Human liver *N*-acetylglucosamine-6-sulphate sulphatase

Purification and characterization

Craig FREEMAN, Peter R. CLEMENTS and John J. HOPWOOD*

Department of Chemical Pathology, Adelaide Children's Hospital, King William Road, North Adelaide, South Australia 5006, Australia

Human *N*-acetylglucosamine-6-sulphate sulphatase was purified at least 50000-fold to homogeneity in 78% yield from liver with a simple three-step four-column procedure, which consists of a concanavalin A-Sepharose/Blue A-agarose coupled step, chromatofocusing and Cu²⁺-chelating Sepharose chromatography. In all, four forms were isolated and partially characterized. Forms A and B, both with a pI greater than 9.5 and representing 30% and 60% respectively of the recovered enzyme activity, were separated by hydroxyapatite chromatography of the enzyme preparation obtained from the Cu²⁺-chelating Sepharose step. Both forms A and B had native molecular masses of 75 kDa. When analysed by SDS/polyacrylamide-gel electrophoresis, form A consists of a single polypeptide of molecular mass 78 kDa, whereas form B contained 48 kDa and 32 kDa polypeptide subunits. Neither form A nor form B was taken up from the culture medium into cultured human skin fibroblasts. The two other forms (C and D), with pI values of 5.8 and 5.4 respectively, represented approx. 7% and 3% of the total recovered enzyme activity. The native molecular masses of forms C and D were 94 kDa and approx. 75 kDa respectively. Form C contained three polypeptides with molecular masses of 48, 45 and 32 kDa. *N*-Acetylglucosamine-6-sulphate sulphatase activity was measured with a radiolabelled disaccharide substrate derived from heparin. The development of this substrate enabled the isolation and characterization of *N*-acetylglucosamine-6-sulphate sulphatase to proceed efficiently. Forms A, B and C had pH optima of 5.0, *K*_m values of 11.7, 14.2 and 11.1 μM respectively and *V*_{max} values of 105, 60 and 53 nmol/min per mg of protein respectively. The molecular basis of the multiple forms of this sulphatase is not known. It is postulated that the differences in structure and properties of the four enzyme forms are due to differences in the state of processing of a large subunit.

INTRODUCTION

The sulphated glycosaminoglycan heparan sulphate is degraded from its non-reducing end by the sequential action of at least nine different lysosomal exo-enzyme activities [1,2]. The clinical expression of the Sanfilippo syndrome (mucopolysaccharidosis type III, MPS III), which classically presents with progressive mental retardation in conjunction with mild skeletal deformities, is a result of a deficiency of any one of four of these nine different enzyme activities, leading to the accumulation of partially degraded heparan sulphate in tissues and its excretion in urine [3]. The de-*O*-sulphation of non-reducing terminal *N*-acetylglucosamine 6-sulphate (GlcNAc6S) residues in heparan sulphate requires a specific exo-sulphatase, namely *N*-acetylglucosamine-6-sulphate sulphatase (6SS), which has been shown to be deficient in Sanfilippo D (MPS IIID) patients [4].

We have previously shown in cultured human skin fibroblasts that the same 6SS enzyme activity will act upon non-reducing terminal GlcNAc6S present in both heparan sulphate [linked α-(1→4) to a uronic acid residue] and keratan sulphate [linked β-(1→3) to a galactose residue] [5]. Keratan sulphate is not detected in

the urine of MPS IIID patients because of an alternative pathway of degradation, which probably proceeds by the action of the A form of β-*N*-acetylglucosaminidase to produce GlcNAc6S, which is not further degraded and is found in urine from these patients [6–8].

A single form of 6SS with activity towards GlcNAc6S at the non-reducing terminal of a heparan sulphate-derived trisaccharide has been purified 136-fold from human urine with a recovery of 1% enzyme activity and shown to have a molecular mass of 97 kDa [9]. Habuchi *et al.* [10] have reported 6SS activity in rat skin towards GlcNAc6S residues at the non-reducing end of heparan sulphate- and keratan sulphate-derived substrates with a molecular mass of 70 kDa. Evidence of a sulphatase activity towards non-reducing terminal 2-sulphamino-glucosamine 6-sulphate residues in heparan sulphate distinct from activity towards GlcNAc6S residues has been reported to be present in bovine kidney extracts [11]. Resolution of questions raised in these studies [5–11] about the physical properties and substrate-specificity of 6SS requires the purification of this low-abundance protein to homogeneity in high yields. We now report the purification to homogeneity of two major forms of 6SS from human liver, both with native molecular masses of

