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Human liver glucuronate 2-sulphatase

Purification, characterization and catalytic properties

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Human glucuronate 2-sulphatase (GAS), which is involved in the degradation of the glycosaminoglycans heparan sulphate and chondroitin 6-sulphate, was purified almost 2000000-fold to homogeneity in 8% yield from liver with a four-step six-column procedure, which consists of a concanavalin A–Sepharose/Blue A–agarose coupled step, a DEAE-Sephacel/octyl-Sepharose coupled step, CM-Sepharose chromatography and gel-permeation chromatography. Although more than 90% of GAS activity had a pI of >7.5, other forms with pI values of 5.8, 5.3, 4.7 and <4.0 were also present. The pI > 7.5 form of GAS had a native molecular mass of 63 kDa. SDS/polyacrylamide-gel-electrophoretic analysis resulted in two polypeptide subunits of molecular mass 47 and 19.5 kDa. GAS was active towards disaccharide substrates derived from heparin {*O*-(β-glucuronic acid 2-sulphate)-(1→4)-*O*-(2,5)-anhydro[1-^{3}H]mannitol 6-sulphate (GSMS)} and chondroitin 6-sulphate {*O*-(β-glucuronic acid 2-sulphate-(1→3)-*O*-(2,5)-anhydro[1-^{3}H]talitol 6-sulphate (GSTS)}. GAS activity towards GSMS and GSTS was at pH optima of 3.2 and 3.0 respectively with apparent K_m values of 0.3 and 0.6 μM respectively and corresponding $V_{max.}$ values of 12.8 and 13.7 μmol/min per mg of protein respectively. Sulphate and phosphate ions are potent inhibitors of enzyme activity. Cu^{2+} ions stimulated, whereas EDTA inhibited enzyme activity. It was concluded that GAS is required together with a series of other exoenzyme activities in the lysosomal degradation of glycosaminoglycans containing glucuronic acid 2-sulphate residues.

INTRODUCTION

The sulphated glycosaminoglycans heparin and heparan sulphate are degraded from their non-reducing terminal by the sequential action of highly specific lysosomal sulphatases and other exoenzyme activities [1,2]. A deficiency in humans of any one of these lysosomal enzyme activities results in tissue accumulation of that enzyme's substrate, giving rise to one of the mucopolysaccharidosis (MPS) group of lysosomal storage disorders [3]. Heparin and heparan sulphate consist of repeating disaccharide units containing alternating residues of uronic acid, either glucuronic acid or iduronic acid, and glucosamine. A large percentage of the disaccharide repeat units consist of iduronic acid that is 2-*O*-sulphated linked α-(1→4) to glucosamine *N*-sulphate, which may also be 6-*O*-sulphated [4]. Iduronate 2-sulphatase has been shown to act specifically upon non-reducing-end iduronic acid 2-sulphate residues, by hydrolysis of the 2-*O*-sulphate from heparin and dermatan sulphate-derived disaccharide substrates [5,6]. Recently it was shown that a small percentage of glucuronic acid residues present in the highly sulphated regions of heparin were 2-*O*-sulphated [7]. Chick-embryo chondrocytes and cultured human skin fibroblasts were shown to contain a specific sulphatase activity, glucuronate 2-sulphatase (GAS), which acts towards glucuronic acid 2-sulphate (GlcA2S) residues present in a disaccharide substrate, *O*-(β-glucuronic acid 2-sulphate)-(1→4)-*O*-(2,5)-anhydro-[1-^{3}H]mannitol 6-sulphate (GSMS). Skin fibroblasts deficient in iduronate 2-sulphatase activity (mucopolysaccharidosis type II, MPS II) were able to desulphate GSMS, proving that different sulphatases are required to desulphate iduronic acid and glucuronic acid residues. It was also shown that GlcA2S residues present in chondroitin 6-sulphate are desulphated by the same enzyme activity that desulphated GSMS [8]. Cultured rat hepatocytes have been shown to secrete heparan sulphate proteoglycan, which is rapidly endocytosed and processed, and free heparan sulphate chains with a high content of sulphated glucuronic acid accumulate in the nuclear pool [9]. The functional significance of GlcA2S residues in both heparan sulphate and in chondroitin sulphate is unclear.

We are particularly interested in the characterization of the enzyme that desulphates GlcA2S residues because of the possibility of a new lysosomal storage disease resulting from a deficiency of GAS. We have previously described the concurrent isolation from human liver of a number of lysosomal enzymes involved in the degrada-

Abbreviations used: GAS, glucuronate 2-sulphatase; GSMS, *O*-(β-glucuronic acid 2-sulphate)-(1→4)-*O*-(2,5)-anhydro[1-^{3}H]mannitol 6-sulphate; ISMS, *O*-(α-iduronic acid 2-sulphate)-(1→4)-*O*-(2,5)-anhydro[1-^{3}H]mannitol 6-sulphate; GSTS, *O*-(β-glucuronic acid 2-sulphate)-(1→3)-*O*-(2,5)-anhydro[1-^{3}H]talitol 6-sulphate; ISTS, *O*-(α-iduronic acid 2-sulphate)-(1→3)-*O*-(2,5)-anhydro[1-^{3}H]mannitol 6-sulphate; GMS, *O*-(β-glucuronic acid)-(1→4)-*O*-(2,5)-anhydro[1-^{3}H]mannitol 6-sulphate; GTS, *O*-(β-glucuronic acid)-(1→3)-*O*-(2,5)-anhydro[1-^{3}H]talitol 6-sulphate; IMS, *O*-(α-iduronic acid)-(1→4)-*O*-(2,5)-anhydro[1-^{3}H]mannitol 6-sulphate; MS, (2,5)-anhydro[1-^{3}H]mannitol 6-sulphate; GlcA2S, glucuronic acid 2-sulphate; MPS, mucopolysaccharidosis.

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tion of sulphated glycosaminoglycans [10–14]. In the present paper we report the extension of this procedure for the purification of GAS from other sulphatases, including iduronate 2-sulphatase, and present details of its physical and catalytic properties.

