Human platelet heparanase: purification, characterization and catalytic activity

Craig FREEMAN[1] and Christopher R. PARISH

Division of Immunology and Cell Biology, John Curtin School of Medical Research, Australian National University, Canberra, ACT 2601, Australia

Heparan sulphate (HS) is an important component of the extracellular matrix (ECM) and the vasculature basal lamina (BL) which functions as a barrier to the extravasation of metastatic and inflammatory cells. Platelet-tumour cell aggregation at the capillary endothelium results in activation and degranulation of platelets. Cleavage of HS by endoglycosidase or heparanase activity produced in relatively large amounts by the platelets and the invading cells may assist in the disassembly of the ECM and BL, and thereby facilitate cell migration. Using a recently published rapid, quantitative assay for heparanase activity towards HS [Freeman, C. and Parish, C. R. (1997), Biochem. J., **325**, 229–237], human platelet heparanase has now been purified 1700-fold to homogeneity in 19% yield by a five column procedure, which consists of concanavalin A-Sepharose, Zn^{2+}-chelating-Sepharose, Blue A-agarose, octyl-agarose and gel filtration chromatography. The enzyme, which was shown to be an endoglucuronidase that degrades both heparin and HS, has a native molecular mass of 50 kDa when analysed by gel filtration chromatography and by SDS/PAGE. Platelet heparanase degraded porcine mucosal HS in a stepwise fashion from a number average molecular mass of 18.5 to 13, to 8 and finally to 4.5 kDa fragments as determined by gel filtration analysis. Bovine lung heparin was degraded from 8.9 to 4.8 kDa while porcine mucosal heparin was degraded from 8.1 kDa to 3.8 and finally to 2.9 kDa fragments. Studies of the enzyme's substrate specificity using modified heparin analogues showed that substrate cleavage required the presence of carboxyl groups, but *O*- and *N*-sulphation were not essential. Inhibition studies demonstrated an absolute requirement for the presence of *O*-sulphate groups. Platelet heparanase was inhibited by heparin analogues which also inhibited tumour heparanase, suggesting that sulphated polysaccharides which inhibit tumour metastasis may act to prevent both tumour cell and platelet heparanase degradation of endothelial cell surface HS and the basal laminar.

INTRODUCTION

Important processes in the tissue invasion by blood-borne malignant tumour cells and leucocytes include their adhesion to the luminal surface of the vascular endothelium, their passage through the vascular endothelial cell layer and the subsequent degradation of the underlying basal lamina (BL) and extracellular matrix (ECM) with a battery of secreted and/or cell surface protease and glycosidase activities [1–3]. The basal lamina and underlying connective tissue stroma consist predominantly of a complex network of fibronectin, laminin, collagen type IV and vitronectin, each of which interact with heparan sulphate (HS) side chains of heparan sulphate proteoglycans (HSPGs) embedded within the matrix [4]. HS chains generally consist of clusters of sulphated disaccharide units (predominantly *N*-sulphated glucosamine linked $1 \rightarrow 4$ to α-L-iduronic acid residues) separated by lowly or non-sulphated regions (predominantly disaccharide units of *N*-acetylated glucosamine linked $1 \rightarrow 4$ to β-D-glucuronic acid) [5,6]. Cleavage of the HS chains by endoglycosidase or heparanase activity produced by invading cells may therefore assist in the disassembly of the ECM and facilitate cell migration. Heparanase activity has been shown to be related to the metastatic potential of murine and human fibrosarcoma and melanoma cell lines [1,7–10]. Heparanase activity has been described in a number of cell types including human platelets [11–13], cultured human skin fibroblasts [14], human neutrophils [15,16], activated but not resting rat T-lymphocytes [17], normal and neoplastic murine β-lymphocytes [18], human monocytes [19] and human umbilical vein endothelial cells (HUVECs) [20,21].

The expression of heparanase activity by platelets, metastatic tumour cells and circulating cells of the immune system have been related to their involvement in their diapedes and extravasation. Studies have shown that while the initial entrapment of metastatic tumour cells by the capillary endothelium is platelet-independent, platelet aggregation which occurs shortly afterwards can lead to platelet activation and degranulation, resulting in gap formation and retraction of endothelial cells exposing the underlying basement membrane to adhesion by the tumour cells [22–24]. Human platelets have been shown to contain high levels of heparanase activity, capable of degrading endothelial cell surface, tumour-derived and ECM-derived HSPG [11,20,25–29] as well as free HS and heparin chains [12,13,27,28,30–32].

Previously, three separate mammalian cell heparanase activities have been reported: mouse melanoma B16 heparanase which cleaves HS only, human platelet heparanase which cleaves both heparin and HS, and a mouse mastocytoma endoglucuronidase which was reported to cleave newly synthesized heparin precursor but not heparin or HS [9,11,33]. More recent studies have indicated that murine melanoma and macrophage extracts are in fact able to degrade both HS and heparin; however, heparin was degraded to a lesser extent than by human platelet extracts [34]. While tumour-derived heparanase was reported to be unable to degrade endothelial cell surface HSPG [35], human platelets have been shown to degrade these HS chains which are more heparin-like in structure [36]. Thus, it is likely that the

Abbreviations used: BL, basal lamina; cHRG, chicken histidine-rich glycoprotein; CPC, cetylpyridinium chloride; ECM, extracellular matrix; GlcA, glucuronic acid; GlcNAc, *N*-acetylated glucosamine; GlcNH, glucosamine; GlcNS, *N*-sulphated glucosamine; GlcNS3S, glucosamine *N*,3-disulphate; HS, heparan sulphate; HSPG, heparan sulphate proteoglycan; HUVEC, human umbilical vein endothelial cells; IdoA, iduronic acid; IdoA2S, iduronic acid 2-sulphate; PBS, phosphate buffered saline.

[1] To whom correspondence should be addressed (e-mail Craig.Freeman@anu.edu.au).

platelet heparanase, which is capable of degrading both cell surface and ECM-associated HSPG, may play a critical role in degrading cell-surface HS in focal adhesion plaques, and aiding the extravasation of blood-borne cells.

Human platelet heparanase was purified 240000-fold from platelet-rich plasma to apparent homogeneity in 6% yield [13]. The enzyme was a 137 kDa single subunit protein which was active towards [^{125}I]heparin and was confirmed to be an endoglucuronidase. Platelet heparanase has been shown to degrade ECM-derived HS chains to 10 kDa [25] and 5 kDa fragments (C. Freeman and M. R. Bartlett, unpublished observations) and porcine mucosal HS was degraded to 4.5 kDa fragments [32]. However, the size of heparin-cleavage products has not been determined [12,13,34]. In contrast, Hoogewerf et al. [11] reported the 4100-fold purification of a 30 kDa human platelet heparanase activity in 8% yield which was shown to be an endoglucosaminidase that cleaved both heparin and HS principally to disaccharides. The activity resided in 8–10 kDa subunit CXC chemokines derived from platelet basic protein, namely connective tissue activating peptide-III (CTAP-III), neutrophil activating peptide-2 (NAP-2) and β-thromboglobulin (β-TG). Graham and Underwood [34] has since shown that the heparanase activity present in human platelet extracts had a M_r of 40–60 kDa.

