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Development of a Colorimetric Assay for Heparanase Activity Suitable for Kinetic Analysis and Inhibitor Screening

Edward Hammond,[a†]* Cai Ping Li[a,b] and Vito Ferro[a,c]*

Subject Category: Enzymatic Assays and Analyses

Short Title: Heparanase Assay for Kinetics and Screening

[a] Drug Design Group, Progen Pharmaceuticals, PO Box 2403, Toowong, Qld 4066, Australia.

Telephone: +61 7 3273 9134; Fax: +61 7 3375 1168; Email: edwardh@progen-pharma.com

[b] Current address: QLD Health Forensic and Scientific Services, Coopers Plains, Qld 4108, Australia.

[c] Current address: Queensland University of Technology, School of Physical and Chemical Sciences, Brisbane, Qld 4001, Australia; Institute for Glycomics, Griffith University, Gold Coast Campus, Qld 4222, Australia.

Telephone: +61 7 3138 1108; Fax: +617 3138 1804; Email: vito.ferro@qut.edu.au; v.ferro@griffith.edu.au

† Author to send proofs to.

* Authors for correspondence.

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Abstract

The role that heparanase plays during metastasis and angiogenesis in tumours makes it an attractive target for cancer therapeutics. Despite this enzyme's significance, most of the assays developed to measure its activity are complex. Moreover, they usually rely upon labelling variable preparations of the natural substrate heparan sulfate which makes comparisons across studies precarious. To overcome these problems, we have developed a convenient assay based on the cleavage of the synthetic heparin oligosaccharide fondaparinux. The assay measures the appearance of the disaccharide product of heparanase-catalysed fondaparinux cleavage colorimetrically using the tetrazolium salt WST-1. Because this assay has a homogenous substrate with a single point of cleavage, the kinetics of the enzyme can be reliably characterised giving a K_m of 46 μM and a k_{cat} of 3.5 s^{-1} with fondaparinux as substrate. The inhibition of heparanase by the published inhibitor, PI-88, was also studied and the K_i of 7.9 nM determined. The simplicity and robustness of this method should, not only, greatly assist routine assay of heparanase activity, but could also be adapted for high-throughput screening of compound libraries with the data generated being directly comparable across studies.

Keywords: heparanase, fondaparinux, colorimetric assay, heparan sulfate, enzyme kinetics.

Introduction

The enzyme heparanase is an endo-β-glucuronidase that cleaves heparan sulfate (HS) in the extracellular matrix (ECM) and basement membranes and thus facilitates metastasis of tumour cells and vascular remodelling associated with angiogenesis [1-3]. It also liberates angiogenic growth factors from the ECM and thereby induces an angiogenic response in vivo [4,5]. Heparanase is preferentially expressed in tumours and its upregulation correlates with

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increased tumour vascularity and poor post-operative survival of cancer patients [6-8]. It is thus an attractive target for the development of cancer drugs with antiangiogenic and antimetastatic activity [9,10]. Although identified over two decades ago as a potential therapeutic target for cancer [11,12] for much of the intervening period heparanase research has been significantly restricted by the lack of a simple, accurate and robust assay for enzyme activity. Various assays for heparanase activity have been developed over the years with a recent proliferation of assays, [13-17] including commercially available kits, [18,19] highlighting the growth in interest in this protein. Most assays use HS or heparin preparations as substrate and generally require prior labelling with radioisotopes, fluorescent labels or biotin to allow detection. These assays are also complicated by the fact that the HS/heparin fragments produced by heparanase catalysis may be inhibitors of the enzyme. Furthermore, most only produce semi-quantitative data making comparisons across studies difficult.

Two assays have recently been developed which utilise a homogeneous oligosaccharide substrate of relatively low molecular weight. The substrate for these assays is fondaparinux (**1,** Figure 1), the fully synthetic methyl glycoside of the antithrombin III (ATIII)-activating pentasaccharide sequence of heparin [20]. These assays build on earlier studies showing that a heparin-derived octasaccharide containing the ATIII-binding sequence is cleaved by heparanase, [21] but make use of a commercially available substrate which avoids the low yielding and laborious total syntheses associated with other HS oligosaccharide substrates for heparanase [22,23]. In the first assay, heparanase activity is determined by measuring the change in anticoagulant activity following the cleavage of substrate using a standard chromogenic assay for anti-factor Xa activity [24]. The second assay utilises an LC-MS method for detecting and quantifying the oligosaccharide cleavage products of the reaction (**2** and **3**) using an internal heparin disaccharide as the standard [25].

