Biochem. J. (1991) 279, 515–520 (Printed in Great Britain)

Human liver *N*-acetylgalactosamine 6-sulphatase

Purification and characterization

Julie BIELICKI and John J. HOPWOOD*

Lysosomal Diseases Research Unit, Department of Chemical Pathology, Adelaide Children's Hospital, 72 King William Road, North Adelaide, South Australia 5006, Australia

Human *N*-acetylgalactosamine 6-sulphatase (EC 3.1.6.14), which is involved in the lysosomal degradation of the glycosaminoglycans keratan sulphate and chondroitin 6-sulphate, was purified more than 130000-fold in 2.8% yield from liver by an eight-step column procedure. One major form was identified with a pI of 5.7 and a native molecular mass of 62 kDa by gel filtration. When analysed by SDS/PAGE, dithioerythritol-reduced enzyme contained polypeptides of molecular masses 57 kDa, 39 kDa and 19 kDa, whereas non-reduced enzyme contained a major polypeptide of molecular mass 70 kDa. It is proposed that active enzyme contains either the 57 kDa polypeptide or disulphide-linked 39 kDa and 19 kDa polypeptides. Minor amounts of other enzyme forms separated during the chromatofocusing step and the Blue A–agarose step were not further characterized. Purified *N*-acetylgalactosamine 6-sulphatase was inactive towards 4-methylumbelliferyl sulphate, but was active, with pH optima of 3.5–4.0, towards 6-sulphated oligosaccharide substrates. K_m values of 12.5 and 50 μM and $V_{max.}$ values of 1.5 and 0.09 μmol/min per mg were determined with oligosaccharide substrates derived from chondroitin 6-sulphate and keratan sulphate respectively. Sulphate, phosphate and chloride ions were inhibitors of enzyme activity towards both substrates, with 50 μM-Na_2SO_4 giving 50% inhibition towards the chondroitin 6-sulphate trisaccharide substrate.

INTRODUCTION

N-Acetylgalactosamine 6-sulphatase (G6S) is one of the lysosomal enzymes involved in the degradation of the glycosaminoglycans keratan sulphate (KS) and chondroitin 6-sulphate (C6S). It catalyses the hydrolysis of the 6-sulphate ester bond present at the non-reducing terminus of C6S oligosaccharides, namely *N*-acetylgalactosamine 6-sulphate, and it also acts on galactose 6-sulphate linkages in KS. The deficiency of this enzyme activity results in the lysosomal storage disorder mucopolysaccharidosis type IVA (Morquio syndrome A), which is inherited as an autosomal recessive trait. The clinical presentation of this disorder is characterized by normal intelligence, dwarfism, corneal clouding, spondyloepiphysial dysplasia and dental abnormalities (Neufeld & Muenzer, 1989). There is storage of partially degraded KS and C6S fragments in tissues as well as excessive urinary excretion of these fragments. As with the other mucopolysaccharidosis disorders, phenotypic variability exists, consistent with the occurrence of different mutant alleles at the G6S locus. Attempts to purify this enzyme from human tissues have been only partially successful. Human placental G6S has been purified about 20000-fold to approx. 70% purity (Glössl *et al.*, 1979) and to 3000-fold (Lim & Horwitz, 1981). In the present paper we report the purification of human liver G6S to more than 130000-fold and some of its physical and kinetic properties.

MATERIALS

Human livers were obtained from autopsies of normal adults with times *post mortem* ranging from 6 to 72 h and were stored at −20 °C.

Concanavalin A–Sepharose 4B (800 mg of lectin/100 ml of gel), PBE94 chromatofocusing medium, phenyl-Sepharose CL-4B, Polybuffer 74, chelating Sepharose 6B and the molecular-mass standard kits for SDS/PAGE gel chromatography were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). E. Merck (Darmstadt, Germany) supplied TSK HW50S Fractogel, and LKB (Bromma, Sweden) supplied TSK G3000SW Ultrapac. Blue A Matrix agarose gel, the DC-2 hollow-fibre concentrator having 10 kDa-cut-off hollow fibres, the ultrafiltration stirred cells (model 8200) and the Diaflo ultrafiltration membrane YM10 were obtained from Amicon (Danvers, MA, U.S.A.). Protein assay reagent was obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Dialysis membrane with a 10–12 kDa cut-off was obtained from Union Carbide Corp. (Chicago, IL, U.S.A.). BSA (crystalline grade) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

METHODS

Assay of G6S activity

G6S activity was assayed under identical conditions with either the trisaccharide substrate (GalNAc6S-GlcA-GalitolNAc6S) derived from C6S or the disaccharide substrate (Gal6S-anM6S) prepared from KS (Hopwood & Elliott, 1983*a*,*b*). The total assay volume of 12 μl included 1 μl, equivalent to 200 μM, of either of the above substrates (specific radioactivity 427 c.p.m./pmol and 775 c.p.m./pmol respectively), 3 μl of 0.2 M-sodium acetate buffer, pH 3.8, containing 2 mg of BSA/ml, 3 μl of 27 mM-2-acetamido-2-deoxy-D-gluconolactone, 4 μl of water and 1 μl of enzyme sample either diluted in 50 mM-sodium acetate buffer, pH 4.0, or dialysed against 50 mM-sodium acetate buffer, pH 4.0, containing 10% (v/v) glycerol and 0.1 mM-dithioerythritol. 2-Acetamido-2-deoxy-D-gluconolactone, which is an inhibitor of β-hexosaminidase, was omitted in the kinetic