Abbreviations used (for simplicity IdOA has been used to abbreviate what is a mixture of reduced uronic acid species containing mostly IdOA): GlcNH6S-IdOA, *O*-(α-glucosamine 6-sulphate)-(1→3)-L-[6-³H]idonic acid; GlcNAc6S-IdOA, *O*-(α-*N*-acetylglucosamine 6-sulphate)-(1→3)-L-[6-³H]idonic acid; GlcNS6S-IdOA, *O*-(α-2-sulphaminoglucosamine 6-sulphate)-(1→3)-L-[6-³H]idonic acid; GlcNAc6S-Galitol, *O*-(β-*N*-acetylglucosamine 6-sulphate)-(1→3)-D-[1-³H]galactitol; 6SS, *N*-acetylglucosamine-6-sulphate sulphatase; MPS, mucopolysaccharidosis.

* To whom correspondence should be addressed.

75 kDa, purified 57000-fold with a recovery of 78% enzyme activity. Two lower-abundance forms have also been partially purified. Enzyme activity was towards an easily prepared ^3H -labelled disaccharide substrate derived from heparin.

MATERIALS AND METHODS

Materials

Human livers, obtained from autopsies of normal adults with intervals *post mortem* ranging from 24 to 72 h, were stored at -20°C . Pharmacia Fine Chemicals (Uppsala, Sweden) supplied concanavalin A–Sephacrose, chelating Sepharose 6B, the chromatofocusing media PBE 94 and Polybuffer 74, Sephadex G-10 and G-25 (superfine grade) and the molecular-mass-standard kits for both gel filtration and SDS/polyacrylamide-gel electrophoresis. Amicon Corp. (Danvers, MA, U.S.A.) supplied Blue A Matrex agarose gel and a DC-2 hollow-fibre concentrator with 10 kDa-cut-off hollow fibres and an ultrafiltration stirred cell (model 8200) with a Diaflo ultrafiltration membrane YM10. Bio-Rad Laboratories (Richmond, CA, U.S.A.) supplied silver stain and protein assay kits, and Dowex AG50W-X8 (100–200 mesh; H^+ form) and Dowex AG1-X4 (100–200 mesh; Cl^- form). Merck (Darmstadt, Germany) supplied Fractogel TSK HW55(S). LKB (Bromma, Sweden) supplied Ultrogel AcA 34 for gel permeation and Ultrogel HA for hydroxyapatite chromatography. Amersham International (Sydney, N.S.W., Australia) supplied NaB^3H_4 (10 Ci/mmol). Beckmann (Gladesville, N.S.W., Australia) supplied Ready Solv EP. Sigma Chemical Co. (St. Louis, MO, U.S.A.) supplied crystalline bovine serum albumin (globulin-free grade) and heparin (grade II prepared from pig intestinal mucosa). BDH chemicals (Poole, Dorset, U.K.) supplied molecular-sieve type 3A pellets and powder. All other chemicals were of the finest grade available. GlcNAc6S-Galitol was prepared from bovine intervertebral-disc keratan sulphate [5]. Fibroblast cultures were established from skin biopsies taken in or provided to this hospital. MPS IIID fibroblasts were obtained from a patient excreting excessive amounts of heparan sulphate and with deficient 6SS activity in leucocytes and cultured fibroblasts [6].

Preparation of 6SS substrates (GlcNAc6S-IdOA and GlcNS6S-IdOA)

A disaccharide fraction was prepared by mild HCl hydrolysis of cetylpyridinium chloride-precipitated heparin [12,13]. This disaccharide has been shown, subsequent to reduction with NaB^3H_4 , to consist of a mixture of glucosamine linked α -(1 $\rightarrow$ 3) to idonic acid, gulonic acid or anhydroidonic acid in the proportions 12:3:5 respectively [12]. The mixture was utilized without separation into its components. The non-reduced disaccharide fraction was 6-sulphated by an adaptation of published methods under conditions that favour sulphation of the primary alcohol groups [14–17]. The formation of the 6-sulphated monosaccharides occurs after reaction at 4°C . Multiple sulphation occurs if the reaction is allowed to reach room temperature. Glucosamine or galactosamine, however, could not be *O*-sulphated by this or other direct sulphation methods. A suggested reason for this difficulty was that the reaction between the free amino and aldehyde groups would lead to the formation of polymers [18]. However,

as shown below, it is possible to *O*-sulphate the above heparin-derived disaccharides directly.