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Whilst this assay represents a significant advance in this area, the amount of protein consumed per assay is relatively high and the assay procedure is labour intensive.

In light of the above limitations, we sought to develop an improved assay using commercially available pentasaccharide **1** as the substrate and based on the detection of the newly formed reducing disaccharide **2**. The quantification of newly formed reducing ends has long been used as a means to determine the enzymatic activity of various glycosidases and, in fact, the dye Tetrazolium Blue, was previously used in this fashion to assay heparanase activity using HS as the substrate [26]. Here we describe the resulting assay which uses colorimetric quantification, allows kinetic analysis and could be used for high-throughput screening of compound libraries to detect heparanase inhibitors in the development of cancer therapeutics.

Materials and Methods

Materials

Unless otherwise stated, all reagents were obtained from Sigma-Aldrich. Assays were carried out in 96 well microplates (Costar 9018 EIA/RIA, Corning) which had been pre-treated with a solution of 4% BSA in phosphate-buffered saline containing 0.05% Tween 20 (PBST) for 2 h at 37°C. The plates were then washed three times with PBST and shaken dry. Pre-treated plates were stored at 4°C for up to 2 weeks before use.

Recombinant human heparanase was expressed in insect cells [27] and then purified from the cell media into which it had been secreted. The media was clarified by centrifugation at 17,700 *g* at 4°C for 30 min before being loaded onto a 100 mL heparin sepharose column (GE Healthcare) pre-equilibrated to 20 mM sodium phosphate pH 7.5. The column was eluted

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with a series of three 150 mL NaCl concentration steps in the same phosphate buffer: 0.3, 0.6 and 0.8 M. Heparanase containing fractions eluting in the 0.8 M step were pooled (typically 70 mL) and dialysed against 2 L of 10 mM sodium phosphate pH 7.0 at 4°C for 20 h. This material was then loaded onto a 55 mL Source 30S column (GE Healthcare) pre-equilibrated to 20 mM sodium phosphate pH 7.0 and eluted with a 900 mL linear NaCl gradient of 0-0.6 M in the same buffer. Fractions containing pure heparanase, determined by silver staining of SDS-PAGE, were pooled, dialysed into 10 mM sodium phosphate pH 7.0, concentrated using centriplus concentrating devices (Millipore) and stored at -80°C until use.

Assay method

Assay solutions (100 μL) comprised 40 mM sodium acetate buffer pH 5.0, 100 mM fondaparinux (GlaxoSmithKline) with or without inhibitor PI-88. Heparanase was added to a final concentration of 140 pM, unless stated otherwise, to start the assay. The plates were sealed with adhesive tape and incubated at 37ºC for 2-24 h before the assays were stopped by the addition of 100 μL of a solution containing 1.69 mM 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate (WST-1, Auspep, Melbourne, Australia) in 0.1 M NaOH. The plates were resealed with adhesive tape and developed at 60ºC for 60 min and the absorbance was measured at 584 nm (Fluostar, BMG Labtech). In each plate, a standard curve constructed with D-galactose as the reducing sugar standard was prepared in the same buffer and volume over the range of 2 to 100 μM. In the case of PI-88, the IC_{50} value was determined and converted to a K_i using the method of Cheng and Prusoff [28]. All curve fitting, whether to determine Michaelis-Menten constants or IC_{50}, was done using Biaevaluation software 4.1 (Biacore).

Results and Discussion

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Selection of reducing end reagent

A variety of compounds were trialled to find the most suitable reagent to quantify the reducing disaccharide **2** formed by heparanase cleavage of the synthetic substrate **1**. The best results, in terms of sensitivity and stability of the end product, were obtained with the water-soluble tetrazolium salt WST-1. When reacted with a reducing sugar, WST-1 gave a response approximately twice that of Nitrotetrazolium Blue (Figure 1 in supplementary material) and because it formed a water-soluble formazan product no precipitate was formed, unlike other reagents such as Tetrazolium Blue and Nitrotetrazolium Blue.

Complete digestion of **1** was accomplished by incubation with 10 nM heparanase in 40 mM sodium acetate buffer pH 5.0 at 37°C for 24 h. Comparison of the WST-1 standard curves of D-galactose and cleaved **1** revealed that the disaccharide product of heparanase catalysis **2** produces a very similar absorbance to that of D-galactose (Figure 2). This is consistent with our expected yield of one reducing end per molecule of **1** cleaved by heparanase. Moreover, it provides us with a convenient standard (D-galactose) to allow the molar quantification of heparanase activity.