MATERIALS AND METHODS

Materials

Human livers, lungs and kidneys, obtained from autopsies of normal adults with intervals *post mortem* ranging from 24 to 72 h, were stored at −20 °C. Cultured skin fibroblasts from normal individuals, were grown and harvested, and the homogenates for enzyme assay were prepared as described previously [15,16]. Pharmacia Fine Chemicals (Uppsala, Sweden) supplied concanavalin A–Sepharose, DEAE-Sephacel, octyl-Sepharose, CM-Sepharose, the chromatofocusing media PBE 94 and Polybuffer 74 and the molecular-mass standard kit for SDS/polyacrylamide-gel electrophoresis. Amicon Corp. (Danvers, MA, U.S.A.), supplied Blue A Matrex agarose gel, a DC-2 hollow-fibre concentrator with 10 kDa-cut-off hollow fibres, an ultrafiltration stirred cell (model 8200), with a Diaflo ultrafiltration membrane YM10, and Centricon 10 Microconcentrators. Bio-Rad Laboratories (Richmond, CA, U.S.A.) supplied silver stain and protein assay kits and Dowex AG1-X4 (100–200 mesh; Cl^- form). LKB (Bromma, Sweden) supplied an Ultrachrom GTI Bioseparation system with an LKB-2135 TSK G-3000 SW Ultrapac column, and molecular-mass standard kit for gel filtration. Amersham International (Sydney, N.S.W., Australia) supplied NaB^3H_4 (10 Ci/mmol). Beckman (Sydney, N.S.W., Australia) supplied Redi-Solv EP scintillation cocktail. Sigma Chemical Co. (St. Louis, MO, U.S.A.) supplied crystalline bovine serum albumin (globulin-free grade), D-saccharic acid 1,4-lactone, heparin (grade II prepared from pig intestinal mucosa) and chondroitin 6-sulphate (type C from shark cartilage). All other chemicals were of analytical quality. Pure human liver iduronate 2-sulphatase was generously given by Ms. J. Bielicki of this Department.

Preparation of GAS substrates (GSMS and GSTS)

ISMS was prepared by HNO_2 deamination of heparin and reduced with NaB^3H_4 (1.0 Ci/mmol) as previously described [15]. GSMS was formed by epimerization of ISMS [8]. Separation of the ISMS and GSMS disaccharides was by descending chromatography on Whatman 3MM chromatography paper developed in butan-1-ol/acetic acid/7 M-NH_3 (2:2:1, by vol.) for 72 h. Areas of radioactivity, detected with a Packard model 7201 radiochromatogram scanner (Packard Instruments, Chicago, IL, U.S.A.), were eluted from the strip in aq. 10% (v/v) ethanol. GSMS and ISMS migrated with mobilities relative to IMS of 0.45 and 0.65 respectively. GSTS was obtained by treatment of chondroitin 6-sulphate with hydrazine for 16 h, followed by HNO_2 degradation (pH 4.0) and reduction with NaB^3H_4 (0.3 Ci/mmol) by the procedure described for the depolymerization of dermatan sulphate [6,17]. The disulphated disaccharide obtained had an elution profile identical with that described previously for ISTS isolated from the dermatan sulphate disaccharide fraction [6]. Descending chromatography showed a single peak with a mobility relative to IMS of 0.42. IMS and GMS were prepared as described previously [15].

GAS enzyme assay

GSMS was used to monitor GAS activities specifically in all the purification steps outlined below and was an adaptation of a previously described method [8]. The standard assay mixture contained 1 μl of enzyme solution [dialysed against 50 mM-sodium acetate buffer, pH 5.5, containing 10% (v/v) glycerol, 250 mM-NaCl and 0.1 mM-dithioerythritol), 20 pmol of GSMS and bovine serum albumin (50 μg/ml) in 60 mM-sodium formate buffer, pH 3.2, in a total volume of 20 μl. The mixture was incubated at 37 °C for a time interval varying from 5 min to 2 h to give between 5 and 20% breakdown of substrate to product. Reactions were stopped by freezing in a solid-CO_2/ethanol bath, and the substrate was separated from the product by high-voltage electrophoresis on Whatman 3MM chromatography paper in 0.74 M-formic acid, pH 1.7, at 45 V/cm for 40 min in a Shandon Southern model L-24 system (Shandon Southern Products, Runcorn, Cheshire, U.K.). Areas of radioactivity were cut from the strip, then placed in 20 ml glass scintillation vials along with 5 ml of water and 10 ml of scintillation cocktail, and their radioactivities were determined in an Isocap 300 liquid-scintillation system (Searle Analytic, Des Plaines, IL, U.S.A.). Enzyme activity was determined from the percentage breakdown of substrate to product (product included GMS and MS resulting from the subsequent action of β-glucuronidase), and was expressed as nmol of product/min per mg of protein or pmol of product/min per μl of enzyme. The pH–enzyme activity profile was determined by using the standard assay mixture in 60 mM-sodium formate buffer, pH 2.4–4.2, and 60 mM-sodium acetate buffer, pH 3.4–6.0. K_m and $V_{max.}$ values were obtained from Lineweaver–Burk plots with GSMS and GSTS assayed within a concentration range of 0.4–20 μM in 60 mM-sodium formate buffer, pH 3.2 and 3.0 respectively.

Other enzyme activities

Sulphamate sulphohydrolase [18], α-L-iduronidase [13], iduronate 2-sulphatase [15], *N*-acetylgalactosamine 4-sulphatase [19], β-*N*-acetylhexosaminidase [20], α-*N*-acetylglucosaminidase [21], β-glucuronidase [22], *N*-acetylgalactosamine 6-sulphatase [23] and *N*-acetylglucosamine 6-sulphatase [12] were assayed by previously reported procedures.