Abbreviations used: G6S, *N*-acetylgalactosamine 6-sulphatase; KS, keratan sulphate; C6S, chondroitin 6-sulphate; GalNAc6S-GlcA-GalitolNAc6S, *N*-acetylgalactosamine 6-sulphate–glucuronic acid–*N*-acetylgalactosaminitol 6-sulphate; Gal6S-anM6S, galactose 6-sulphate–anhydromannitol 6-sulphate.

* To whom correspondence should be addressed.

studies with 'pure' enzyme. Assay tubes were incubated at 37 °C for 1 h with the C6S-derived trisaccharide substrate or for 5–8 h with the KS-derived disaccharide substrate to give less than 30 % breakdown of substrate to product. The reaction was stopped by freezing the assay mixture, and the substrate and product were separated by using high-voltage electrophoresis according to a previously described method (Bielicki *et al.*, 1990). Enzyme activity was determined from percentage breakdown of each substrate to product and was expressed as pmol of product/min per μl.

Assay of other lysosomal enzyme activities

α-L-Iduronidase, with the fluorogenic substrate 4-methylumbelliferyl α-L-iduronide (Clements *et al.*, 1985*b*), glucosamine 6-sulphatase (Freeman *et al.*, 1987), *N*-acetylgalactosamine 4-sulphatase (Hopwood *et al.*, 1986) and iduronate 2-sulphatase (Bielicki *et al.*, 1990) were assayed by previously reported procedures. β-Hexosaminidase activity towards GalNAc-GlcA-GalitolNAc6S was determined in the absence of 2-acetamido-2-deoxy-D-gluconolactone (Hopwood *et al.*, 1986). Arylsulphatase activity was measured at pH 5.7 against 4-methylumbelliferyl sulphate (Chang *et al.*, 1981).

Purification of G6S

All procedures were carried out at 4 °C unless otherwise stated.

Step 1: liver extraction. Approx. 900 g of liver was homogenized in 3 vol. of 15 mM-sodium dimethylglutarate buffer, pH 6.0, containing 0.5 M-NaCl and 0.1 mM-dithioerythritol (buffer A), delipidated with carbon tetrachloride and centrifuged further at 27000 *g* for 30 min (Clements *et al.*, 1983; Mahuran *et al.*, 1983*a*).

Step 2: concanavalin A–Sepharose – Blue A–agarose chromatography. The step 1 clear supernatant (1.5–2 litres) was applied to a 300 ml concanavalin A–Sepharose column equilibrated in buffer A containing 1 mM-$MnCl_2$ and 1 mM-$CaCl_2$ and washed extensively with 6 litres of buffer A. The enzyme was eluted from concanavalin A–Sepharose according to a method described elsewhere (Mahuran *et al.*, 1983*a*; Clements *et al.*, 1983). Unbound proteins were displaced with 2 litres of buffer A, and this fraction (2–2.5 litres), from now on referred to as the concanavalin A recycling eluate (CARE), was concentrated to 250–300 ml on a Amicon hollow-fibre concentrator. Six such CARE fractions were combined and concentrated to 84 ml in an Amicon stirred cell and then dialysed for 16 h against 5 litres of 30 mM-Tris/HCl buffer, pH 7.2, containing 10 % (v/v) glycerol, 0.1 mM-dithioerythritol and 3 mM-NaN_3 (buffer B). No significant loss of enzyme activity occurred from this processing and subsequent centrifugation in step 3.

Step 3: chromatofocusing chromatography (Fig. 1). The dialysed fraction from step 2 was centrifuged at 1800 *g* for 10 min, and the supernatant was loaded on to a PBE94 column (45 cm × 1.5 cm) equilibrated in buffer B (flow rate 1 ml/min) and washed with 200–300 ml of buffer B until absorption at 280 nm approached baseline level. Bound proteins were eluted with 1 litre of Polybuffer 74 that had been diluted 1 in 18 in water, the pH was adjusted to 4.0 with HCl and the solution was made 10 % (v/v) in glycerol, 0.1 mM in dithioerythritol and 3 mM in NaN_3 (buffer C). The column was then eluted with 300 ml of buffer C containing 0.25 M-NaCl. Fractions containing G6S were pooled as shown in Fig. 1.