The resolution of the reported differences in the molecular size (8–137 kDa), the substrate specificity (whether the enzyme is an endo-glucoronidase or endo-glucosaminidase activity), and the size of the substrate cleavage products (to disaccharides or 5–10 kDa) requires purification of the enzyme(s) to homogeneity in high yield. While some of its substrate specificities have been reported [12,13,33], little has been reported on the use of sulphated polysaccharides to inhibit the platelet enzyme. Similar studies of the use of modified heparins to inhibit tumour cell heparanase activity has led to the development of sulphated polysaccharide inhibitors of metastasis [7–9,37–39]. Because platelets play an important role during the initial stages of tumour cell extravasation, the inhibition of platelet heparanase may also inhibit metastasis. We have recently described a rapid quantitative assay for the determination of heparanase activity in human platelets, and in human, rat and murine metastatic and non-metastatic tumour cell lines and tissues [32]. We now describe a new procedure for the purification of human platelet heparanase. In contrast to previous studies [11,13], the platelet enzyme is a single subunit, 50 kDa endoglucuronidase. Details of the enzyme's substrate specificity and inhibition by heparin analogues are described and compared with those reported for the tumour cell associated heparanase activities.

MATERIALS AND METHODS

Heparin-derived molecular mass ($M_{r,av}$) standards of 16.7, 10.6, 6.7 and 3.1 kDa were a generous gift from Nova Nordisk (Gentofte, Denmark) (see Kristensen et al. [40]). Porcine mucosal HS (ORG 553) was a generous gift from Organon Int. Bv. (Oss, The Netherlands). Pharmacia Fine Chemicals (Uppsala, Sweden) supplied PD-10 columns, DEAE-Sepharose, concanavalin A-Sepharose 4B, chelating-Sepharose 4B, Superose 12 HR 10/30 gel chromatography columns, molecular mass standard kits for standardizing gel filtration columns and SDS/PAGE gels, and octyl-Sepharose. Sigma Chemical Co. (St. Louis, MO, U.S.A.) supplied crystalline bovine serum albumin (BSA), bovine lung and porcine mucosal heparin, bovine kidney HS (13 kDa), dextran sulphate (5 kDa), pentosan polysulphate, Cibacron blue 3GA, chondroitin sulphate C, chondroitin ABC-lyase, D-glucuronic acid, 3,3-dimethylglutaric acid, cetylpyridinium chloride (CPC), Blue A-agarose, octyl-agarose, CHAPS, zinc acetate octyl glucoside, hydrazine hydrate and hydrazine sulphate. Suramin was a gift from Bayer AG (Leverkusen, Germany). A highly sulphated porcine mucosal HS (11 kDa) was a gift from Dr. Barbara Mulloy (Hertfordshire, U.K.). Amicon Corp. (Beverly, MA, U.S.A.) supplied Centricon 100 and 30 mini-concentrators and a Diaflow ultrafiltration stirred cell concentrator with a PM 30 kDa cutoff. [^{3}H]Acetic anhydride (500 mCi/mmol) in toluene solution, [^{3}H]$NaBH_4$ (10 Ci/mmol), Hybond-C nitrocellulose membrane and an ECL Western blotting analysis system were obtained from Amersham International (Sydney, Australia). Beckmann (Gladesville, Australia) supplied Ready Safe scintillation fluid. Dowex AG50W-X8 (100–200 mesh; H^+ form), precast PAGE (10 and 15% acrylamide) gels, silver staining kit and protein assay kit were obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Stratagene U.S.A. (La Jolla, CA, U.S.A.) supplied StrataClean Resin. Toronto Research Chemicals (North York, Ontario, Canada) supplied L-iduronic acid (IdoA). Recombinant *N*-glycosidase F, BSA-free *O*-glycosidase and Neuraminidase (*Vibrio cholera*) were obtained from Boehringer Mannheim (Sydney, Australia). Human β-thromboglobulin, human platelet factor 4 and sheep polyclonal antibodies specific for the proteins were generous gifts from Dr. Duncan Pepper (Edinburgh, U.K.). Dako Corp. (Carpenteria, CA, U.S.A.) supplied peroxidase-conjugated rabbit antibody to sheep immunoglobulins.

Preparation of radiolabelled substrates and modified heparins

Porcine intestinal mucosal heparan sulphate (ORG 553), porcine intestinal mucosal and bovine lung heparin were radiolabelled by the method of Freeman and Parish [32] by *N*-deacetylation with hydrazine and re-*N*-acetylation with [^{3}H]acetic anhydride. Carboxyl-reduced-HS and carboxyl-reduced lung heparin were prepared by the method of Karamanos et al. [41] and radiolabelled with [^{3}H]acetic anhydride as described above for the preparation of radiolabelled HS [32]. Desulphated bovine lung heparin, its re-*N*-sulphated analogue and de-*N*-sulphated bovine lung heparin were prepared by the method of Belford et al. [42] and were reductively radiolabelled with [^{3}H]$NaBH_4$ [43]. De-*N*-sulphated heparin, and de-sulphated heparin were radiolabelled with [^{3}H]acetic anhydride [32] to yield their respective *N*-acetylated derivatives. Each of the substrates were purified by desalting on a PD10 column and were stored at -20 °C in 10% (v/v) ethanol. D-Glucuronic and L-iduronic acid were radiolabelled with [^{3}H]$NaBH_4$ [43] to yield gulonic and idonic acid respectively.

Heparanase assays

Assay 1

Human platelet heparanase activity was determined towards HS and the products separated from the substrate using the cHRG-Sepharose beads separation method of Freeman and Parish [32]. Briefly, up to 2 μl of sample was added to an incubation mixture consisting of 90 pmol of radiolabelled HS in 0.05 M-sodium acetate buffer, pH 5.1, containing 5 mM-*N*-acetylmannosamine and 0.1 mg/ml BSA in a total volume of 20 μl and incubated at 37 °C for the appropriate time to yield between 5 and 20% breakdown of the substrate to products. Enzyme activity was expressed as pmol product formed per hour per mg protein.

Assay 2

Heparanase activity towards 2 μg of the radiolabelled HS or heparin analogues was detected by incubation in 0.05 M sodium acetate buffer, pH 5.1, containing 5 mM *N*-acetylmannosamine

and 0.1 mg/ml BSA in a total volume of 20 μl and incubated at 37 °C for 16 h, and the incubation mixture analysed by gel filtration chromatography on Superose 12. The column was equilibrated in, and developed with, PBS at a flow rate of 0.25 ml/min with 0.5 ml fractions being collected directly into scintillation vials, to which 3 ml of scintillation fluid was added, the radioactivity determined and the number average molecular mass calculated [44]. The columns were calibrated with [^{3}H]*N*-acetylglucosamine (221 Da), bovine lung heparin (12.5 kDa) and heparin-derived $M_{r,av}$ standards (16.7, 10.6, 6.7 and 3.1 kDa) which had been partly de-*N*-acetylated and re-*N*-acetylated with [^{3}H]acetic anhydride as described for the preparation of radiolabelled HS [32].