Tissue contents of GAS

Human liver, kidney and lung (10 g) were homogenized in 3 vol. of 15 mM-sodium dimethylglutarate buffer, pH 6.0, containing 500 mM-NaCl and 0.1 mM-dithioerythritol in Ultra-Turrax homogenizer. Cultured human skin fibroblasts were harvested at confluency and freeze–thawed [16]. GAS activity was assayed at 37 °C for 1 h (liver, kidney and lung) or 4 h (skin fibroblasts).

Purification and solubilization of GAS from human liver

All steps were performed at 4 °C and all buffers contained 0.1 mM-dithioerythritol, and, with the exception of buffer A, or where ethylene glycol was present, all buffers contained 10% (v/v) glycerol. Human liver (800 g) was homogenized in 3 vol. of 15 mM-sodium dimethylglutarate buffer, pH 6.0, containing 500 mM-

NaCl (buffer A) and centrifuged as previously described [10,11].

Concanavalin A–Sepharose/Blue A–agarose chromatography. The 27000 *g* supernatant of the homogenate was applied to a 300 ml column of concanavalin A–Sepharose equilibrated in and subsequently washed with 7 litres of buffer A. The enzyme was transferred from the concanavalin A–Sepharose column to a 300 ml column of Blue A–agarose by using a method described elsewhere [10,11]. Briefly, the two columns were connected and the enzyme was eluted on to the Blue A–agarose by the recycling of 750 ml of 15% (w/v) methyl α-mannoside in buffer A for 24 h at a flow rate of 2 ml/min over the joined columns. The columns were washed free of methyl α-mannoside with 2 litres of buffer A. The Blue A–agarose column was washed in turn with 1.5 litres of 30 mM-Tris/HCl buffer, pH 7.5 (buffer B), containing 0.5 M-NaCl and 1.5 litres of buffer B containing 1 M-NaCl, before GAS activity was eluted with 1.5 litres of buffer B containing 1.7 M-NaCl. This fraction containing GAS activity was concentrated to 200 ml by an Amicon hollow-fibre concentrator and further concentrated to 20 ml in an Amicon ultrafiltration stirred cell containing a YM10 membrane. The concentrates from three separate liver preparations that had been stored at 4 °C for less than 3 weeks were pooled for the next step.

DEAE-Sephacel/octyl-Sepharose chromatography (Fig. 1). The concentrated enzyme solution was dialysed overnight against 5 litres of 20 mM-Tris/HCl buffer, pH 7.5 (buffer C), any precipitate was removed by centrification at 4000 *g* for 10 min, and the supernatant was applied to a DEAE-Sephacel ion-exchange column (1 cm × 10 cm) coupled to an octyl-Sepharose hydrophobic column (0.7 cm × 10 cm), both of which were equilibrated in buffer C. The combined columns were washed with a further 60 ml of buffer C. The octyl-Sepharose column was washed in turn with 50 ml of 15 mM-sodium dimethylglutarate buffer, pH 7.4, containing 30 mM-NaCl (buffer D) and 50 ml of buffer D containing 15% (v/v) ethylene glycol, and GAS activity was eluted with 20 ml of buffer D containing 60% (v/v) ethylene glycol. Approx. 8% of the GAS activity applied to the coupled columns remained bound to the DEAE-Sephacel column and was eluted with 50 ml of buffer C containing 500 mM-NaCl.

CM-Sepharose chromatography (Fig. 2). The eluate from octyl-Sepharose containing GAS activity was diluted 1:1 (v/v) with buffer D and applied to a CM-Sepharose column (0.9 cm × 5 cm) equilibrated in buffer D. The column was washed with 30 ml each of buffer D and buffer D containing 75 mM-NaCl. GAS activity was eluted with 12 ml of buffer D containing 300 mM-NaCl.

TSK G-3000 SW chromatography (Fig. 3). The fraction containing GAS activity was concentrated to approx. 200 μl in an Amicon Centricon 10 Microconcentrator, 2 ml of 50 mM-sodium acetate buffer, pH 5.6, containing 500 mM-NaCl (buffer E) was then added and the solution was again concentrated to approx. 200 μl. This was applied to a TSK G-3000 SW Ultrapac column (0.8 cm × 30 cm) equilibrated in and subsequently developed in buffer E. The LKB Ultrachrom GTI Bioseparation system was run at 1.5 MPa pressure at a flow rate 0.5 ml/min with fractions collected every 30 s. Protein was detected at 280 nm.

Chromatofocusing chromatography of GAS activity (Fig. 4)

The 27000 *g* supernatant resulting from the homogenization of 50 g of human liver was applied to a 25 ml column of concanavalin A–Sepharose equilibrated in buffer A as described above. The column was washed with 200 ml of buffer A and eluted with 200 ml of buffer A containing 15% (w/v) methyl α-mannoside. The eluate was concentrated to 30 ml in an Amicon ultrafiltration stirred cell containing a YM10 membrane, and was dialysed overnight against 1 litre of buffer C and applied to a chromatofocusing PBE 94 column (0.9 cm × 15 cm) equilibrated in buffer C. The column was washed with 45 ml of buffer C before the application of 160 ml of a 1:8 dilution of Polybuffer 74/HCl, pH 4.0, followed by 50 ml of the same buffer containing 500 mM-NaCl (Fig. 4*a*).

A 1-litre volume of morning void urine from an adult male was concentrated to 50 ml, and dialysed overnight against 2 litres of buffer A, before application to concanavalin A–Sepharose and the chromatofocusing column as described above (Fig. 4*b*). Cultured human skin fibroblasts from four 50 ml culture bottles were harvested at confluency as previously described [16], freeze–thawed six times in 500 μl of buffer A and dialysed overnight against 200 ml of buffer C. The suspension was centrifuged at 4000 *g* for 10 min and the supernatant was applied to a 3 ml PBE 94 column equilibrated in buffer C. The column was washed with 15 ml of buffer C before the application of 30 ml of a 1:8 dilution of Polybuffer 74/HCl, pH 4.0, followed by 6 ml of the same buffer containing 500 mM-NaCl (Fig. 4*c*).