A 5 ml portion of pyridine (dried over type 3A molecular sieves) was added to the desalted and freeze-dried disaccharide (7.4 mg, $21\ \mu\text{mol}$), and the mixture was rotary-evaporated to dryness in a pear-shaped flask. This procedure was repeated twice, before 5 ml of dry pyridine, 10 ml of dry dimethylformamide and approx. 50 mg of type 3A molecular-sieve powder were added and the stoppered flask was chilled in an ice bath. Chlorosulphonic acid ($150\ \mu\text{l}$, 2 mmol) in dried redistilled chloroform ($150\ \mu\text{l}$) was added in three equal portions over 10 min, and the mixture was stirred at 0°C for 1 h and then at room temperature (22°C) for a further 1 h. Next 10 ml of water was added, the mixture was centrifuged at 2000 g for 10 min and the supernatant was neutralized with 1 M-NaOH and rotary-evaporated to dryness. Then 20 ml of water was added, and the solution was neutralized and applied to a 5 ml column of Dowex 1 (Cl^- form), which was washed with 50 ml of water and then with 20 ml of 1 M-LiCl. The LiCl eluate was concentrated to 4 ml by rotary evaporation and desalted on a Sephadex G-10 column (100 cm $\times$ 1 cm diam.) developed in 10% (v/v) ethanol in water. The uronic acid-positive fractions, determined by the method of Bitter & Muir [19], were pooled and concentrated to 1 ml by rotary evaporation, $50\ \mu\text{l}$ of 1 M- BaCl_2 was added and the precipitate (BaSO_4) was removed by centrifugation, and the procedure was repeated until a precipitate was no longer observed. The supernatant was applied to a 2 ml column of Dowex 50 (H^+ form) and washed with 10 ml of water, and the eluate was concentrated and then desalted on a Sephadex G-10 column, as described above, to give a 6-sulphated disaccharide mixture (2.8 mg, $8\ \mu\text{mol}$) in 38% yield on recovered uronic acid. The freeze-dried 6-sulphated disaccharides were radiolabelled by reduction with $100\ \mu\text{Ci}$ of NaB^3H_4 (10 Ci/mmol) by a method described [13] to give a disaccharide mixture consisting of *O*-(α -2-glucosamine 6-sulphate)-(1 $\rightarrow$ 3)-L-[6- ^3H]idonic acid, *O*-(α -2-glucosamine 6-sulphate)-(1 $\rightarrow$ 3)-D-[6- ^3H]gulonic acid and *O*-(α -2-glucosamine 6-sulphate)-(1 $\rightarrow$ 3)-2,5-anhydro-L-[6- ^3H]idonic acid (for convenience abbreviated GlcNH6S-IdOA) with a specific radioactivity of 10 Ci/mmol of uronic acid. GlcNH6S-IdOA was *N*-acetylated [15] and purified by preparative high-voltage electrophoresis on Whatman 3MM chromatography paper in 1.74 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 at approx. 12 cm towards the anode from the origin, detected with a Packard model 7201 radiochromatogram scanner (Packard Instruments, Chicago, IL, U.S.A.), were eluted from the strip in 10% (v/v) ethanol in water. The eluate was freeze-dried and reconstituted in 10% (v/v) ethanol of water to give the substrate GlcNAc6S-IdOA. GlcNAc6S-IdOA was also prepared by 6-sulphation of the *N*-acetylated disaccharide fraction from heparin and then reduction. GlcNH6S-IdOA was *N*-sulphated [15] and purified by preparative high-voltage electrophoresis (as above) to yield GlcNS6S-IdOA. GlcNAc6S-IdOA was completely desulphated by incubation with cultured human skin fibroblast homogenates, but there was no detected hydrolysis with homogenates of skin fibroblasts derived from an MPS IIID patient. These results, taken with the

Fig. 1. Chromatofocusing chromatography of step 1 fractions (Table 1)

For experimental details see the Materials and methods section. The arrow indicates the start of Polybuffer elution (-----, pH). Fractions indicated were assayed for 6SS activity (●) and protein (○).

further characterization of the initial disaccharide [12,13], are very strong evidence in support of the GlcNAc6S-IdOA structure proposed for this substrate. Care was taken to remove free sulphate by precipitation with BaCl_2 , as the 6SS activity towards the disaccharide substrate is 50% inhibited by $30 \mu\text{M-Na}_2\text{SO}_4$. GlcNS6S-IdOA and GlcNAc6S-Galitol were also used as substrates for 6SS. However, since GlcNS6S-IdOA and GlcNAc6S-Galitol are also hydrolysed by sulphamate sulphonydrolase and β -*N*-acetylhexosaminidase respectively [5,13], they were only used in preliminary studies of enzyme substrate-specificity with purified forms A, B, C or D.