Testing the assay

Having established that the product of heparanase cleavage of **1** can be quantified using WST-1, experiments were conducted to confirm that the velocity of heparanase catalysis could be quantified using this method. First, the linearity of heparanase-dependant product formation over time was assessed by initiating numerous identical assays and stopping them at different times, ranging from 2 to 24 h. Similar experiments were conducted at different heparanase concentrations. The rate of product formation and, therefore, heparanase-catalysed **1** cleavage was linear up to 24 h for the range of heparanase

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concentrations, 56 to 224 pM (Figure 3). This indicates that the assays are not substrate limiting, nor are they being inhibited by product accumulation, nor is heparanase stability affecting the results.

The slopes of the linear regressions fitted to the data in Figure 3 indicate that the rate of **1** cleavage is proportional to the amount of heparanase added but to confirm this, further experiments were conducted. The results of the two sets of experiments indicate that the assay is heparanase dependent at least up to 22.4 fmol/assay (or 224 pM) as shown by the linear response of measured velocity with respect to enzyme concentration (Figure 4).

Kinetic studies

This heparanase assay allows kinetic analysis of the enzyme and its inhibitors, something usually not possible with previous assays. Using this assay, the substrate dependency of heparanase activity was examined and found to conform to Michaelis-Menten kinetics (Figure 5). The K_m (46 ± 14 μM) and k_{cat} (from V_{max}, 3.5 ± 0.3 s^{-1}) for heparanase-catalysed **1** cleavage were determined by fitting the Michaelis-Menten equation to this data. The K_m was previously reported to be 33 μM [29] but upon further analysis the result of 46 μM was determined. This is significantly higher than the K_m published for HS, 1.64 μM determined using the Microcon size exclusion assay [30], indicating that heparanase has a lower affinity for **1** than HS. Such a conclusion is consistent with previous observations that an oligosaccharide in which the 3-*O*-sulfo group on the central D-glucosamine residue of **1** is replaced with H, and which represents a sequence more commonly found in HS, significantly increases heparanase activity [23].

The assay was tested by measuring the heparanase inhibition of PI-88, a competitive inhibitor of the enzyme [30]. The K_i determined for PI-88 was 7.9 ± 0.6 nM (Figure 6) which is considerably lower than the value of 240 ± 30 nM obtained using the Microcon assay [30].

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This difference is probably due to loss of PI-88 onto BSA which is added to the Microcon assay as a carrier to stabilise heparanase. BSA, however, is not required in the sample mixture of this assay making it more suitable for screening inhibitors because it will reduce the number of false negative results caused by compound loss onto carrier proteins. This assay has been designed to measure heparanase activity in well characterised samples and should excel in screening for heparanase inhibitors and in examining the kinetics of the enzyme. It may not be suitable for assaying some crude biological samples for heparanase because there may be components in these samples that interfere with the WST-1 detection and lead to erroneous results.

Conclusion

This report describes a simple, accurate and robust biochemical assay for heparanase activity that uses a commercially available, homogeneous substrate (fondaparinux) with a single enzymatic cleavage point and, thus, does not have the problems associated with using HS-based assays. Detection is easily carried out by incubation with the water-soluble tetrazolium salt WST-1, which reacts with the newly created reducing disaccharide to produce a blue colour. The assay is suitable for testing heparanase inhibitors and could easily be adapted for use in high-throughput screening applications. Moreover, if widely adopted its use will allow the direct comparison of inhibitors by the determination of their K_i values.

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Abbreviations used

BSA, bovine serum albumin; HS, heparan sulfate; LC-MS, liquid chromatography-mass spectrometry; PBST, phosphate-buffered saline plus 0.05% Tween 20; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; WST-1, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate.

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Figure Legends

Figure 1. Cleavage of fondaparinux (**1**) by heparanase to produce the reducing disaccharide (**2**) and the non-reducing trisaccharide (**3**).

Figure 2. Comparison of the absorbance responses at 584 nm obtained following reaction of WST-1 with D-galactose and disaccharide (**2**) formed from complete digestion of fondaparinux with 10 nM heparanase at 37ºC for 24 h. Error bars are SE (n = 4).