Purification of platelet heparanase

Expired platelet-rich plasma was obtained from the Canberra Hospital, washed twice with normal saline by centrifuging the suspended platelets at 1600 ***g*** for 15 min at 20 °C and the pellet stored frozen at −80 °C. All the following procedures were performed at 4 °C unless otherwise stated. Heparanase activity towards HS was determined using assay 1. In each step (except for the 0.8 M NaCl wash of Blue A-agarose in Step 3), the buffer washes of the columns continued until no more protein was detected in the column eluate using a Bio-Rad protein assay kit.

Step 1. Solubilization of enzyme activity

Fifty units of frozen, washed human platelets were allowed to thaw by suspension in 4 volumes of 15 mM sodium dimethyl glutarate buffer, pH 6.0, containing 0.5 M NaCl (buffer A), and the sample freeze/thawed three times by immersion in a solid CO_2/ethanol bath. The suspended platelet homogenate was centrifuged at 35000 ***g*** for 60 min. The pelleted material was resuspended in 200 ml of buffer A, freeze/thawed again and centrifuged as before. The combined supernatants were used in Step 2. The pelleted material was resuspended in 200 ml of buffer A, freeze/thawed and centrifuged as before. This procedure was repeated once again before the final pellet was homogenized in buffer A containing 1 % (v/v) CHAPS and centrifuged as before. Heparanase activity in the CHAPS supernatant was purified exactly as described below except that Triton X-100 was not added to the extract in Step 2.

Step 2. Concanavalin A-Sepharose chromatography

The supernatant from Step 1 was made 0.2 % in Triton X-100 (v/v) and 1 mM in $CaCl_2$ and $MnCl_2$ and applied at approx. 1 ml/min to a concanavalin A-Sepharose column (1.5 × 5.0 cm) equilibrated in buffer A containing 0.2 % Triton X-100 (buffer B). The column was washed in turn with 25 ml of buffer B and 80 ml of buffer A at 4 °C before being washed with 40 ml of buffer A which had been prewarmed to 20 °C. Heparanase activity was eluted by the application of 40 ml of buffer A containing 20 % α-methyl mannoside (w/v), which had been prewarmed to 20 °C. The eluate was collected into a container held in an ice bucket.

Step 3. Zn^{2+}-chelating Sepharose/Blue A-agarose chromatography

The enzyme solution from Step 2 was applied at 1 ml/min to a Zn^{2+}-chelating Sepharose column (1.0 × 10.0 cm) connected in series with a Blue A-agarose column (1.0 × 2.0 cm). The combined columns were washed with 50 ml of buffer A, before the Blue A-agarose column was disconnected and washed in turn with 20 ml of Tris buffer [60 mM-Tris/HCl buffer, pH 7.2, with 10 % (v/v) glycerol] containing 0.5 M NaCl, and 1.5 ml of Tris buffer containing 0.8 M NaCl.

Step 4. Blue A-agarose/octyl-agarose chromatography

The Blue A-agarose column in Step 3 was connected to an octyl-agarose column (1.0 × 1.5 cm) which was equilibrated in Tris buffer containing 2 M NaCl. The Blue A-agarose column was eluted at 0.5 ml/min by 15 ml of Tris buffer containing 2 M NaCl. The octyl-agarose eluate was immediately concentrated to 5 ml in an Amicon ultrafiltration stirred cell containing a PM30 membrane before being concentrated to 0.2 ml in volume by Centricon 30 centrifugation. Buffer A was added and the enzyme solution concentrated again to 0.4 ml.

Step 5. Superose 12 10/30 chromatography

The concentrated enzyme solution from Step 4 was applied in two separate lots of 200 μl to two Superose 12 HW 10/30 columns connected in series and equilibrated in, and developed with, buffer A at 0.5 ml/min. Fractions of 0.25 ml were collected, assayed for heparanase activity and fractions containing heparanase activity were concentrated to 0.4 ml by Centricon 30 centrifugation. The purified enzyme was stable for at least 2 months when stored in buffer A at 4 °C.

Step 6: Centricon 100 and 300 centrifugation

For platelet preparations obtained from less than 20 units of washed platelets, the 2 M NaCl eluate from the octyl-agarose column in Step 4 was concentrated in a Centricon 100 mini-concentrator and the filtrate containing the heparanase activity was concentrated and dialysed against buffer C using a Centricon 30 concentrator as described in Step 4.

Native and subunit M_r

The native M_r of purified human platelet heparanase activity was determined by chromatography on two Superose 12 HW 10/30 columns connected in series as described in Step 5 above. The columns were calibrated with the following M_r standards: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), fructose-biphosphate aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa) and ribonuclease A (13 kDa).

Discontinuous SDS/PAGE (10 % acrylamide) were run according to the method of Laemmli [45] and stained with Coomassie brilliant blue R250. Gels were calibrated with the following M_r standards: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soya-bean trypsin inhibitor (20 kDa) and α-lactalbumin (14 kDa). Samples were concentrated by the addition of 10 μl of StrataClean Resin, vortexing and centrifuging the suspension according to the manufacturer's instructions. The pelleted material was reduced by boiling in dithiothreitol, and the suspension applied to the stacking gel by the standard procedure. A discontinuous SDS/PAGE (15 % acrylamide) of reduced purified human platelet heparanase (4 μg) was silver stained using a Bio-Rad silver stain kit.

Purified human platelet heparanase (4 μg), β-thromboglobulin (0.2 μg) and platelet factor 4 (0.2 μg) were each reduced by boiling in dithiothreitol, the proteins separated by discontinuous SDS/PAGE (10 % acrylamide) and transferred to nitrocellulose. Western blotting was performed at 4 °C according to the following protocol: the membrane was coated with blocking buffer [3 % (w/v) BSA in PBS] for 16 h, probed for 2 h with

either sheep anti-human β-thromboglobulin or sheep anti-human platelet factor 4 {1:1000 dilution in PBST [blocking buffer containing 0.3% (v/v) Tween 20]}, washed three times with PBST for 5 min, probed for 2 h with peroxidase-conjugated rabbit anti-sheep immunoglobulin (1:1000 dilution in PBST), washed for 5 min three times in PBST and the immunoreactive proteins detected using an ECL Western blotting analysis system.

Deglycosylation of human platelet heparanase

Purified heparanase (20 μg) in 20 μl of 200 mM-sodium phosphate buffer, pH 7.4, containing 1% (w/v) SDS was denatured by boiling for 2 min, diluted with 90 μl of 0.56% (w/v) octylglucoside and the mixture boiled for another 2 min. The mixture was divided in half and *N*-glycosidase F (0.4 units) or a cocktail of *N*-glycosidase F (0.4 units), *O*-glycosidase (2.5 mU) and neuraminidase (2 mU) were added to each fraction which was incubated at 37 °C for 15 h before 10 μl of each sample was reduced by boiling in dithiothreitol and analysed by discontinuous SDS/PAGE (10% acrylamide) and the resulting gel was stained with Coomassie blue.