6SS enzyme assay

GlcNAc6S-IdOA was routinely used to monitor 6SS activities specifically in all the purification steps outlined below.

The standard assay mixture contained $1 \mu\text{l}$ of enzyme solution [dialysed against 20 mM-Tris/HCl buffer, pH 7.2, containing 10% (v/v) glycerol, 0.5 M-NaCl and 0.1 mM-dithioerythritol], 0.2 nmol of GlcNAc6S-IdOA, and bovine serum albumin ($50 \mu\text{g/ml}$) in 60 mM-sodium acetate buffer, pH 5.0, in a total volume of $20 \mu\text{l}$. The mixture was incubated at 37°C for a time interval varying from 10 min to 12 h to give between 5 and 20% breakdown of substrate to product. Fibroblast 6SS was assayed by using the above incubation mixture with added fibroblast homogenate prepared [5] and adjusted to give a total of $10 \mu\text{g}$ of protein per incubation. 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 1.74 M-formic acid, pH 1.7. Areas of radioactivity were cut from the strip, placed in 20 ml glass scintillation vials along with 5 ml of water and 10 ml of scintillation cocktail and their radioactivities determined in an Isocap 300 liquid-scintillation system (Searle Analytic, Des Plaines, IL, U.S.A.) with a counting efficiency of 19%. Enzyme activity was determined from the percentage breakdown of substrate to product and expressed as nmol/min per mg of protein or pmol/min per μl of enzyme.

The pH-enzyme activity profile was determined by using the standard assay mixture in 60 mM-sodium acetate varying in pH from 3.0 to 6.8. K_m and $V_{\max}$ values were obtained from Lineweaver-Burk plots with a GlcNAc6S-IdOA concentration range of 1–100 μM .

Purification of 6SS

All procedures were performed at 4°C and all buffers contained 10% (v/v) glycerol and 0.1 mM-dithioerythritol.

Step 1. Concanavalin A-Sepharose/Blue A-agarose chromatography. Preparation of liver (0.8 kg) homogenate and high-speed supernatant was as described elsewhere [21,22] and modified as in ref. [23]. The joined columns were washed with 2 litres of 15 mM-sodium dimethylglutarate buffer, pH 6.0, containing 0.5 M-NaCl (buffer A). This was combined with the reservoir (methyl α -mannoside recycling) buffer, and concentrated to approx. 200 ml in a hollow-fibre Amicon DC-2 concentrator and the volume reduced further to approx. 40 ml in an Amicon ultrafiltration stirred cell (model 8200) containing a YM10 membrane. For convenience concentrates from three separate 0.8 kg liver preparations, which had been stored at 4°C for no longer than 3 weeks, were pooled for use in step 2.

Step 2. Chromatofocusing chromatography (Fig. 1). The concentrated enzyme solution was dialysed overnight against 2 litres of 30 mM-Tris/HCl buffer, pH 7.4, and applied to a PBE 94 chromatofocusing column ($45 \text{ cm} \times 1.5 \text{ cm}$ diam.) equilibrated in the same buffer. The column was washed with 150 ml of the same buffer, and this was combined with the flow-through material (obtained from the sample application) for further processing in Step 3. Application of 600 ml of a 1-in-18 dilution of Polybuffer 74/HCl, pH 4.0, eluted more 6SS activity with peak activity at pH 5.8 (form C) and pH 5.4 (form D). Further purification of form C is described in steps 5 and 6 below.

Step 3. Cu^{2+} -chelating Sepharose chromatography (Fig. 2). Chelating Sepharose, in a column ($10 \text{ cm} \times 1 \text{ cm}$

Fig. 2. Cu^{2+} -chelation Sepharose chromatography of the form A plus form B pool from step 2 (Table 1 and Fig. 1)

For experimental details see the Materials and methods section. Arrows indicate buffer pH changes. Fractions indicated were 6SS activity (●) and protein (○).

Fig. 3. Hydroxyapatite chromatography of 6SS-positive fractions from step 3 (Table 1 and Fig. 2)

For experimental details see the Materials and methods section. ----, Conc. of NaCl. Fractions indicated were assayed for 6SS activity (●).

diam.), was complexed with Cu^{2+} ions and equilibrated in buffer A adjusted to pH 6.7. The unbound fraction from the chromatofocusing step was made 0.5 M in NaCl, concentrated to 20 ml, and dialysed in an ultrafiltration stirred cell against buffer A adjusted to pH 6.7 and applied to the Cu^{2+} -chelating Sepharose column. The column was washed with 50 ml of the same buffer and the 6SS activity was eluted with 150 ml of buffer A adjusted to pH 5.7.