Figure 3. Time course of fondaparinux cleavage at seven heparanase concentrations. Assays (100 μL) comprised 40 mM sodium acetate pH 5.0, 100 μM fondaparinux and indicated heparanase concentrations at 37ºC for indicated periods of time. Error bars are SE (n = 6 to 8).

Figure 4. Measured fondaparinux cleavage velocity is proportional to heparanase content. Slope data from Figure 3 in open symbols. Additional data in solid symbols are means of three replicates assayed at 37ºC for 20 h (error bars are SE). Assays (100 μL) comprised 40 mM sodium acetate pH 5.0, 100 μM fondaparinux and indicated heparanase contents at 37ºC.

Figure 5. Velocity of fondaparinux cleavage by heparanase conforms to Michaelis-Menten kinetics. Curve fit to data estimates the K_m at 46 ± 14 μM and V_{max} at 3.5 ± 0.3 fmol fmol^{-1} s^{-1} (Biaevaluation software). Assays (100 μL) comprised 40 mM sodium acetate pH 5.0, indicated fondaparinux concentrations and 140 pM heparanase at 37ºC for 20 h. Error bars are SE (n = 4 or 5).

Figure 6. Inhibition of heparanase by the competitive inhibitor PI-88. Curve fit to data gives an IC_{50} of 25 ± 2 nM (Biaevaluation software) which was converted to a K_i of 7.9 ± 0.6 nM using the method of Cheng and Prusoff [28]. Assays (100 μL) comprised 40 mM sodium acetate pH 5.0, 100 μM fondaparinux, 140 pM heparanase and indicated PI-88 concentrations at 37ºC for 20 h. Data are expressed as % relative to controls containing no PI-88. Error bars are SE (n = 4).

Figure 1

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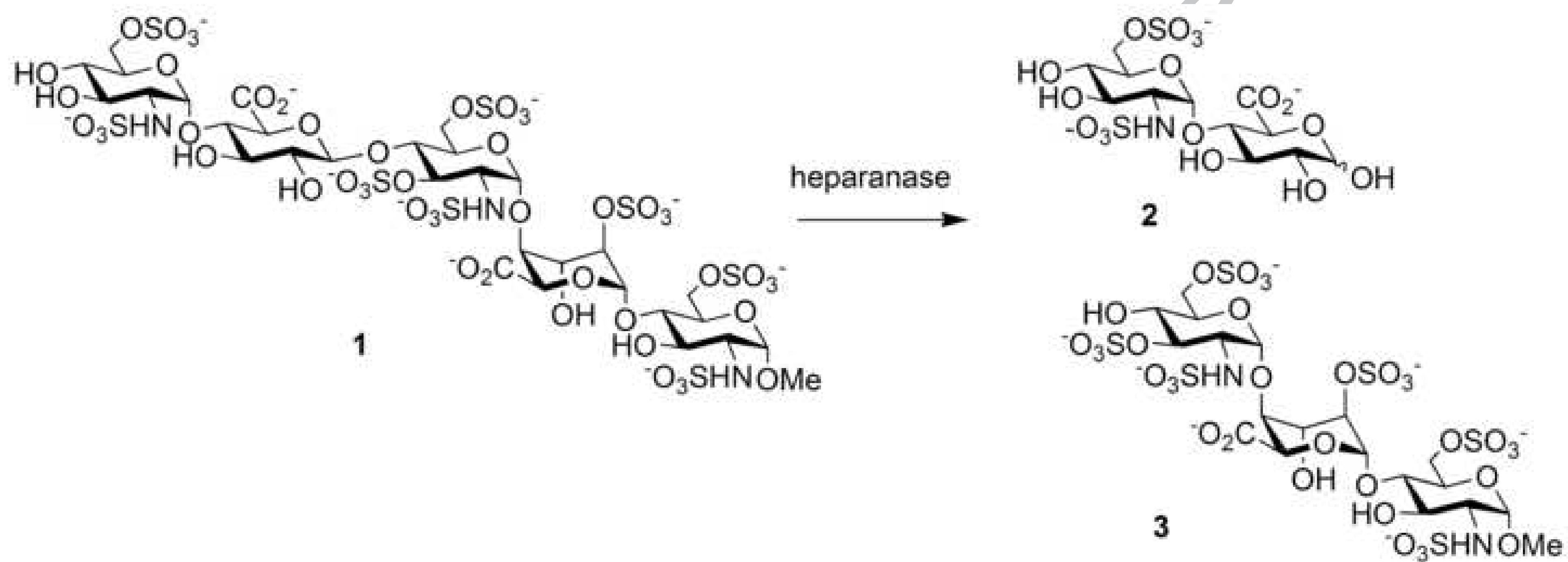