Determination of heparanase-susceptible linkage

HS, mucosal heparin and lung heparin (100 mg) were each dissolved in 10 ml of 1% cetylpyridium chloride (CPC) containing 0.63 M NaCl (for heparin) or 0.27 M NaCl (for HS), and the solution incubated at 37 °C for 30 min. Solid material was pelleted by centrifugation at 5000 ***g*** for 30 min, dissolved in 2 ml of 2 M NaCl, and precipitated with the addition of 8 ml of ethanol. The precipitate was collected by centrifugation, dissolved in 2 M NaCl, precipitated again with ethanol and the pelleted material lyophilized before being reduced with $NaBH_4$ [43]. Following treatment with purified human platelet heparanase, the newly produced reducing terminals were radiolabelled with [^{3}H]$NaBH_4$ and hydrolysed with trifluoroacetic acid and nitrous acid [46]. The reducing terminal residue was determined by separation of neutral from acidic radiolabelled products by Dowex 1 (formate form) chromatography by the method of Bame and Robson [47] and the acidic fraction analysed by descending chromatography on Whatman P81 phosphocellulose paper developed for 12 h in ethyl acetate/pyridine/5 mM boric acid (3:2:1, by vol.). Radiolabelled gulonic and idonic acid were run as standards. The strips were cut into 1 cm pieces, placed in scintillation vials containing 0.5 ml of water, 3.5 ml of scintillation fluid added and the radioactivity determined.

RESULTS AND DISCUSSION

Purification of platelet heparanase

Human platelet heparanase was purified 1700-fold to homogeneity in 19% yield by a five column procedure, which consisted of concanavalin A-Sepharose, Zn^{2+}-chelating-Sepharose, Blue A-agarose, octyl-agarose and gel filtration chromatography. Approx. 75% of the total heparanase activity was solubilized by repeated freeze/thawing of the platelet homogenate (Table 1). The remaining membrane bound enzyme could only be released by homogenization in buffer A containing 1% (v/v) CHAPS. Homogenization of the membranes with buffer A containing 1% Triton X-100 solubilized the enzyme, but it rapidly lost activity. The membrane bound enzyme was purified from 20 units of washed platelets by the same procedure used to isolate the soluble enzyme, except Step 5 gel filtration was replaced by differential filteration using Centricon 100 and 30 concentrators (Step 6).

Table 1 Purification of heparanase from human platelets

For experimental details, see 'Methods'.

	Step	Heparanase activity (nmol/h)	Total protein (mg)	Specific activity (nmol · h^{-1} · mg^{-1})	Purification (-fold)	Yield (%)
1	Homogenate	12192	3465	3.52	—	—
	Supernatant	9898	2098	4.72	1	100.0
	Pellet	4480	552	8.12	(1.7)	—
2	Concanavalin A-Sepharose	5523	63.1	87.53	18.5	55.8
3	Zn-Sepharose/ Blue-agarose					
4	Blue-agarose/ Octyl-agarose	2579	0.37	6970.3	1476.7	26.1
5	Superose 12	1873	0.24	7804.2	1653.4	18.9

All of the enzyme activity applied to the concanavalin A-Sepharose column was tightly bound. Washing the column by application of buffer A at 20 °C removed more non-specifically bound protein than by washing the column at 4 °C alone and the recovery of enzyme activity and speed of elution (in less than five column volumes of eluant) was greatly enhanced by elution of the column at 20 °C, compared to elution at 4 °C. Heparanase activity was purified by 19-fold with a recovery of 56% from the original platelet supernatant (Table 1). The recovery of enzyme activity from this step was generally within the range 55–70%. No further heparanase activity was eluted from the column by buffer containing α-methylmannoside and 0.1% Triton X-100 at 20 °C.

The use of chelating-Sepharose and dye-matrix chromatography, which has been so successfully used to purify HS-degrading exo-enzyme activities [48–52], has now been applied to purify a mammalian heparanase activity. The enzyme, which did not bind to the zinc-chelating column, was tightly bound to the dye column which permitted a complete separation of heparanase from the heparin-degrading exo-glycosidase activities α-L-iduronidase, β-D-glucuronidase and α-N-acetylglucosaminidase (results not shown). No heparanase activity was detected in the unbound fraction, or in the Tris buffer washes of the dye column. Following elution of heparanase activity from the Blue-agarose column, most of the contaminating protein bound to the octyl-agarose column, while the unbound heparanase was concentrated in an almost pure form for a final purification by gel chromatography. Heparanase activity was purified a further 80-fold over step 2, giving an overall purification of 1500-fold with a recovery of 26% of activity. No further activity was recovered from the chelating-Sepharose column or from the octyl-agarose column following elution of the rest of the bound protein from the columns with buffer A containing 0.1 M imidazole and 1% Triton X-100 respectively.

Gel filtration chromatography (Figure 1) resulted in a further 1.1-fold purification of the enzyme over Step 4, resulting in a final 1700-fold purification with 19% recovery of activity from the original platelet supernatant. This compares with a 240000-fold purification in 6% yield and 4100-fold purification in 8% yield from platelet-rich plasma [11,13]. Smaller preparations of soluble platelet heparanase (from < 20 units of washed platelets), could be prepared within 2 days in < 30% yield using proportionally smaller columns and the gel filtration Step 5 replaced by filtering the octyl-agarose eluate through a Centricon 100 to be concentrated by a Centricon 30 filter. No heparanase activity

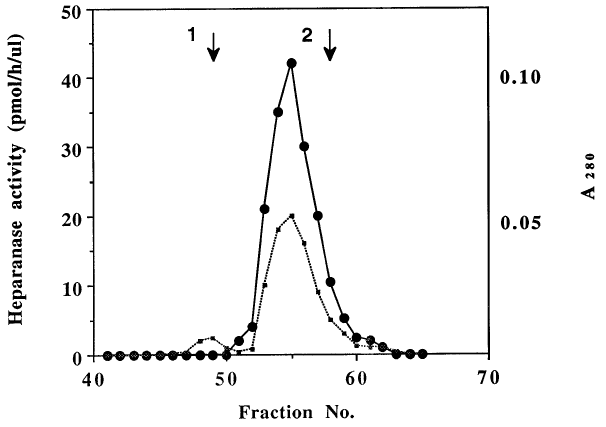

Figure 1 Superose 12 chromatography of human platelet heparanase

Human platelet heparanase from Step 4 was purified by gel filtration as described in the Methods section. Fractions were assayed for heparanase activity (●) and monitored for protein (-----A_{280}). Arrows 1 and 2 indicate V_e for bovine serum albumin and ovalbumin respectively.