Step 4. Hydroxyapatite chromatography (Fig. 3). The fractions from step 3 containing 6SS activity (Fig. 2) were pooled and concentrated to 20 ml, dialysed in an ultrafiltration stirred cell against 20 mM-Tris/HCl buffer, pH 7.4, and applied to a hydroxyapatite column (30 cm $\times$ 1.5 cm diam.) equilibrated in and subsequently washed with 200 ml of the same buffer. 6SS activity

was eluted with a 2 $\times$ 600 ml linear gradient of 30–1200 mM-NaCl in 20 mM-Tris/HCl buffer, pH 7.4.

Step 5. Gel-filtration chromatography of form C. Fractions containing form C (Fig. 1) were pooled, concentrated to 2 ml, dialysed against buffer A and applied to an Ultrogel AcA 34 column (80 cm $\times$ 2.5 cm diam.) equilibrated in the same buffer and eluted at a rate of 20 ml/h.

Step 6. Cu^{2+} -chelating Sepharose chromatography of form C. The fractions containing 6SS activity from step 5 were pooled, concentrated to 20 ml and dialysed against buffer A adjusted to pH 6.7, and the procedure outlined in step 3 was utilized.

Other enzyme activities

Enzyme activity towards sulphamate sulphonylhydrolase [24], α -L-iduronidase [23], sulphiduronate sulphonylhydrolase [25], arylsulphatase B [26], β -N-acetylhexosaminidase [5], α -N-acetylglucosaminidase [27], acetyl-CoA:glucosamine N-acetyltransferase [28], β -glucuronidase [29] and N-acetylgalactosamine-6-sulphate sulphatase [30] substrates were assayed by previously reported procedures.

Uptake of 6SS into enzyme-deficient fibroblasts

Fibroblasts from a MPS IIID patient were grown in culture as described previously [5]. Solutions of forms A and B (equivalent to 150 times the enzyme units present in an equivalent number of normal fibroblasts; 16 pmol/3 ml of medium per 50 ml culture bottle) in culture medium were sterilized by filtration and added to the culture flasks. Cultures of the cells were harvested at intervals over 48 h and washed thoroughly with 0.15 M-NaCl, and the 6SS activity in cell homogenates was determined by using GlcNAc6S-IdOA incubations as described above.

Gel-permeation chromatography

A column (80 cm $\times$ 2.5 cm diam.) of Ultrogel AcA 34 equilibrated in buffer A had a 2 ml sample of enzyme in the same buffer loaded and eluted at a flow rate of 20 ml/h. A column (83 cm $\times$ 2.5 cm diam.) of Fractogel TSK HW55(S) in buffer A was eluted at a flow rate of 60 ml/h. The columns were calibrated with the following molecular-mass-standard proteins: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), fructose-bisphosphate aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa) and ribonuclease A (13 kDa).

SDS/polyacrylamide-slab-gel electrophoresis

Samples were prepared by using a deoxycholate/trichloroacetic acid co-precipitation method [31] and, unless otherwise stated, reduced by boiling in dithioerythritol. Discontinuous SDS/polyacrylamide-gel electrophoresis was run according to the method of Laemmli [32], stained with Coomassie Brilliant Blue R250 and with the silver staining method of Merrill *et al.* [33]. Gels were calibrated with a low-molecular-mass electrophoresis calibration kit consisting of 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).

Table 1. Purification of 6SS from 2400 g of human liver

For full experimental details see the Materials and methods section

Step		Total activity (nmol/min)	Total protein (mg)	Specific activity (nmol/min per mg)	Purification (fold)	Recovery (%)
	Homogenate	309	111 000	0.003	1	
	Supernatant	240	56 000	0.004	1.3	77
1.	Concanavalin A-Sepharose/Blue A-agarose	500	787	0.635	212	162
2.	Chromatofocusing					
	pI > 7.5 (forms A and B)	262	66	4.0	1323	85
	pI 5.8 (form C)	20	28	0.7	240	6.5
	pI 5.4 (form D)	10	275	0.036	12	3.2
3.	Cu ²⁺ -chelating Sepharose chromatography of step 2 (forms A and B)	241	1.4	172	57360	78
4.	Hydroxyapatite chromatography of step 3					
	Form A	17.5	0.076	230	76730	8.0
	Form B	48.7	0.231	254	83330	16.9
5.	Gel-filtration chromatography of step 2 (form C)	16.4	6.4	2.6	867	5.3
6.	Cu ²⁺ -chelating Sepharose chromatography of step 5	10.2	0.1	102	33666	3.3

