On the other hand, 500 ng of HS was sufficient to degrade HCII in the presence of 0.5 ng of dextran sulfate (Fig. 2B, lane 7).

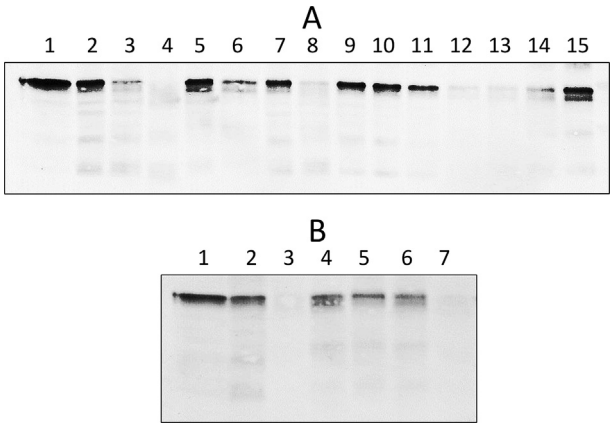

Fig. 2. Effect of HS and dextran sulfate on the degradation of HCII. Panel A, various sources and concentrations of HS and dextran sulfate. Increasing amounts of HS (lanes 2–8), hyaluronic acid (lanes 9 and 10) and dextran sulfate (lanes 11–15) were incubated with 5 ng of HCII with 0.02% ASB-14 in 10 μL of TB 8.5 at 55 °C for 20 h and subjected to 10% SDS-PAGE and Western blotting. Lane 1, HCII only; lanes 2–4, bovine kidney HS; lane 2, 0.05 μg; lane 3, 0.2 μg; lane 4, 1 μg; lanes 5–6, porcine mucosa HS from Iduron: lane 5, 0.2 μg; lane 6, 1 μg; lanes 7–8, porcine mucosa HS from Sigma: lane 7, 0.2 μg; lane 8, 1 μg; lane 9, 0.2 μg; lane 10, 1 μg; lane 11, 0.2 ng; lane 12, 1 ng; lane 13, 5 ng; lane 14, 25 ng; lane 15, 125 ng. Panel B, additive effect of HS and dextran sulfate on the degradation of HCII. Lane 1, HCII only; lane 2, HS 50 ng; lane 3, HS 50 ng and dextran sulfate 5 ng: lane 4, HS 50 ng and dextran sulfate 500 ng; lane 5, dextran sulfate 0.5 ng; lane 6, dextran sulfate 0.5 ng and HS 50 ng; lane 7, dextran sulfate 0.5 ng and HS 500 ng.

3.3. Confirmation of HCII degradation

Besides Western blotting, the degradation of HCII by HS and dextran sulfate was also investigated by SDS-PAGE and silver staining. When HCII was treated with HS and dextran sulfate, HCII bands, as detected by Western blot and silver staining (Fig. 3, lanes 1 and 3, respectively) completely disappeared (lanes 2 and 4), indicating that HCII was degraded into small pieces that were not resolved by SDS-PAGE. HS and dextran sulfate did not cleave specific sites of HCII but decomposed it at random (data not shown).

3.4. Effect of heparinase on HS

In order to clarify that the degradation of HCII was not initiated by a contaminating protease, HS was pretreated with heparinase II prior to its incubation with HCII. Heparinase II degrades both heparin and HS by cleaving the bond between N-sulfo/N-acetyl glucosamine and glucuronic/iduronic acid. When HS was treated with 0.1 unit of heparinase II, degradation of HCII (Fig. 4, lane 2) was prevented as shown in lane 4 of Fig. 4. These data clearly show that

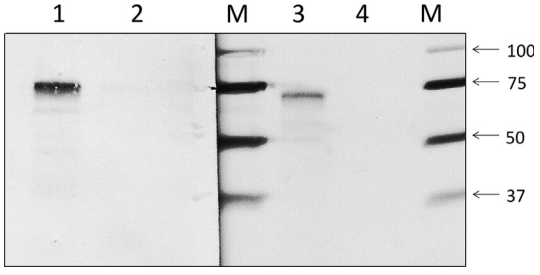

Fig. 3. Degradation of HCII by HS and dextran sulfate. HCII (1 μg) was incubated with or without 5 μg of HS and 15 ng of dextran sulfate in 15 μL of TB 8.5 containing 0.02% ASB-14 at 55 °C for 20 h. A 0.5 μL aliquot was removed for Western blotting (lanes 1 and 2) and the remaining samples were stained with silver (lanes 3 and 4) after 10% SDS-PAGE. Lanes 1 and 3, intact HCII; lanes 2 and 4, HS/dextran sulfate-treated samples. M indicates marker protein lanes and the molecular weights (kDa) are on the right.