SDS/polyacrylamide-gel electrophoresis (Fig. 5)

Samples were prepared by a deoxycholate/trichloroacetic acid co-precipitation method [24], and reduced by boiling with dithioerythritol. Discontinuous SDS/polyacrylamide gels (12.0% acrylamide) [25] were stained with Coomassie Brilliant Blue R250 or with silver [26].

RESULTS

Preparation of substrates for GAS activity

The disaccharide substrates GSMS and GSTS for the assay of GAS activity towards heparin, heparan sulphate and chondroitin 6-sulphate were prepared by previously described procedures [8,17]. However, separation of GSMS from its C-5 epimer, ISMS, was accomplished by using conditions for the descending chromatography that allowed separation of the substrates with only one passage of buffer for 72 h, instead of the reported nine 20 h descents [8]. Separation of GSTS from the less-sulphated disaccharides was accomplished by chromatography on Dowex 1, similarly to the preparation of ISTS from chondroitin 4-sulphate [6]. Purified GAS desulphated both GSMS and GSTS, to give GMS and GTS respectively, which have been previously described [6,15]. Purified iduronate 2-sulphatase was not active towards either GSMS or GSTS when assayed under conditions that would normally desulphate ISMS 100 times over [15].

Table 1. Tissue contents of GAS activity

For full experimental details see the Materials and methods section.

Tissue source	Enzyme activity (pmol/min per mg of protein)
Liver	3.3
Lung	2.2
Kidney	7.1
Skin fibroblasts ($n = 2$)	3.1, 7.3

Purification of GAS

Each tissue assayed had similar activities when expressed as pmol/min per mg of protein (Table 1). Liver was chosen because of its on-going use for purification of a number of lysosomal enzymes involved in the degradation of heparan sulphate and dermatan sulphate [10,12–14,27,28]. Complete solubilization of GAS activity required homogenization in buffer containing 0.5 M-NaCl similar to that reported for the isolation of α-L-iduronidase [13]. Only 10% of the total GAS activity was solubilized by buffer containing only 40 mM-NaCl or 40 mM-NaCl and 3% (v/v) Triton X-100. All of the applied GAS activity bound to the concanavalin A–Sepharose column, demonstrating the glycoprotein nature of the enzyme. GAS activity was bound tightly to the Blue A–agarose column, requiring 1.7 M-NaCl for complete elution of activity, giving a 1450-fold purification with 37% recovery of enzyme activity from the original liver homogenate (Table 2). Although over 90% of the total β-glucuronidase, β-*N*-acetylhexosaminidase, α-*N*-acetylglucosaminidase and iduronate 2-sulphatase did not bind to Blue A–agarose, small but significant amounts (1–2% of total) of these enzyme activities co-purified with GAS at this step. To maintain enzyme stability during the latter stages of the purification procedure, it was necessary to pool the Blue A–agarose eluates from three separate liver preparations. The application of enzyme to coupled DEAE-Sephacel/octyl-Sepharose columns resulted in most of the applied GAS activity passing through the DEAE-Sephacel column to be bound by the octyl-Sepharose column (Fig. 1), whereas most of the iduronate 2-sulphatase activity remained bound to the DEAE-Sephacel column (Table 2), allowing an almost complete separation of sulphatase activities that act towards glucuronic acid and iduronic acid residues. Elution of the octyl-Sepharose column resulted in a 32000-fold purification of GAS activity (Table 2). Elution of the DEAE-Sephacel column with buffer containing 0.5 M-NaCl allowed a recovery of at least three more acidic forms of GAS activity (approx. 8% of the GAS activity applied to the column). These acidic forms were not further characterized because of their low abundance and problems with enzyme stability (see below). β-Glucuronidase activity paralleled GAS activity in that 90% of the applied activity passed through the DEAE-Sephacel column, whereas at least three minor forms remained bound. The remaining β-glucuronidase and iduronate 2-sulphatase activity was separated from GAS activity by chromatography on CM-Sepharose (Fig. 2), resulting in a 700000-fold purification of GAS activity (Table 2). Gradient elution of GAS activity resulted in identical profiles of GAS activity and eluted protein. Final purification of GAS to homogeneity was achieved by gel-

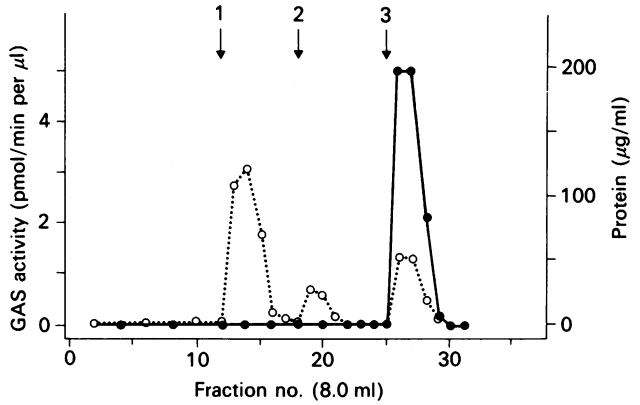

Fig. 1. Octyl-Sepharose chromatography of GAS activity after separation of the column from the DEAE-Sephacel column

For experimental details see the Materials and methods section. The arrows indicate buffer changes: buffer D containing (1) 0%, (2) 15% and (3) 60% (v/v) ethylene glycol. Fractions indicated were assayed for GAS activity (●) and protein (○).

Table 2. Purification of GAS from 2400 g of human liver

For full experimental details see the Materials and methods section. Abbreviation: N.D., none detected.

Step	Total GAS activity (nmol/min)	Total protein (mg)	Specific activity (nmol/min per mg)	Purification (fold)	Recovery (%)	Total iduronate 2-sulphatase activity (nmol/min)
Homogenate	642	182000	0.0035	1	100	4253
Supernatant	375	64000	0.006	1.7	58	4536
Concanavalin A–Sepharose/ Blue A–agarose	237	46.8	5.1	1450	37	65
DEAE-Sephacel/octyl-Sepharose	102	0.92	111	31700	16	1.5
CM-Sepharose	66	0.03	2460	703000	10.3	N.D.
TSK G-3000 SW	53	0.008	6650	1900000	8.3	N.D.