Figure 2

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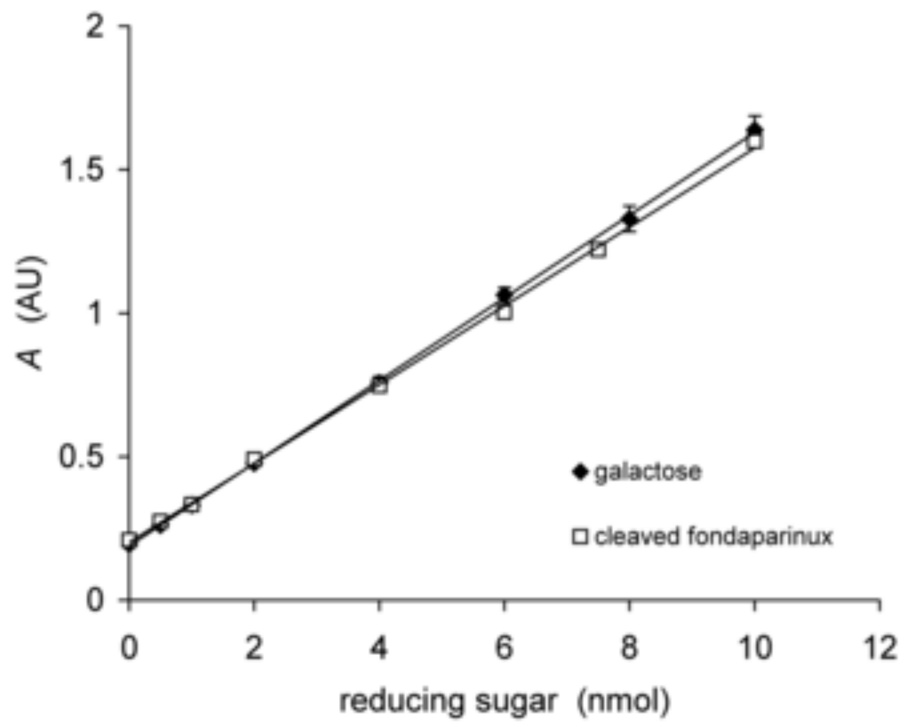

Figure 3

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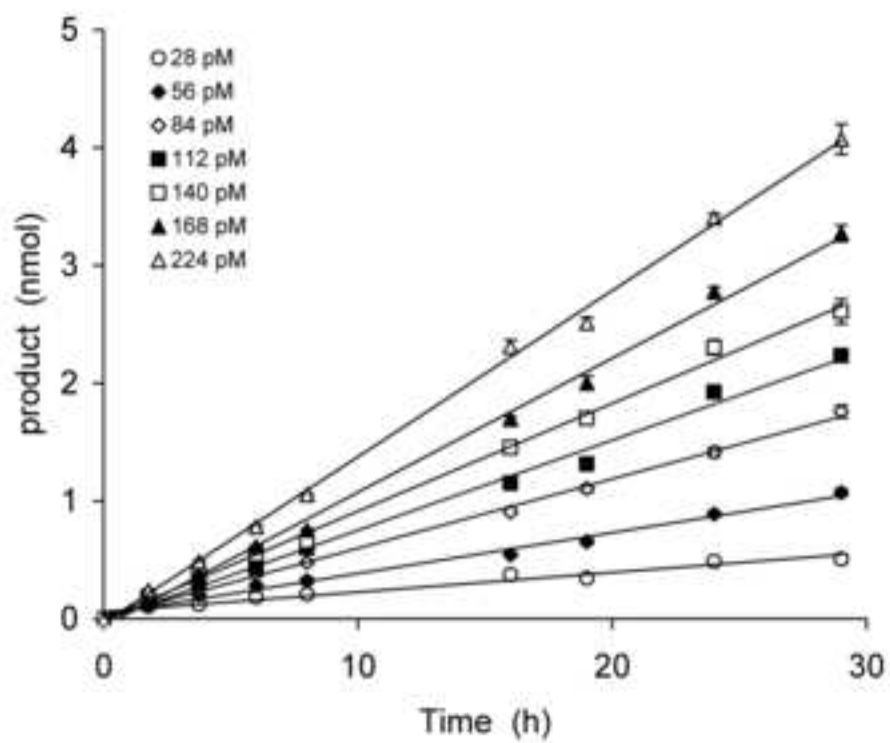