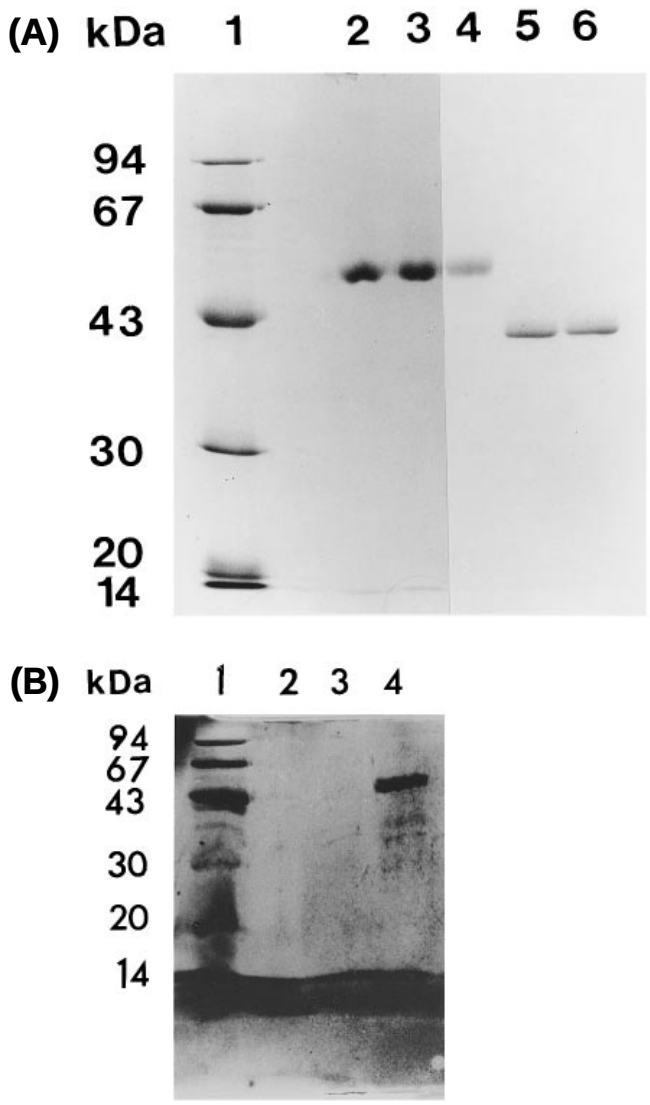

Figure 2 SDS/PAGE of purified human platelet heparanase

(**A**) Purified platelet heparanase was reduced with dithioerythritol, electrophoresed in a 10% polyacrylamide gel and stained with Coomassie brilliant blue R250. Lane 1, M_r standards; lane 2, Step 5 heparanase; and lane 3, Step 5 membrane-associated heparanase. Step 5 platelet heparanase was incubated with (*a*) no enzyme (lane 4), (*b*) *N*-glycosidase F (lane 5) and (*c*) *N*-glycanase F, O-glycosidase and neuraminidase (lane 6). (**B**) Purified platelet heparanase was reduced with dithioerythritol, electrophoresed in a 15% polyacrylamide gel and stained with silver. Lane 1, M_r standards; lanes 2 and 3, buffer blank and lane 4, Step 5 heparanase.

was detected in the filtrate following Centricon 30 concentration of the pure enzyme in contrast to that reported by Hoogewerf et al. [11].

Between 10 and 25% of the total platelet heparanase activity was observed to be associated with the pelleted membrane fraction and required detergent to solubilize the enzyme activity. The membrane-associated enzyme was purified with 25% recovery of enzyme activity and exhibited the same chromatographic properties and had a final specific activity of 6600 $nmol \cdot h^{-1} \cdot mg^{-1}$ of protein which is similar to the soluble form of the enzyme. It is not known how the enzyme is associated with the membrane. Platelet heparanase may therefore exist in both soluble and membrane associated forms similar to the lysosomal enzyme acid phosphatase, which has been described in platelets, is transported to the lysosome by a mannose-6-phosphate independent pathway and has a 2 kDa C-terminal transmembrane peptide which results in the enzyme existing as both membrane bound and soluble forms [53]. In contrast, cathepsin D is synthesized as a soluble protein, and becomes membrane associated by a mannose-6-phosphate-independent mechanism which is prevented by proteolytic processing [54,55], while glucocerebrosidase is a membrane associated enzyme which lacks a transmembrane spanning domain [55,56]. Previously, Gallagher et al. [57] reported the presence of a rat liver membrane heparanase activity which was inactive at pH values less than 7.5, unlike the enzyme reported here (see below).

Native and subunit molecular mass

Gel filtration analysis of both purified heparanase (Step 5) and of crude enzyme eluted from concanavalin A-Sepharose either at 4°C or at 20 °C (Step 2) demonstrated that the enzyme had an apparent M_r of 50 kDa (Figure 1). No enzyme activity was detected in either case at M_r values corresponding to 8–10 kDa or dimers or tetramers of the chemokine sized platelet heparanase activity [11], nor was any activity detected that corresponded to an apparent M_r of 137 kDa [13]. Previously, it was observed that the buffer pH and salt concentration could influence the state of aggregation and apparent M_r for several HS-degrading exoenzyme activities [51,52]. The buffer conditions used in this study (0.5 M NaCl and pH 6.0) would be expected to minimize aggregation, therefore it is unlikely the apparent M_r of 50 kDa was a result of aggregation of smaller (8 kDa) subunits. All of the platelet heparanase activity was retained by Centricon 30 kDa membranes (even in the presence of 2 M NaCl) in contrast to that reported by Hoogewerf et al. [11]. The results presented in this report are similar to the M_r values of 40–60 kDa observed for human platelet extracts [34] and 45 kDa and 50 kDa reported for purified human placental heparanase [58] and for partly purified human spleen heparanase [19] respectively, but differs from the M_r value of 94 kDa reported for the murine melanoma enzyme [59].

SDS/PAGE analysis of the purified enzyme indicated a single Coomassie blue staining band corresponding to a M_r value of 50 kDa under both reducing (Figure 2A) and non-reducing conditions (results not shown), suggesting that the single subunit

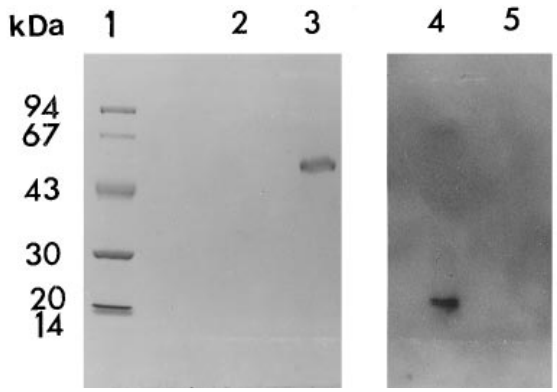

Figure 3 Absence of β-thromboglobulin immunoreactivity on a Western blot of purified human heparanase

Purified platelet heparanase (4 μg) and β-thromboglobulin (0.2 μg) were reduced with dithioerythritol, electrophoresed in a 10% polyacrylamide gel and stained with Coomassie brilliant blue R250 (lanes 1–3) or transferred to a nylon membrane and probed for immunoreactivity towards sheep anti-human β-thromboglobulin antibodies (lanes 4 and 5). Lane 1, M_r standards; lanes 2 and 4, β-thromboglobulin; and lanes 3 and 5, Step 5 heparanase. For full experimental details, see 'Methods'.