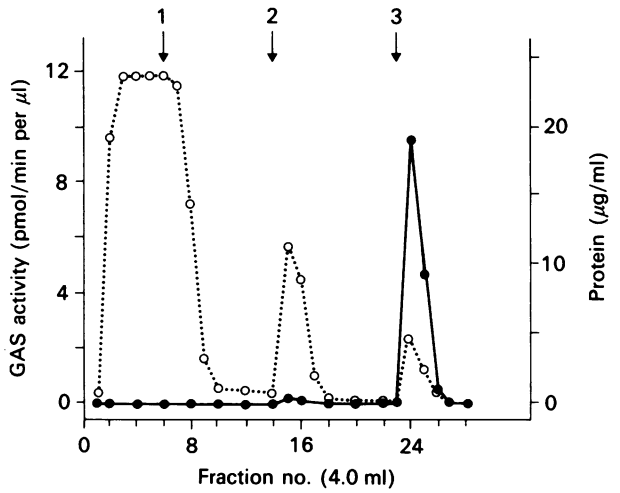

Fig. 2. CM-Sepharose chromatography of GAS activity

For experimental details see the Materials and methods section. The arrows indicate buffer changes: buffer D containing (1) 30 mM-, (2) 75 mM- and (3) 300 mM-NaCl. Fractions indicated were assayed for GAS activity (●) and protein (○).

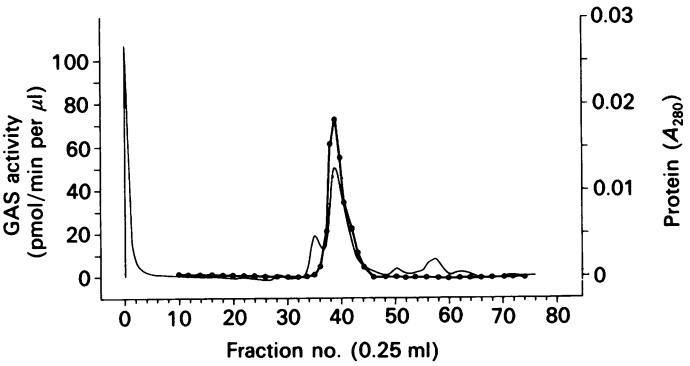

Fig. 3. TSK G-3000 SW chromatography of GAS activity

For experimental details see the Materials and methods section. Fractions indicated were assayed for GAS activity (●) and protein (A_{280}, ——). An LKB molecular-mass calibration kit consisting of γ-globulin (160 kDa), human serum albumin (68 kDa), ovalbumin (49 kDa) and myoglobulin (17 kDa) was used.

permeation chromatography on TSK G-3000 SW (Fig. 3), giving an almost 2000000-fold purification with an 8% recovery of GAS activity from the original liver homogenate. No other glycosaminoglycan-degrading lysosomal enzyme activities were detected in the final enzyme preparation.

Chromatofocusing chromatography of GAS activity

The application of GAS activity from human liver to a chromatofocusing column resulted in most (>90%) of the GAS activity passing through the column, indicating an apparent pI value greater than 7.5 (Fig. 4*a*). Elution of the column with Polybuffer resulted in the separation of three minor forms with apparent pI values of 5.8, 5.3 and 4.7. No further GAS activity was eluted by the addition of 0.5 M-NaCl. GAS activity from human urine behaved differently from the liver enzyme, as a small proportion of enzyme activity passed through the chromatofocusing column, whereas most GAS activity was eluted in approximately equal amounts as acidic forms with apparent pI values of 5.8, 5.2 and 4.6 and a more acidic form (pI < 4.0) eluted by the addition of 0.5 M-NaCl (Fig. 4*b*). Human skin fibroblast GAS activity was different again, with the major form with a pI value of 6.5 and minor forms with pI values of greater than 7.5, 5.8, 5.4 and a more acidic form (pI < 4.0) eluted by the addition of 0.5 M-NaCl (Fig. 4*c*).

Native and subunit molecular mass of GAS

TSK G-3000 SW chromatography resulted in the elution of a single peak of GAS activity with a molecular mass of 63 kDa. SDS/polyacrylamide-gel electrophoresis under reducing conditions showed the presence of two subunits with molecular masses of 47 and 19.5 kDa (Fig. 5), which is consistent with the observed native molecular mass.

Storage stability of GAS activity

GAS preparations eluted from the Blue A–agarose, DEAE-Sephacel and octyl-Sepharose steps retained at least 90% of their initial activity after 1 month stored in buffer E at 4 °C. However, both the CM-Sepharose and TSK G-3000 SW eluates were unstable, losing half their activity during 48 h. The addition of bovine serum albumin at a concentration of 0.1 mg/ml stabilized the enzyme activity for up to 1 month. Small portions of GAS preparations were stored with bovine serum albumin before kinetic studies.

Assay of GAS activity with GSMS and GSTS

Purified human liver GAS displayed pH optima of 3.2 and 3.0 towards GSMS and GSTS respectively when assayed in sodium formate buffer in the presence of bovine serum albumin at a final concentration of 50 μg/ml. Enzyme activity was linear with respect to time for incubations up to 15 h and for incubations containing between 20 pg and 1 ng of enzyme protein per assay in the presence of bovine serum albumin (50 μg/ml). Lineweaver–Burk plots resulted in apparent K_m values of 0.3 and 0.6 μM for activity towards GSMS and GSTS respectively, and with corresponding $V_{max.}$ values of 12.8 and 13.7 μmol/min per mg of protein respectively (Fig. 6). GAS activity present in the Blue A–agarose eluate also had an apparent K_m value of 0.3 μM at a pH optimum of 3.2 towards GSMS both in the presence and in the absence of added bovine serum albumin (50 μg/ml). Purified GAS preparations displayed substrate inhibition when GSMS and GSTS were assayed at substrate concentrations above 4 μM (Fig. 6). Similar substrate inhibition occurred for GAS activity present in the Blue A–agarose eluate. We have previously observed substrate inhibition by highly sulphated substrates acted upon by sulphamate sulphohydrolase [27] and *N*-acetylglucosamine 6-sulphatase [28]. Increasing the concentration of bovine serum albumin from a final value of 50 μg to 1 mg/ml delayed the onset of substrate inhibition towards both substrates from 4 μM to 15 μM. No change in pH optima was observed with increased albumin concentration. The apparent K_m was unaffected by the increased albumin concentration for both substrates; however, the $V_{max.}$ increased by 50%. GAS activity present in the Blue A–agarose eluate was similarly stimulated by the increased concentration of albumin without affecting the