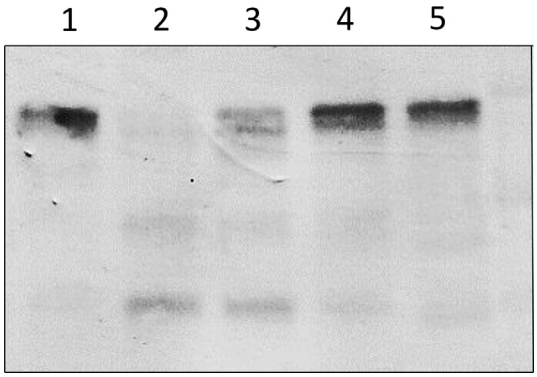

Fig. 4. Effect of heparinase treatment on HS. HS (0.5 μg) was incubated with 0.01 unit (lane 3) or 0.1 unit (lane 4) of heparinase II at 37 °C for 16 h in 10 μL of TB 8.5, then further incubated at 55 °C for 24 h with 5 ng of HCII and 0.02% ASB-14. Lane 2, HS without heparinase II treatment; lanes 1 and 5, HCII only.

HCII was specifically degraded by HS activity and not by contaminating proteases.

3.5. Inhibition of thrombin by HCII and ATIII

Thrombin is a key enzyme in the coagulation cascade and is inhibited by serpin molecules, such as ATIII, HCII and protein C inhibitor [13,14,16,17]. When HCII or ATIII is degraded by HS, the thrombin inhibitory activity of these serpins is postulated to disappear. A thrombin inhibitory assay, using a chromogenic substrate, was used to confirm the degradation of HCII and ATIII by the action of HS and dextran sulfate. As shown in Table 1, thrombin activity after treatment with HCII and ATIII, which had been incubated at 55 °C, decreased to 32% and 15% of untreated control, respectively. Thrombin activity was restored to that of intact thrombin when HCII and ATIII were pretreated with HS and dextran sulfate. This indicated that the serpin molecules degraded or denatured. HCII and ATIII, which, in turn, lost their thrombin inhibitory activity. When HS and dextran sulfate were included, the amidolytic activity of thrombin increased by 10%–30% under these assay conditions.

4. Discussion

The basic domain structures of HS and heparin are similar; however, heparin shows highly N-sulfated glucosamines, while HS has more varied N-substitution [3]. HS and heparin enhance the anticoagulant activity of plasma serpins by binding to HCII, ATIII and protein C inhibitor [13,18,19]. However, HS, but not heparin, degraded serpin molecules as shown in Fig. 1. Dermatan sulfate, as well as HS and heparin, binds to HCII and inhibits thrombin but it did not degrade HCII and ATIII. We have previously shown that HS was similar to dermatan sulfate rather than heparin with respect to its interaction with plasma proteins [20]. We have also previously reported that dermatan sulfate bound comparable amounts of plasma proteins to HS, and both dermatan sulfate and HS activated plasma kallikrein [20,21].

Table 1
Thrombin inhibitory activity by HCII and ATIII.

HCII	–	+	+	–	–	–
ATIII	–	–	–	+	+	–
HS/Dextran sulfate	–	–	+	–	+	+
Relative activity (%)	100	32	102	15	98	112

The remaining amidase activity of thrombin was measured with S-2366 as described in the text.

Dextran sulfate is structurally different from HS, but both molecules exhibited HCII-degrading activity as shown in Fig. 2. Yamagishi et al. measured the second-order rate constant of the HCII-thrombin reaction in the presence of dextran sulfate samples having different sulfur contents; the highly sulfated sample (18%, MW 37,000) exhibited the highest value, at 0.1–5 μg/mL concentrations, and the value decreased at higher concentrations [14]. Colwell et al. reported 0.5–1000 μg/mL of dextran sulfate (MW 500,000) was effective for thrombin inhibition by HCII, with inhibition also abolished at higher concentrations (17). Similarly, as shown in Fig. 2, degradation of HS was observed with 0.1–0.5 μg/mL of dextran sulfate, while excess dextran sulfate interfered with the degradation. Studies by de Raucout et al. investigated the anticoagulant activity of dextran derivatives, as well as heparin and dextran sulfate, and showed that the desulfation of dextran derivatives increased thrombin time several hundredfold [22]. They also measured the kinetic constants of the thrombin-HCII interaction and found that dextran sulfate showed a comparable value to heparin.

Of major concern during experiments was whether HCII degradation was induced unequivocally by acidic carbohydrates, and not by contaminating proteases. However, contamination by proteases was ruled out for the following reasons: Firstly, the incubation temperature of 55 °C used in these experiments was unfavorable for conventional protease enzyme reactions. Degradation of HCII was observed at 37 °C, but the reaction proceeded slowly (data not shown). Secondly, three different sources of HS exhibited HCII degradation activity, although each activity was somewhat different. Thirdly, only dextran sulfate within a narrow range of concentrations showed degradation activity. Additionally, if a protease contaminant was present in dextran sulfate samples, a high amount of dextran sulfate would exhibit high proteolytic activity, which was definitely not noted. Moreover, five different samples of dextran sulfate of different molecular weights were examined and all found to degrade HCII. Finally, degradation activity was lost by heparinase pretreatment of HS, while amidolytic activity of thrombin was restored by treatment of HCII with HS and dextran sulfate.

