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Development of both colorimetric and fluorescence heparinase activity assays using fondaparinux as substrate

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ABSTRACT

Because tumors and other diseases are characterized by increased heparanase levels, human heparanase is a promising drug target and diagnostic marker. Therefore, methods are needed to determine heparanase activity and to examine potential inhibitors. Because of substrate comparability, we used the bacterial enzyme heparinase II (heparinase) for the assay development. Usually the substrate of heparanase assays is heparan sulfate, which has several disadvantages. Because of that, we used fondaparinux, which is being cleaved by both heparanase and heparinase. Two concepts to detect its degradation were examined: measurement of anti-factor Xa activity of fondaparinux and its direct quantification with the fluorescent sensor polymer-H. Using fondaparinux as substrate, the anti-factor Xa assay was shsown to be appropriate to determine heparinase activity. The detection with polymer-H was easier and even faster to perform. Linearity was given with fondaparinux as well as heparan sulfate, and heparin as substrates, but fondaparinux turned out to be most suitable. By modifications (incubation time, fondaparinux concentration, and polymer-H concentration), the limit of quantification and the linear range can be adapted to the respective requirements. In conclusion, a simple, accurate, and robust heparinase assay was developed. It is suitable for heparinase quality control and testing heparinase inhibitors and could be adapted to heparanase.

Mammalian heparanase is an endoglycosidase that degrades heparan sulfate (HS)[1] proteoglycan on cell surfaces in basement membrane and extracellular matrix. Thereby, it affects various biological processes, including morphogenesis, angiogenesis, inflammation, tumor invasion, and metastasis [1,2].

Increased levels of human heparanase (heparanase) were found in sera and urine of metastatic tumor-bearing cancer patients as well as in patients with inflammatory diseases such as rheumatoid arthritis and diabetes [3–5]. The heparanase expression was found to correlate with the malignancy of a tumor, its ability to metastasize, and a poor prognosis of patients [1,6–8].

Because heparanase was shown to be involved in tumor invasion and metastasis in many different types of cancer [7–10], heparanase inhibitors are considered as promising candidates for tumor therapy. Therefore, a variety of inhibitory molecules have been developed, including antibodies [11], peptides [12], and modified heparin-like glycol-split heparins [13] as well as several other polyanionic molecules such as laminarin sulfate [14], suramin [15], and the phosphosulfomannan PI-88 [16]. Because PI-88 proved its efficacy as an antitumor agent in several clinical trials, analogues with improved properties are currently under investigation [17].

Moreover, heparanase has been proposed as a diagnostic marker in tumor diseases [18–20]. Consequently, there is a need for simple and rapid screening methods to determine heparanase activity.

The first heparanase activity assay was the method by Nakajima and coworkers [21] using solid-phase-bound radiolabeled HS as substrate. Since then, most of the developed assays still use the natural substrate HS [22–29]. But due to its high structural complexity and diversity, HS is hard to standardize and is also expensive. In addition, the substrate HS must be labeled to measure the extent of its degradation. Only a few newer methods use fluorescent [28,30] or biotin-based [21,26,29] markers instead of the conventional radiolabeled HS [23,25–29,31,32]. However, labeling with such markers is not only elaborate but also prone to batch-to-batch variability and may change the substrate properties.

Furthermore, to determine the HS degradation, the labeled HS degradation products must be separated from residual labeled HS. For this, gel chromatography is a frequently applied method

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[1] *Abbreviations used:* HS, heparan sulfate; ELISA, enzyme-linked immunosorbent assay; aFXa, anti-factor Xa; UV, ultraviolet; FPX, fondaparinux; AT, antithrombin; FXa, factor Xa; FI, fluorescence intensity; NaAc, sodium acetate; BSA, bovine serum albumin; CaAc, calcium acetate; UFH, unfractionated heparin; NaOH, sodium hydroxide; NaCl, sodium chloride; Tris, tris(hydroxymethyl) aminomethane; Na_2EDTA, dinatrium ethylenediaminetetraacetic acid; OD, optical density.

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[22,23,27,31,32]. It is also possible to immobilize the substrate on a surface [21,26,28,29] or to use ultrafiltration for separation [25]. Sophisticated but quite expensive is the assay by Enomoto and coworkers, which obviates the need for separation by using HS labeled with both biotin and europium cryptate and measuring the time-resolved fluorescence after the addition of fluorescence acceptor-labeled streptavidin [30]. Finally, the enzyme-linked immunosorbent assay (ELISA) technique was used not only to quantify heparanase protein but also to detect the activity by using two different antibodies [33].

Only 20 years after the first assay, Ahn and coworkers presented a colorimetric heparinase I assay using heparin instead of HS as substrate [34]. Heparinase I activity was measured by the loss of its anti-factor Xa (aFXa) activity in a chromogenic aFXa assay. Ahn and coworkers chose heparinase I as a commercially available model enzyme for human heparanase [34]. The bacterial heparinases I, II, and III have been used for many years as valuable tools for structure elucidation of heparin [35]. According to that main application, there are only few activity assays described in the literature [34,36–38]. The simplest method is based on the increasing ultraviolet (UV) absorption at 232 nm by the products generated by these lyases [35].

However, the heterogeneous and variable composition of heparin may represent a relevant disadvantage for its use as a substrate because its susceptibility to cleavage by heparanase/heparinases depends on its fine structure and it was found to contain even inhibitory motifs for heparanase [39].