Figure 4

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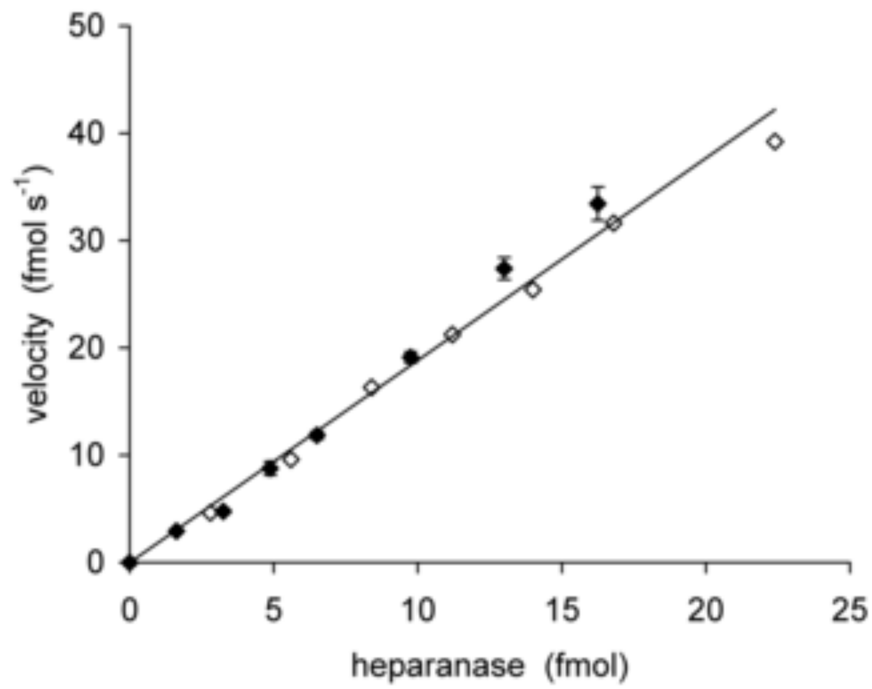

Figure 5

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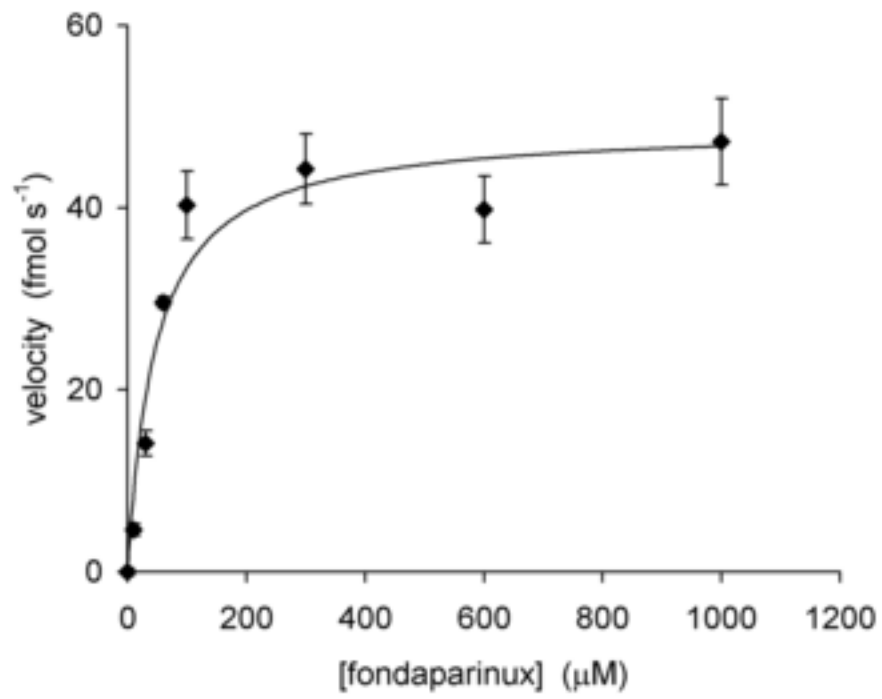

Figure 6

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