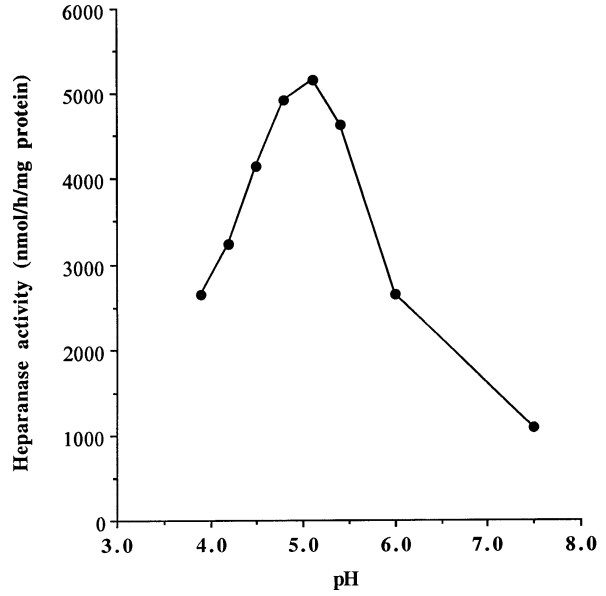

Figure 4 Hydrolysis of HS as a function of pH

Heparanase activity towards radiolabelled porcine mucosal HS (ORG 553) was determined using assay 1. For experimental details, see 'Methods'.

protein has no significant intramolecular disulphide bridging. This size was identical to the value obtained by gel filtration analysis. The preparation was determined to be homogeneous following excessive silver staining of the SDS/PAGE gel which resulted in non-specific staining of the lower part of the enzyme and buffer blank containing lanes of the gel (Figure 2b). Western blot analysis of an SDS/PAGE of the purified enzyme with antibodies against human β-thromboglobulin (an *N*-truncated derivative of platelet basic protein and CTAP-111) (Figure 3), or platelet factor 4 (results not shown) demonstrated that neither of these 8 kDa platelet-derived HS-binding proteins were present in the purified platelet heparanase preparation. The enzyme was deglycosylated by *N*-glycosidase F which resulted in an apparent size of 40 kDa by SDS/PAGE analysis (Figure 2A). This is consistant with a predicted cDNA size of 42 kDa with six putative *N*-glycosylation sites (C. Freeman and C. R. Parish, unpublished results). No further reduction in the apparent size of the *N*-deglycosylated material was observed following concurrent *O*-glycosidase and neuraminidase treatment (Figure 2A). The purified membrane bound enzyme also had a native M_r and subunit M_r of 50 kDa as determined by gel filtration and SDS/PAGE analysis under reducing conditions (Figure 2A).

Substrate specificity of purified platelet heparanase

Purified human platelet heparanase activity towards HS, as determined by assay 1, was maximal at pH 5.1, with 48 and 21% of maximal activity observed at pH 4.0 and 7.5 respectively (Figure 4), which was similar to that reported for the platelet homogenate heparanase activity [32]. Superose 12 gel filtration analysis (assay 2) was used to study the substrate specificity of heparanase towards HS and heparin-derived analogues. The purified enzyme cleaved both HS and heparin at pH 5.1. Activity at pH 4.0 or 6.0 resulted in less formation of smaller M_r products compared to assay at pH 5.0 for all the substrates tested. The 18.5 kDa porcine mucosal HS was cleaved to 4.5 kDa fragments (Figure 5a), as were 13 kDa bovine kidney HS and a highly sulphated 11 kDa porcine mucosal HS (results not shown). Previously, porcine mucosal HS was shown to be cleaved by platelet homogenate heparanase activity in a stepwise fashion from 18.5 to 13 to 8 and finally to 4.5 kDa fragments [32]. No activity was detected towards carboxyl-reduced HS (results not shown), demonstrating an absolute requirement for the presence of unmodified carboxyl groups. Such dependence on the presence of the carboxyl groups has been previously shown for placental heparanase [60] and for the HS-degrading exoglycosidases α-L-iduronidase and β-D-glucuronidase [61].

Mucosal heparin was cleaved from 8.1 to 2.9 kDa while the more highly sulphated lung heparin was cleaved from 8.9 kDa to 4.8 kDa fragments (Figures 5b and 5c, respectively). Mucosal heparin appeared to be cleaved twice in a stepwise fashion compared to the singularly cleaved lung heparin. Whereas desulphated lung heparin was not a substrate (Figure 5d), platelet heparanase cleaved both the re-*N*-acetylated and re-*N*-sulphated analogues of desulphated heparin (Figures 5e and 5f, respectively) and also cleaved both de-*N*-sulphated heparin and its re-*N*-acetylated analogue (Figures 5g and 5h, respectively). Oldberg et al. [12] had previously demonstrated that partly purified platelet heparanase had a requirement for substrates containing sulphamino groups and could not cleave the heparin biosynthetic precursor heparan (a polymer consisting of alternating -GlcNAc-GlcA- residues). In a more detailed study, this report demonstrates that neither the presence of *N*- nor *O*-sulphation are an absolute requirement for substrate cleavage since the enzyme will in fact cleave a non-sulphated (GlcNAc-containing) substrate, providing that IdoA residues are also present and the glucosamine residues are substituted as demonstrated by the cleavage of re-*N*-acetylated/desulphated heparin. Without having the appropriate M_r standards for the various modified heparins, it is difficult to estimate the size of the modified heparin degradative products. However, it is clear from the gel filtration profiles that the modified heparins, like heparin itself, are cleaved with a minimal number of cleavages and the modifications have probably not introduced any new cleavage sites. For cleavage to occur, the GlcNAc, GlcA and IdoA residues must therefore occur within distinct sequences. We [32] and others [3,62–65] have observed