An attractive alternative heparanase substrate seems to be fondaparinux (FPX), which is the synthetic chemically defined sulfated pentasaccharide corresponding to the antithrombin (AT)-binding site of heparin and has been approved as an antithrombotic drug in 2001 [40]. An octasaccharide containing the pentasaccharide sequence was shown to be cleaved by heparanase between the glucuronic acid and the 3-O-sulfated glucosamine moiety [39]. Bisio and coworkers developed an online ion pair reversed-phase high-performance liquid chromatographic/electrospray ionization mass spectrometric method that sensitively detects the degradation of FPX by heparanase, resulting in a di- and trisaccharide, and thus is suitable for screening potential heparanase inhibitors [41]. However, this technique needs an experienced staff and expensive laboratory equipment.

Hammond and colleagues presented a microplate assay for the heparanase inhibitor screening, which detects the degradation of FPX by the reducing capacity of the disaccharides measured in a chromogenic reaction [42]. A limitation of this assay is the interference with the detection reaction by any reducing or oxidizing compounds in the sample.

Due to the advantages of FPX as chemically defined heparanase substrate requiring no labeling, we aimed to develop a simple, rapid, and inexpensive microplate assay using FPX as substrate as well that is suitable to determine both the activity and the inhibition of heparanase.

Because FPX inhibits factor Xa (FXa) by binding to AT, thereby accelerating its inhibition by AT, the cleavage of FPX by heparanase as well as bacterial heparinases (I and II) should lead to a loss of its AT-mediated aFXa activity. Therefore, we initially evaluated the determination of the bacterial heparinase II (heparinase) activity by measuring the degradation of FPX in this chromogenic aFXa assay. The latter is a well-known and widely used assay for both quality control of heparins and clinical monitoring of low-molecular-weight heparins and FPX [43]. During our work, we found the patent of Petitou and Driguez [44], who presented novel aza-sugars as heparanase inhibitors that they had tested by using FPX and an aFXa assay as well. But in this way, we could demonstrate that the same assay principle is applicable for both bacterial heparinase and human heparanase.

Then a method detecting FPX by the fluorescent sensor polymer-H was elaborated. This was based on the observation that FPX increases the fluorescence intensity (FI) of polymer-H, a sensor molecule for the direct quantification of sulfated glycans [45] that is also applied in a purity assay to detect heparin falsifications [46–48]. In this way, the detection method for the heparinase activity could be advanced from a three-step to a single-step pipetting method. Here we describe both heparinase activity assays differing in their mode of FPX detection.

Materials and methods

Materials

Heparinase II (heparinase)

Heparinase II (*Flavobacterium heparinum*, H 6512, 155 Sigma units (SU)/mg conforming to 0.258 IU/mg) obtained from Sigma (St. Louis, MO, USA) was dissolved in sodium acetate buffer (pH 7.0, 100 mmol/L sodium acetate [NaAc] containing 0.01% bovine serum albumin [BSA]). The stock solution (100 mIU/ml) was further diluted with sodium acetate buffer containing 10 mmol/L calcium acetate (CaAc) instead of BSA.

Heparinase substrates

Fondaparinux sodium (Arixtra (R), relative molecular mass (M_r) = 1728) was a kind donation from Glaxo Smith Kline (Notre Dame de Bondville, France).

Heparan sulfate (sodium salt from bovine kidney) was purchased from Sigma.

Unfractionated heparin (UFH, from porcine intestinal mucosa) was a kind donation from Novartis (Nürnberg, Germany).

Fluorescent sensor: Polymer-H

Polymer-H, a sensor for sulfated glycans, was synthesized as described previously [49]. In short, three comonomer units—methacrylamide derivatives of (i) dansyl, (ii) *ortho*-aminomethylphenylboronic acid, and (iii) ethylammonium moieties—were subjected to conventional radical copolymerization, which resulted in polymer-H containing the three functional monomers in the ratios 0.3 (i), 1.0 (ii), and 2.0 (iii). Its hydrodynamic volume (MW_{HD}) amounts to 116,000 as determined by aqueous gel filtration chromatography (defined polyethylene glycols as standards) [49].

Assay materials for chromogenic aFXa assay

Bovine FXa (assay solution 3.55 nkat/ml, cat. no. 41218), human AT (assay solution 0.1 IU/ml, cat. no. 41220), and the chromogenic substrate S 2222 (assay solution 1.0 mmol/L, cat. no. 41201) all were purchased from Haemochrom Diagnostica (Essen, Germany).

Further compounds

Galactose (cat. no. G0750), BSA (lyophilized powder, essentially fatty acid and globulin free, ~99%, cat. no. A1173), and all of the used salts (analytical degree) were purchased from Sigma.

Methods

Degradation of FPX with heparinase

The degradation was performed in the wells of a microplate (cat. no. 269620, Nunc, Langenselbold, Germany). According to the established standard procedure, 15 µl of FPX (100 µg/ml) were mixed with 135 µl of heparinase (1 m IU/ml) for 1 min by shaking (600 rpm, MS-1 Minishaker, IKA, Staufen, Germany), resulting in 10 µg/ml FPX. After incubation for 30 min at 37 °C (Incubator/Shaker AM89C, Dynex, Denkendorf, Germany), the samples were