Both HS and dextran sulfate bind to HCII within 1–5 min to exert anticoagulant activity [14,16]. In contrast, the degradation of HCII by HS required more than 1 h at 55 °C (data not shown). Therefore, it is postulated that HS acts as an anticoagulant in the initial stages of the reaction, but becomes procoagulant in the long run by degrading HCII. The biological meaning of the degradation of serpin molecules by HS and dextran sulfate is not presently clear. The elucidation of the initial cleavage site of HCII by HS and dextran sulfate would be a prerequisite to revealing the reaction mechanism involved as well as its biological significance.

Conflict of interest

The author declares no conflict of interest.

References

[1] R.V. Iozzo, Matrix proteoglycans: from molecular design to cellular function, Annu. Rev. Biochem. 67 (1998) 609–652.
[2] J.D. Esko, S.B. Selleck, Order out of chaos: assembly of ligand binding sites in heparan sulfate, Annu. Rev. Biochem. 71 (2002) 435–471.
[3] U. Lindahl, M. Kusche-Gullberg, L. Kjellen, Regulated diversity of heparan sulfate, J. Biol. Chem. 273 (1998) 24979–24982.
[4] J.R. Bishop, M. Schuksz, J.D. Esko, Heparan sulfate proteoglycans fine-tune mammalian physiology, Nature 446 (2007) 1030–1037.
[5] M.A. Nugent, J. Zaia, J.L. Spencer, Heparan sulfate-protein binding specificity, Biochemistry (Mosc) 78 (2014) 726–735.
[6] J.C. Rankin, A. Jeanes, Evaluation of the periodate oxidation method for structural analysis of dextrans, J. Am. Chem. Soc. 76 (1954) 4435–4441.
[7] M. Burstein, H.R. Scholnick, R. Morfin, Rapid method for the isolation of lipoproteins from human serum by precipitation with polyanions, J. Lipid Res. 11 (1970) 583–595.
[8] G.M. Wahl, M. Stern, G.R. Stark, Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextran sulfate, Proc. Natl. Acad. Sci. 76 (1979) 3683–3687.
[9] A. Saito, Glycosaminoglycan and collagen facilitate the degradation of pigment epithelium-derived factor by chymotrypsin, Biosci. Biotechnol. Biochem. 77 (2013) 1628–1632.
[10] A. Saito, Diversity of human plasma protein C inhibitor, Thromb. Res. 130 (2012) 661–666.
[11] D.M. Tollefsen, M.K. Blank, Detection of a new heparin-dependent inhibitor of thrombin in human plasma, J. Clin. Invest. 68 (1981) 589–596.
[12] D.M. Tollefsen, D.W. Majerus, M.K. Blank, Heparin cofactor II: purification and properties of a heparin-dependent inhibitor of thrombin in human plasma, J. Biol. Chem. 257 (1982) 2162–2169.
[13] H.C. Whinna, F.C. Church, Interaction of thrombin with antithrombin, heparin cofactor II, and protein C inhibitor, J. Protein Chem. 12 (1993) 677–688.
[14] R. Yamagishi, M. Niwa, N. Sakuragawa, Thrombin inhibition activity of heparin cofactor II depends on the molecular weight and sulfate amount of dextran sulfate, Thromb. Res. 44 (1986) 347–354.
[15] D.M. Tollefsen, C.A. Pestka, W.J. Monafo, Activation of heparin cofactor II by dermatan sulfate, J. Biol. Chem. 258 (1983) 6713–6716.
[16] R. Yamagishi, M. Niwa, S. Kondo, N. Sakuragawa, T. Koide, Purification and biological property of heparin cofactor II: activation of heparin cofactor II and antithrombin III by dextran sulfate and various glycosaminoglycans, Thromb. Res. 36 (1984) 633–642.
[17] N.S. Colwell, M.J. Grupe, D.M. Tollefsen, Amino acid residues of heparin cofactor II required for stimulation of thrombin inhibition by sulphated polyanions, Biochim. Biophys. Acta 1431 (1999) 148–156.
[18] J.A. Huntington, Mechanisms of glycosaminoglycan activation of the serpins in hemostasis, J. Thromb. Haemost. 7 (2003) 1535–1549.
[19] C.M. Rein, U.R. Desai, F.C. Church, Serpin-glycosaminoglycan interactions, Methods Enzymol. 501 (2011) 105–137.
[20] A. Saito, H. Munakata, Analysis of plasma proteins that bind to glycosaminoglycans, Biochim. Biophys. Acta 1770 (2007) 241–246.
[21] A. Saito, Plasma kallikrein is activated on dermatan sulfate and cleaves factor H, Biochem. Biophys. Res. Commun. 370 (2008) 646–650.
[22] E. de Raucourt, S. Mauray, F. Chaubet, O. Maiga-Revel, M. Jozefowicz, A.M. Fischer, Anticoagulant activity of dextran derivatives, J. Biomed. Mater. Res. 41 (1998) 49–57.