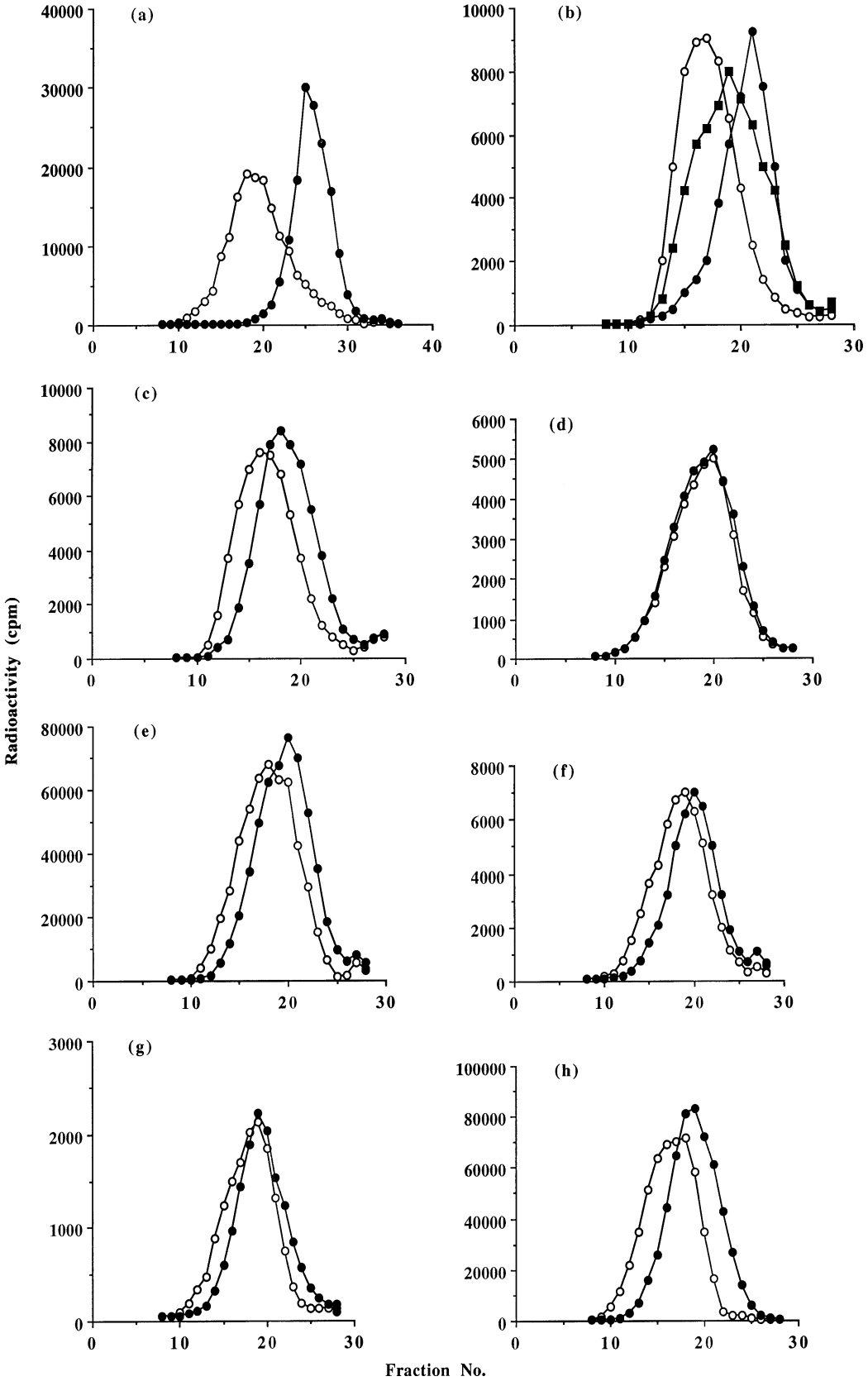

Figure 5 Gel filtration analysis of heparanase degradation of HS and heparin analogues

Radiolabelled (**a**) porcine mucosal HS (ORG 553), (**b**) porcine mucosal heparin, (**c**) bovine lung heparin, and bovine lung heparin which had been (**d**) desulphated, (**e**) desulphated and re-*N*-acetylated, (**f**) desulphated and re-*N*-sulphated, (**g**) de-*N*-sulphated, and (**h**) de-*N*-sulphated and re-*N*-acetylated were incubated with platelet heparanase for 4 h (■), for 16 h (●) or with no enzyme (○). The incubation mixtures were analysed by Superose 12 gel chromatography. For experimental details, see 'Methods'.

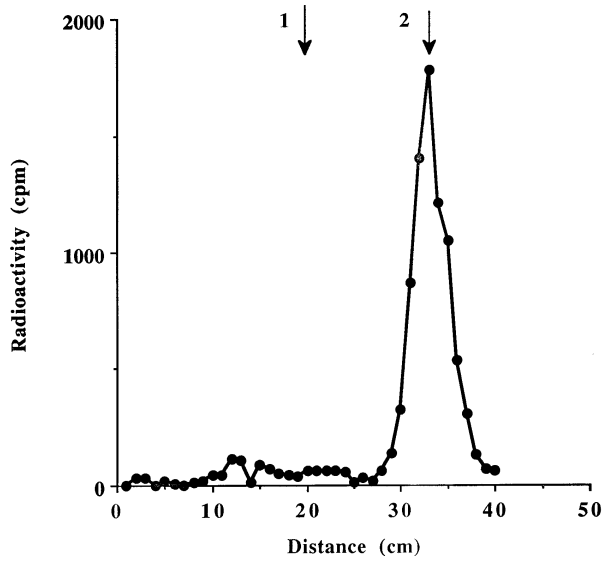

Figure 6 Phosphocellulose paper chromatography of acid hydrolysed radiolabelled products of heparanase degradation of HS

Following heparanase digestion of HS, the products were reductively radiolabelled, hydrolysed by trifluoroacetic and nitrous acid and the acidic material following Dowex 1 chromatography separated by descending chromatography on phosphocellulose paper developed for 12 h in ethyl acetate/pyridine/5 mM-boric acid (3:2:1, by vol.). Arrows 1 and 2 indicate the positions of idonic and gulonic acid respectively. For experimental details, see 'Methods'.

that several different types of HS are cleaved by heparanases from a variety of cell sources in a stepwise fashion to approx. 10 and to 5 kDa fragments. Unlike the report of Hoogewerf et al. [11], however, we did not observe any evidence of the production of the predominately disaccharide products resulting from the cleavage of HS or heparin (or modified heparins) following prolonged incubations of either purified enzyme (see Figure 5) or platelet heparanase homogenates (results not shown), even after pre-treatment of the enzyme with reducing and oxidizing agents as described by these authors. To date, no detailed study of the ability of tumour-derived heparanases to cleave modified heparins has been reported, although murine melanoma cell heparanase (which did not cleave mucosal heparin) was reported to cleave *N*-acetylated/de-*N*-sulphated mucosal heparin, presumably following the introduction into the substrate of heparanase-susceptible linkages [7].

Identification of heparanase-susceptible linkages

To determine the residue platelet heparanase cleaves adjacent to, sodium borohydride-reduced HS, mucosal and lung heparin were each cleaved by purified platelet heparanase and the fragments radiolabelled by reduction of the newly produced reducing terminals. Dowex-1 chromatography of the trifluoroacetic and nitrous acid hydrolysed material revealed that 90% of the radiolabelled material was acidic and almost all of this co-ran with gulonic acid following phosphocellulose paper chromatography confirming that the newly heparanase-generated reducing terminal in each case was glucuronic acid (Figure 6). The radiolabelled neutral material not bound by the Dowex-1 column was not further investigated. These results confirm previous reports that the human platelet heparanase is an endo-glucuronidase [12,13], but are different to those of Hoogewerf et al. [11] who reported the purification of a platelet heparanase endo-glucosaminidase. Interestingly, Klein et al. [66] reported that stored HS in fibroblasts obtained from mucopolysaccharidoses patients (deficient in HS exo-degrading enzymes) was smaller than extracellular HS (indicating that endo-glycosidase activity was functioning) but up to 80% of the stored HS had reducing terminal glucosamine residues, suggesting the existence of both endo-glucuronidase and endo-glucosaminidase activities.

Table 2 Inhibition of platelet heparanase activity towards HS

The inhibition of purified human platelet heparanase activity towards HS by modified heparins and other sulphated carbohydrates was determined by heparanase assay 1. For full details, see 'Methods'.