RESULTS

Purification of 6SS

All 6SS activity was bound to concanavalin A-Sepharose, demonstrating the glycoprotein nature of the enzyme. However, 6SS did not bind to the Blue A-agarose following its elution with methyl α -mannoside from the lectin column. A total of 162% 6SS activity was recovered in this step, representing a 212-fold purification with a 98% decrease in protein (Table 1). The apparent increase in the total 6SS activity recovered at this stage may be due to the removal of a non-diffusible inhibitor.

The chromatofocusing step separated 6SS forms A and B, which did not bind to the column at pH 7.4, from forms C and D, which remained bound to the column along with 90% of the applied protein (Fig. 1). After application of Polybuffer, 6SS activity was eluted in two broad peaks, at pH 5.8 (form C) and pH 5.4 (form D). Forms C and D comprised 10% of the total 6SS activity recovered from this step. The unbound forms (A plus B) did not bind if re-applied to a regenerated chromatofocusing column at pH 7.4 or at pH values up to 9.5, indicating that these forms had a pI greater than 9.5. The enzyme was unstable in buffer at higher than pH 9.5. Application of the 6SS fractions from step 1 to a DEAE-Sepharose column at pH 7.4 and elution with a salt gradient gave a similar separation of forms to that observed with the chromatofocusing column (results not shown).

Chromatography of the chromatofocusing-column unbound fraction (forms A and B) on chelating Sepharose charged with Cu²⁺ resulted in separation of 6SS from all other proteins (Fig. 2), including other lysosomal enzymes involved in glycosaminoglycan degradation (including no detectable activity of α -L-iduronidase,

β -*N*-acetylglucosaminidase, sulphamate sulphonydrolase, arylsulphatase B, sulphoiduronate sulphonydrolase, α -*N*-acetylglucosaminidase, *N*-acetylgalactosamine-6-sulphate sulphatase, β -glucuronidase and acetyl-CoA:glucosamine *N*-acetyltransferase in the eluate) to give a final 57000-fold purification of 6SS containing both forms A and B with a 78% recovery of the total enzyme activity found in the original liver homogenate. SDS/polyacrylamide-gel-electrophoresis analysis revealed three Coomassie-Blue-stained bands at apparent molecular-mass values of 78, 48 and 32 kDa (Figs. 4 and 5) whether the samples were pretreated under reducing (with dithioerythritol) or non-reducing conditions. No other polypeptides were observed after staining with the more sensitive silver staining method of Merrill *et al.* [33]. Two faint bands often observed on the gels at apparent molecular-mass values of 60 and 54 kDa were attributable to dithioerythritol as described by Tasheva & Dessev [20], and were present in the buffer blank as well as in all other samples that had been reduced with dithioerythritol.

The 6SS fraction from step 3 was separated into form A and form B by hydroxyapatite chromatography (Fig. 3). This step, in which the 6SS activity was spread over much of the profile, resulted in a considerable loss of activity, possibly from dilution of the enzyme. Phosphate (100 mM) and 1.5 M-NaCl washes of the hydroxyapatite column did not contain 6SS activity. Generally we have found hydroxyapatite chromatography of lysosomal enzymes to result in poor yields compared with other chromatography steps [21,23]. Forms A and B have similar native molecular masses, but differ in SDS/polyacrylamide-gel-electrophoresis subunit composition (Fig. 4). Form A, which was eluted at 0.32 M-NaCl, revealed a single band at molecular mass 78 kDa, whereas form B, which was eluted at 0.64 M-NaCl, contained 48 kDa and

Fig. 4. SDS/polyacrylamide-gel electrophoresis of 6SS from various steps of the purification scheme (Table 1)

All samples were reduced with dithioerythritol and electrophoresed in a 12% polyacrylamide gel. For experimental details see the Materials and methods section. Lanes 1 to 6 and lanes 7 to 9 were on separate electrophoresis runs. Lane 1, molecular-mass standards for lanes 2 to 6; lane 2, step 2, chromatofocusing fraction (forms A and B); lane 3, step 3, Cu^{2+} -chelating Sepharose chromatography fraction (forms A and B); lane 4, step 4, hydroxyapatite chromatography fraction (form A); lane 5, step 4, hydroxyapatite chromatography fraction (pool 2); lane 6, step 4, hydroxyapatite chromatography fraction (form B); lane 7, step 5, gel-filtration chromatography of form C; lane 8, step 6, Cu^{2+} -chelating Sepharose chromatography of form C; lane 9, positions of molecular-mass standards for lanes 7 and 8.