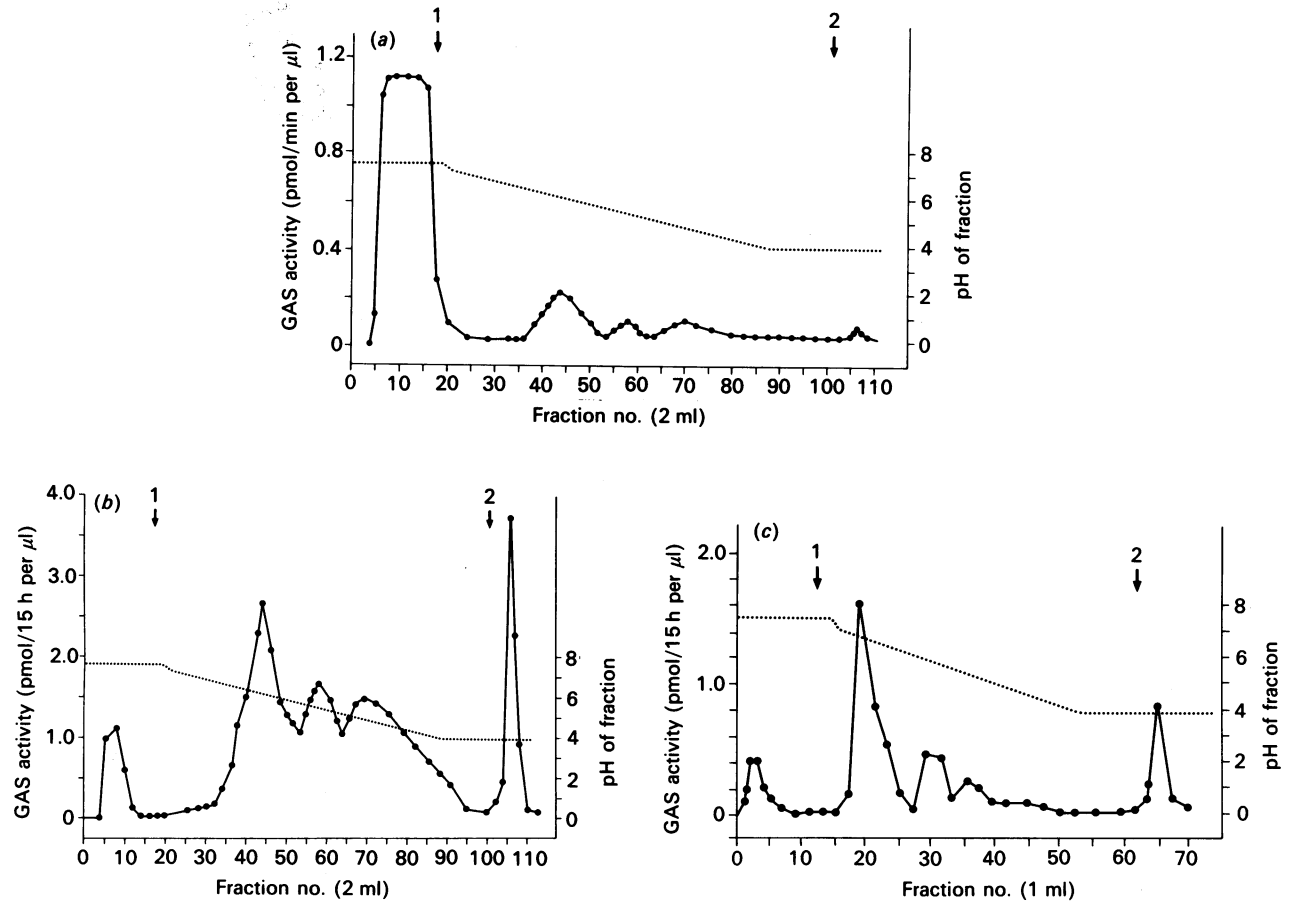

Fig. 4. Chromatofocusing chromatography of GAS activity from (*a*) human liver, (*b*) human urine and (*c*) cultured human skin fibroblasts.

For experimental details see the Materials and methods section. Arrows indicate (1) the start of Polybuffer elution (·····, pH) and (2) Polybuffer containing 500 mM-NaCl. Fractions were assayed for GAS activity (●).

apparent K_m. The stimulation and stabilization of enzyme activity by bovine serum albumin has been reported for other lysosomal sulphatases [27,28]; however, the mechanism by which albumin acts is unknown.

Effects of salts and substrate analogues on GAS activity towards GSMS

We examined the effect of a range of NaCl, Na_2SO_4, NaH_2PO_4 and $CuCl_2$ concentrations upon purified GAS activity during incubation at its optimal pH of 3.2. A 50% inhibition of GAS activity was observed at 75 mM-NaCl. Both Na_2SO_4 and NaH_2PO_4 were potent inhibitors of GAS activity, with 50% inhibition of enzyme activity at concentrations of 80 and 250 μM respectively. $CuCl_2$ and $ZnCl_2$ (10 mM) stimulated GAS activity by 30%. Previously $CuCl_2$ has been reported as either a potent inhibitor of lysosomal enzyme activity [13,27] or to have no effect [28]. $MnCl_2$, $MgCl_2$ and $CaCl_2$ at 10 mM had no detectable effect on GAS activity. EDTA at a concentration of 2 mM inhibited GAS activity by 50%, suggesting the possibility of an endogenous cation cofactor. ISMS, which was not a substrate for GAS activity, inhibited by 50% at 6 μM-ISMS. GAS activity was inhibited by its product GMS by 50% at 1 μM-GMS. D-Saccharic acid 1,4-lactone, an inhibitor of β-glucuronidase, did not inhibit GAS activity at a concentration of 1 mM.