Inhibitor	IC 50 (μg/ml)
Lung heparin	1.2
Carboxyl-reduced heparin	2
De-sulphated heparin	> 300
De-sulphated/*N*-acetylated heparin	> 300
De-sulphated/*N*-sulphated heparin	> 300
De-*N*-sulphated heparin	15
De-*N*-sulphated/*N*-acetylated heparin	5
3.1 kDa mucosal heparin	18
Suramin	7
Cibacron blue 3GA	20
Pentosan polysulphate	5
Dextran sulphate	4
Dermatan sulphate	280
Chondroitin sulphate A	> 300
Chondroitin sulphate C	> 300

Inhibition of platelet heparanase activity with modified heparins

Although platelet heparanase probably plays an important role in the extravasation of tumour cells, no detailed study has reported the inhibition of platelet heparanase activity towards HS by heparin analogues, similar to that reported for the inhibition of rat hepatocyte and murine melanoma cell heparanase extracts [38,67,68]. Using our rapid, quantitative heparanase assay 1, we have determined the IC_{50} (the quantity of inhibitor required to inhibit purified platelet heparanase activity towards HS by 50%) for each heparin analogue (Table 2). Platelet heparanase was inhibited by 50% in the presence of 1.2 μg/ml of lung heparin. Carboxyl-reduced heparin was nearly as potent an inhibitor as heparin. De-*N*-sulphated heparin and its *N*-acetylated derivative were 12- and 4-fold poorer inhibitors than heparin itself. De-*N*-sulphation would effectively reduce the overall negative charge of the inhibitor and this effect was partially relieved by *N*-acetylation of the inhibitor. De-sulphated heparin and its *N*-acetylated and *N*-sulphated analogues did not inhibit the enzyme at the concentrations assayed, demonstrating the absolute requirement for the presence of *O*-sulphate groups for efficient inhibition of the enzyme. In this regard, we have observed that the inhibition of heparanase activity by small non-heparinoid oligosaccharides was related to its size and the degree of *O*-sulphation of the inhibitor (C. Freeman and C. R. Parish, unpublished observations). The smaller, but more highly sulphated dextran sulphate (5 kDa) and pentosan polysulphate

were 3–5-fold poorer inhibitors than heparin, while the larger, but less sulphated polysaccharides dermatan sulphate and chondroitin 4- and 6-sulphate were quite poor inhibitors. The type of linkage between the sugar residues may also be an important factor for inhibiting heparanase activity. Dermatan sulphate, which contains IdoA residues, was a better inhibitor than either of the GlcA-containing chondroitin sulphates. The polysulphonated naphthylene compounds suramin and Cibacron blue 3GA were moderately potent inhibitors, again demonstrating the influence of *O*-sulphate groups.

Although some of the lung heparin-analogues were inhibitors of the enzyme, they were also shown in the previous section to be substrates for the enzyme. For example, lung heparin was cleaved to 4.8 kDa fragments (Figure 5c). However, the cleaved products may themselves still be potent inhibitors of heparanase activity. A 3.1 kDa mucosal heparin fragment, which was not a substrate for the enzyme (results not shown), was an inhibitor of the platelet enzyme (Table 2). Vlodavsky et al. [68] has also observed that murine melanoma heparanase was potently inhibited by heparin-derived tetradesaccharides. In animal models of metastasis, although heparin itself may be cleaved by platelet heparanase, its products may still inhibit HS degradation and metastasis.

The results observed in our study show that, in general, the same heparin-derived compounds which inhibited the cleavage of HS by the platelet heparanase have also been reported to inhibit murine B16 melanoma, mouse lymphoma and rat hepatoma-derived heparanases and metastasis in animal models [3,7–9,37–39,67–70]. Previously heparin has been shown to be a potent inhibitor of tumour cell-derived heparanase activity. Eldor et al. [39] demonstrated that mucosal heparin, *N*-acetylated/*N*-desulphated heparin and *N*-sulphated/desulphated heparin each inhibited human platelet and human neutrophil heparanase activity towards ^{35}S-labelled ECM with similar potency. Nakajima et al. [9] also observed heparanase was inhibited by *N*-sulphated/desulphated but not by de-*N*-sulphated mucosal heparin, in contrast to the results in this study. HS degradation by heparanase was observed to be potently inhibited by carboxyl-reduced heparin by Lapierre et al. [67], as was shown in this study, in contrast to the lack of inhibition toward murine melanoma heparanase activity reported by Irimura et al. [38]. The differences in inhibition by the heparin-analogues may be attributed to differences in the enzyme source (including purified and non-purified enzyme), the substrate used and the source of the heparin (i.e. mucosal heparin or the more highly sulphated lung heparin).

GENERAL DISCUSSION

Human platelets were shown to contain very high amounts of heparanase activity compared to metastatic tumour cells and other cell types when expressed as activity/mg of protein [32]. The exact role of platelet heparanase in the diapedesis and extravasation of tumour cells has not been determined. Tumour cells have been shown to interact with platelets at the site of tumour cell extravasation through the capillary wall. In rats, a reduction in pulmonary metastases was observed following induction of experimental immune thrombocytopenia or by treatment of rats with antibodies which prevent the binding of platelets to the tumour cells [22,71–73]. The level of lung metastases was restored by platelet transfusions. Studies have also shown that following entrapment of the tumour cells by the capillary endothelium (which is platelet independent), platelet aggregation occurs in the vicinity of the bound tumour cells, followed by platelet activation and degranulation. Following a breaching of the endothelial–endothelial cell junctions, retraction of the endothelial cells exposes the underlying basement membrane to which the tumour cells adhere to and eventually degrade. Human platelets have been shown to contain catalytically inactive heparanase which was activated by soluble factors secreted by tumour and other cell types [30,74]. Therefore, tumour cells may not only be involved in the recruitment and activation of platelets, but may regulate platelet heparanase activity.

Human platelet heparanase has been reported to differ in its substrate specificity from tumour and other blood-borne cell heparanases with regards to its ability to degrade both heparin and HS [9,37]. The partial detachment of endothelial cells from the underlying basement membrane during gap formation is likely to require degradation of the endothelial cell surface HS which may interact with ECM HS-binding adhesive molecules. Endothelial cell surface HS has been shown to be distinct from basement membrane HS, being more 'heparin-like' in its structure [26,75]. In this regard, Hennes et al. [35] has reported that tumour cell heparanase can degrade ECM-derived HS, but was unable to degrade endothelial cell surface HS. Thus platelet heparanase may play an initial role in gap formation by degrading cell surface HS, exposing the basal laminar, and subsequently aid in its degradation.

There have been many studies using modified heparins to inhibit tumour-derived heparanase activity and metastasis in animal models [3,7–9,37–39,67–70]. Our results show that in general, these same heparin-derived compounds inhibit the cleavage of HS by platelet heparanase. Therefore, these anti-metastatic compounds may be active in not only inhibiting tumour degradation of the basement membrane, but also platelet heparanase degradation of both the endothelial cell HS (and the subsequent process of gap formation) and degradation of the underlying basement membrane.

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