32 kDa polypeptides when subjected to SDS/polyacrylamide-gel electrophoresis under reducing or non-reducing conditions. Pool 2 (Fig. 3) by SDS/polyacrylamide-gel electrophoresis appeared to be a mixture of forms A and B.

Partial purification of form C

Form C from the chromatofocusing column (Fig. 1), when subjected to gel-permeation chromatography on Ultrogel AcA 34, was eluted with a molecular-mass value of 94 kDa. This step, beside removing other proteins, separated 6SS from the non-diffusible Polybuffer, which, if it remained, stripped Cu^{2+} from the Cu^{2+} -chelating Sepharose during the next stage of purification. Form C was eluted from the Cu^{2+} -chelating Sepharose column under conditions similar to those observed for forms A and B. These steps resulted in a 34000-fold purification of form C. SDS/polyacrylamide-gel-electrophoresis analysis revealed three major Coomassie-Blue-stained bands corresponding to molecular-mass values of 48, 45 and 32 kDa (Figs. 4 and 5).

Native molecular masses of forms A, B, C and D (Fig. 5)

Fractogel TSK chromatography of 6SS (containing forms A and B) from the step 3 Cu^{2+} -chelating Sepharose procedure resulted in the elution of a single peak of activity with a molecular-mass value of 75 kDa. SDS/polyacrylamide-gel-electrophoresis assay of frac-

tions through the elution profile clearly showed co-elution of the 6SS activity with a constant ratio of the three polypeptides (78, 48 and 32 kDa). Ultrogel AcA 34 gel filtration of form A or B resulted in elution of 6SS activity, each with an apparent molecular-mass value of 75 kDa. Chromatography of form C on Ultrogel AcA 34 gave a molecular mass of 94 kDa. Chromatography of a mixture (from combination of fractions from the chromatofocusing step) of forms C and D on Ultrogel AcA 34 resulted in an elution profile suggestive of two separate peaks of 6SS activities, with peak elutions corresponding to molecular-mass values of 94 kDa and 75 kDa respectively.

Storage stability of forms A and B

Both form A and form B retained 95% of their initial enzyme activity after 2 months at 4 °C when stored in buffer A adjusted to pH 5.7. Both forms were stable between pH 5 and pH 7.5, but were completely inactive after 24 h at pH values less than 4.5 or more than 9.5.

Assay of 6SS activity with GlcNAc6S-IdOA

Forms A, B and C each displayed a pH optimum of 5.0 when assayed for activity towards GlcNAc6S-IdOA in sodium acetate buffer in the presence or absence of bovine serum albumin. Enzyme activity was linear with respect to time for incubations up to 15 h and with respect to enzyme protein for incubations containing between 20 and 200 ng of protein/assay in the presence of albumin (50 µg/ml). In preliminary studies forms A, B and C displayed similar affinities towards GlcNAc6S-IdOA; Lineweaver-Burk plots resulted in apparent K_m values of 11.7, 14.2 and 11.1 µM respectively with apparent V_{max} values of 105, 60 and 53 nmol/min per mg of protein respectively. Incubation of forms A, B, C and D with 10 µM-GlcNAc6S-Galitol and GlcNS6S-IdOA at pH 5.0 demonstrated 6SS activity towards both substrates. The relative level of 6SS activity towards GlcNS6S-IdOA was similar to that observed with GlcNAc6S-IdOA for each form, whereas the relative 6SS activity of forms A, B, C and D towards GlcNAc6S-Galitol was about 100-fold less than that observed towards GlcNAc6S-IdOA for each form.

Uptake of 6SS forms A and B into 6SS-deficient fibroblasts

6SS activity was not taken up into MPS IIID fibroblasts from culture medium containing form A or form B.