DISCUSSION

We have purified GAS from human liver by almost 2000000-fold with a net recovery of 8% activity by a six-column four-step procedure that resulted in the complete separation of sulphatase activities towards 2-sulphated iduronic acid and glucuronic acid. The purification of GAS was an extension of a procedure used to purify several other lysosomal enzymes involved in glycosaminoglycan degradation from the same liver preparation [10–14,27,28]. Purified GAS activity towards the heparin-derived and chondroitin 6-sulphate-derived disaccharide substrates GSMS and GSTS respectively was maximal at pH values of 3.2 and 3.0 respectively, indicating its probable lysosomal localization. Rat liver GAS activity had a similar subcellular localization in Percoll gradients to β-*N*-acetylhexosaminidase, α-L-iduronidase and other lysosomal exoenzyme activities (C. Freeman, unpublished work).

Chromatofocusing chromatography of GAS activity confirmed the observation that several of the lysosomal glycosaminoglycan-degrading enzymes (GAS, *N*-acetylglucosamine 6-sulphatase, sulphamate sulphohydrolase and β-glucuronidase) exist in human liver predominately in higher-pI forms (pI > 7.5) than are found in other tissues or urine ([10,12,13]; C. Freeman, unpublished work). Forms of GAS that have apparent pI values of

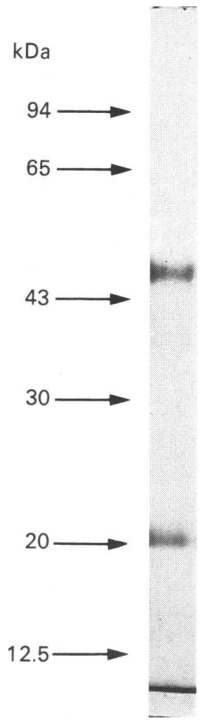

Fig. 5. SDS/polyacrylamide-gel electrophoresis of purified GAS activity

For experimental details see the Materials and methods section. The gel shown was stained with silver and the tight intense band seen below the 12.5 kDa marker represents the dye-front position of the gel electrophoresis. Arrows indicated positions of the molecular-mass 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 (12.5 kDa).

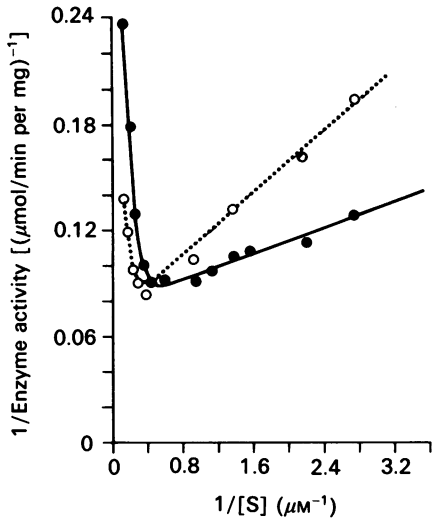

Fig. 6. Hydrolysis of GSMS (●) and GSTS (○) by purified GAS as a function of substrate concentration

For experimental details see the Materials and methods section.

less than 7.5 were more predominant in other tissues or urine, suggesting that these tissues may process lysosomal enzymes differently.

To date, patients deficient in GAS activity have not been described. On the basis of an expected low concentration of stored endogenous heparan sulphate substrate in their tissues, these patients would be expected to show a mild Sanfilippo (MPS III) clinical phenotype. The low abundance of GAS enzyme protein (approx. 10–30 μg per adult liver) may be related to the low abundance of GlcA2S reported to be present in heparan sulphate and chondroitin 6-sulphate [7,17,29,30]. The relative concentrations of GlcA2S found during subcellular fractionation of cultured hepatocyte heparan sulphate were reported to vary considerably, depending upon the growth stage of the cells, with the highest concentrations occurring in the nuclear pool during the confluency stage of cell growth [9,31]. It has been suggested that sulphation of glucuronic acid residues in heparan sulphate may inhibit the action of endo-β-glucuronidase activity, and allow diversion of heparan sulphate fragments with a high GlcA2S content to the nucleus [2,9]. The role of GlcA2S in chondroitin 6-sulphate is not known. Chondroitin AC lyase I from *Flavobacterium heparinium* acts more slowly on hexosaminidic linkages adjacent to sulphated glucuronic acid residues than when adjacent to non-sulphated glucuronic acid [29]. The proportions of GlcA2S residues in chondroitin 6-sulphate of different tissues varied considerably from being absent in sturgeon notochord to between 14 and 21% of the uronic acid present in shark fin chondroitin 6-sulphate [29]. Shark scapular cartilage (commercial chondroitin sulphate type C used here for the preparation of GSTS) contained approx. 8% of the glucuronic acid residues 2-sulphated [29]. King-crab cartilage chondroitin sulphate has been reported to contain glucuronic acid 3-sulphate (GlcA3S) residues as distinct from GlcA2S residues found in shark cartilage [32]. To date, no enzyme capable of desulphation of GlcA3S residues has been described. We have shown that purified GAS will desulphate GlcA2S residues presumed to be present in both heparin-derived and chondroitin 6-sulphate-derived substrates. The high substrate specificity of the different lysosomal sulphatases that we have studied [2,27,28] thus far suggests that it would be unlikely that a 2-sulphatase enzyme would act towards 3-sulphated residues.

We have postulated [2,12,13] the existence of specific interactions between lysosomal enzymes that act upon a common substrate (e.g. heparan sulphate). This association into a multienzyme complex would allow the compartmentalization of intermediate substrates whereby the product of one enzyme is immediately available to become the substrate for the next enzyme in the degradative pathway. The heparan sulphate-degrading enzymes may be located in a large complex within close proximity to acetyl-CoA:glucosamine *N*-acetyltransferase, an integral lysosomal membrane protein, which requires cytosolic-derived acetyl-CoA to enable heparan sulphate degradation to proceed [2,33]. There are several observations in our study of GAS that support the hypothesis of a coupled process for glycosaminoglycan degradation. Firstly, the requirements of high NaCl concentrations for complete solubilization of GAS activity is similar to that reported for other heparan sulphate-degrading enzyme activities [12,13], and is an indication of strong interaction with the lysosomal membrane. Secondly, kinetic studies of GAS activity

have shown the presence of both substrate (GSMS) and product (GMS) inhibition, as well as inhibition by related substrates (ISMS) for other lysosomal enzymes but that GAS does not hydrolyse. Substrate and product inhibition with other heparan sulphate-degrading enzymes have also been reported [13,27,28]. An ordered multienzyme complex would protect these enzymes from substrate, product and other substrate inhibitory influences. Thirdly, co-purification of GAS with β-glucuronidase, β-*N*-acetylhexosaminidase and α-*N*-acetylglucosaminidase activities (the preceding and subsequent enzymes to GAS activity towards heparan sulphate or chondroitin sulphate degradation) through the early stages of the GAS purification procedure is consistent with the proposal that specific interaction between the enzymes to form a co-operative complex would achieve the most efficient turnover of complex substrates such as heparan sulphate to monosaccharides and sulphate. Whether this clearly speculative model is close to cellular reality or not remains to be determined.

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REFERENCES

1. Rodén, L. (1980) in The Biochemistry of Glycoproteins and Proteoglycans (Lennarz, W. J., ed.), pp. 267–371, Plenum Press, New York
2. Hopwood, J. J. (1989) in Heparin (Lane, D. & Lindahl, U., eds.), Edward Arnold, London, in the press
3. McKusick, V. A. & Neufeld, E. F. (1983) in The Metabolic Basis of Inherited Disease (Stanbury, J. B., Wyngaarden, J. B., Fredrickson, D. S., Goldstein, J. L. & Brown, M. S., eds.), 5th edn., pp. 751–771, McGraw-Hill, New York
4. Gallagher, J. T., Lyon, M. & Steward, W. P. (1986) Biochem. J. **236**, 313–325
5. Lim, T. W., Leder, I. G., Bach, G. & Neufeld, E. F. (1974) Carbohydr. Res. **37**, 103–109
6. Hopwood, J. J. & Muller, V. J. (1983) Carbohydr. Res. **122**, 227–239
7. Bienkowski, M. J. & Conrad, H. E. (1985) J. Biol. Chem. **260**, 356–365
8. Shaklee, P. N., Glaser, J. H. & Conrad, H. E. (1985) J. Biol. Chem. **260**, 9146–9149
9. Fedarko, N. S. & Conrad, H. E. (1986) J. Cell Biol. **102**, 587–599
10. Mahuran, D. J., Clements, P. R. & Hopwood, J. J. (1983) Biochim. Biophys. Acta **757**, 359–365
11. Clements, P. R., Mahuran, D. J. & Hopwood, J. J. (1983) J. Chromatogr. **261**, 77–82
12. Freeman, C., Clements, P. R. & Hopwood, J. J. (1987) Biochem. J. **246**, 347–354
13. Clements, P. R., Brooks, D. A., Saccone, G. T. P. & Hopwood, J. J. (1985) Eur. J. Biochem. **152**, 21–28
14. Gibson, G. J., Saccone, G. T. P., Brooks, D. A., Clements, P. R. & Hopwood, J. J. (1987) Biochem. J. **248**, 755–764
15. Hopwood, J. J. (1979) Carbohydr. Res. **69**, 203–216
16. Hopwood, J. J., Muller, V. J. & Pollard, A. C. (1979) Clin. Sci. **56**, 591–599
17. Shaklee, P. N. & Conrad, H. E. (1986) Biochem. J. **235**, 225–236
18. Hopwood, J. J. & Elliott, H. (1982) Clin. Chim. Acta **123**, 241–250
19. Hopwood, J. J., Elliott, H., Muller, V. J. & Saccone, G. T. P. (1986) Biochem. J. **234**, 507–514
20. Hopwood, J. J. & Elliott H. (1983) Biochem. Int. **6**, 141–148
21. Hopwood, J. J. & Elliott, H. (1982) Clin. Chim. Acta **120**, 77–86
22. Muller, V. J. & Hopwood, J. J. (1982) Clin. Chim. Acta **123**, 357–360
23. Hopwood, J. J. & Elliott, H. (1983) Clin. Sci. **65**, 325–331
24. Mahuran, D., Clements, P. R., Carella, M. & Strasberg, P. M. (1983) Anal. Biochem. **129**, 513–516
25. Laemmli, U. K. (1970) Nature (London) **227**, 680–685
26. Merril, C. R., Goldman, D., Sedman, S. A. & Ebert, M. H. (1981) Science **211**, 1437–1438
27. Freeman, C. & Hopwood, J. J. (1986) Biochem. J. **234**, 83–92
28. Freeman, C. & Hopwood, J. J. (1987) Biochem. J. **246**, 355–365
29. Yamagata, M., Kimata, K., Oike, Y., Tani, K., Maeda, N., Yoshida, K., Shimomura, Y., Yoneda, M. & Suzuki, S. (1987) J. Biol. Chem. **262**, 4146–4152
30. Marcum, J. A., Atha, D. H., Fritze, M. S., Nawroth, P., Stern, D. & Rosenberg, R. D. (1986) J. Biol. Chem. **261**, 7507–7517
31. Ishihara, M., Fedarko, N. S. & Conrad, H. E. (1986) J. Biol. Chem. **261**, 13575–13580
32. Seno, N. & Murakami, K. (1982) Carbohydr. Res. **103**, 190–194
33. Bame, K. J. & Rome, L. H. (1985) J. Biol. Chem. **260**, 11293–11299

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