DISCUSSION

We have purified 6SS from human liver by more than 55000-fold with a net recovery of at least 78% after the Cu^{2+} -chelating Sepharose step. This is an extremely simple purification protocol utilizing a rapid three-column procedure that includes a combined concanavalin A-Sepharose/Blue A-agarose recycling step and separate chromatography steps of chromatofocusing and Cu^{2+} -chelating Sepharose chromatography. Remarkably, the major forms A and B of 6SS bind only to two columns (concanavalin A-Sepharose and Cu^{2+} -chelating Sepharose). The procedure has the added advantage that other lysosomal enzymes involved in glycosaminoglycan degradation can be conveniently obtained from the same liver [21–23,34].

Fig. 5. Determination of (a) subunit molecular mass and (b) native molecular mass of the various forms of 6SS

For experimental details see the Materials and methods section.

The Cu^{2+} -chelating Sepharose step removed all contaminating proteins to yield 6SS that consists of three polypeptides of 78, 48 and 32 kDa. Hydroxyapatite chromatography of 6SS from the Cu^{2+} -chelating Sepharose step separated two forms (A and B) both with native molecular masses of 75 kDa but with differing subunit compositions. Whereas form A contained only 78 kDa subunit, form B consisted of two polypeptides (48 and 32 kDa). Forms A and B represent approx. 30% and 60% respectively of the total 6SS activity present at step 3 (Table 1). Two low-abundance forms (C and D) also separate at the chromatofocusing step. The subunit stoichiometry of 6SS, although uncertain at this point, is likely to be that form B consists of two different subunits that interact to give an active complex of apparent molecular size and pI similar to form A. It is also possible that form B is derived from form A by at the least a single peptide-bond break or at most by the loss of a relatively small polypeptide fragment from central region of form A. The composition of form C, however, is somewhat more complex in that the 48 kDa, 45 kDa and 32 kDa subunits interact to give an active complex with a larger apparent molecular size (94 kDa) and a much lower pI (5.8 compared with > 9.5) than observed for form A or B. It seems likely that the 45 kDa polypeptide is responsible for these differences between form C and form A or B. We have not yet purified form D to homogeneity and are therefore unable to provide details about its subunit composition. Our findings should be considered alongside the reports by Basner *et al.* [9] and Habuchi *et al.* [10] describing 6SS isolated from human urine and rat skin respectively. Human urine 6SS isolated in 1% yield had a molecular mass of 97 kDa and a broad pI spectrum between pH 5.4 and pH 8.3 with a maximum enzyme activity at pH 7.7 [9]. Partially purified rat skin 6SS had a molecular mass of 70 kDa and, although most activity did not bind to DEAE-cellulose at pH 7.2, a small amount did bind and was eluted by a salt gradient, but was not further characterized [10]. These different physical properties for 6SS possibly reflect the different sources of enzyme and/or their relatively low recoveries, resulting in the selective isolation of one form over others that may be present in the initial tissue extract.

The presence of multiple forms of other lysosomal enzymes involved in the degradation of glycosaminoglycans {e.g. α -L-iduronidase [23], arylsulphatase B [35,36], α -N-acetylglucosaminidase [37], sulphiduronate sulphohydrolase (J. Bielicki, P. R. Clements & J. J. Hopwood, unpublished work) and β -N-acetylhexosaminidase [38]} or in the degradation of other glycoconjugates [39,40] have been reported. Cheng *et al.* [40] purified and identified two forms of α -D-mannosidase from human liver that differed in both their subunit composition and their ability to be taken up into cultured human skin fibroblasts. The major forms (A and B) of 6SS were not taken up by fibroblasts under similar conditions to those described by Cheng *et al.* [40] and to those in which we have been able to demonstrate uptake of arylsulphatase B and α -L-iduronidase prepared from the same human livers (G. J. Gibson, P. R. Clements, C. Freeman & J. J. Hopwood, unpublished work). Different forms of the same enzyme activity may represent isoenzyme involvement in separate glycoconjugate-degradation pathways or result from proteolysis that has occurred during the period *post mortem* or during the purification procedure, or represent stages of enzyme maturation. We consider the production of four forms of the enzyme by proteolytic degradation during 6SS isolation to be unlikely, since the relative proportions of all four forms to each other remained relatively constant throughout at least ten different preparations in which donor age, liver periods *post mortem* and times at and between the various purification steps varied. Preliminary studies demonstrate that all four forms present in human liver are active to the same extent towards substrates derived from keratan sulphate and heparin. We have been unable to demonstrate separate or different 6SS activities towards substrates with either α - or β -linked *N*-acetylglucosamine 6-sulphate or α -linked 2-sulphaminoglycosamine 6-sulphate non-reducing end residues. This aspect, together with the effect of aglycone structure on 6SS activity, has recently been studied in depth with the various enzyme forms described above [41].

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