Step 4: TSK HW50S Fractogel chromatography. The A pool of fractions from step 3 was concentrated to 2.0 ml in an Amicon ultrafiltration stirred cell and the buffer was changed to 15 mM-sodium dimethylglutarate buffer, pH 6.0, containing 0.5 M-NaCl, 10 % (v/v) glycerol, 0.1 mM-dithioerythritol and 3 mM-NaN_3 (buffer D). The sample was loaded on to the TSK HW50S Fractogel column (110 cm × 2.5 cm) and eluted with buffer D with a flow rate of 0.5 ml/min. G6S active fractions were pooled, the pH was adjusted to pH 6.5 with 0.1 M-NaOH and the preparation was applied to the next column.

Step 5: Cu^{2+}-chelation Sepharose chromatography (Fig. 2). This column (3 cm × 1 cm) was saturated with copper acetate and equilibrated in buffer D minus dithioerythritol. The sample from step 4 was loaded, washed on with the above buffer and eluted with a decreasing linear pH gradient made from two solutions of buffer D, one of which was at pH 6.5, the other at pH 3.5. Fractions containing enzyme activity were pooled as shown in Fig. 2.

Step 6: phenyl-Sepharose CL-4B chromatography. This hydrophobic column (3 cm × 1 cm) was equilibrated in buffer D and run at room temperature (20–25 °C). The sample from step 5 was concentrated to 13 ml, the buffer was changed to buffer D, and it was loaded and washed on with three 10 ml portions of buffer D. The column was eluted with three 10 ml portions of buffer D (without glycerol) for each concentration of ethylene glycol starting at 20 % (v/v) and increasing to 25 %, 30 %, 35 %, 40 %, 45 % and 50 % (v/v). The eluate was collected into tubes standing in ice. Both the flow-through and the first 20 %-(v/v)-ethylene glycol wash, which contained enzyme activity, were pooled and concentrated to 1.0 ml.

Step 7: TSK G3000SW Ultrapac chromatography (Fig. 3). The concentrated sample was applied at room temperature to an LKB Ultrachrom GTi f.p.l.c. system with a TSK G3000SW Ultrapac column (30 cm × 0.8 cm), equilibrated and eluted in buffer D at flow rate of 0.5 ml/min and pressure of 150 kPa. A 100 μl sample of each fraction from 18 to 26 was separately treated with deoxycholate/trichloroacetic acid for SDS/PAGE. The remainder of each fraction showing G6S was pooled as shown in Fig. 3 and loaded directly on to the next column.

Step 8: second Cu^{2+}-chelation Sepharose chromatography (Fig. 4). A column (1 cm × 1 cm) was saturated with copper acetate and equilibrated in buffer D without dithioerythritol. The enzyme sample was applied, washed with 20 ml of the above buffer and then eluted with 10 ml volumes of the same buffer, but at decreasing pH, starting at pH 4.6, then pH 4.0 and finally pH 3.5. Fractions (2 ml) were collected, and those containing enzyme activity were pooled as shown in Fig. 4.

Gel-permeation chromatography

Native protein molecular-mass estimations were attempted by using both TSK HW50S Fractogel (110 cm × 2.5 cm) and the f.p.l.c. system with a TSK G3000SW Ultrapac column (30 cm × 0.8 cm). Both columns were calibrated in buffer D with, as protein standards, thyroglobulin (660 kDa), ferritin (440 kDa), catalase (232 kDa), fructose-bisphosphate aldolase (158 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), chymotrypsinogen A (25 kDa) and RNAase (13.7 kDa).

SDS/PAGE

Samples for electrophoresis were prepared by precipitation with deoxycholate/trichloroacetic acid, as described by Mahuran *et al.* (1983*b*). Discontinuous SDS/polyacrylamide slab gels with 12 % acrylamide were run according to the method of Laemmli (1970). All samples were denatured before electrophoresis by incubation for 2 min at 100 °C in the presence or in the absence (where indicated) of 0.1 mM-dithioerythritol. Gels were stained with Coomassie Brilliant Blue R250. Pharmacia molecular-mass standards were used for gel calibration and included the following proteins: phosphorylase *b* (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa),

soya-bean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa).

Kinetic experiments

Enzyme solution containing 500 μg of BSA/ml was dialysed overnight at 4 °C against 2 litres of 50 mM-sodium acetate buffer, pH 4.0, containing 10% (v/v) glycerol, 0.1 mM-dithioerythritol and 3 mM-NaN_3. The pH–activity profiles were obtained with a 12 μl assay containing substrate and 50 mM-sodium acetate or 50 mM-sodium formate buffer (pH as indicated). Reactions were initiated by the addition of 1 μl of dialysed enzyme (equivalent to 1.25 ng to 15 ng of G6S protein) and incubated at 37 °C. Incubation times were optimized to give a percentage breakdown of substrate to product in the range 5–30%, which was linear with respect to time. Reactions were terminated by plunging the reaction tubes into an ethanol/solid CO_2 bath. The substrate and product were separated according to the procedure described above under 'Assay of G6S activity'.

Kinetic data (K_m and $V_{max.}$) were obtained with assay conditions similar to those for the pH–activity profiles. Substrate concentrations ranged from 2 to 42 μM. Incubation time was adjusted to within the linear range for substrate hydrolysis. Incubations were at the optimum pH for both substrates. K_m and $V_{max.}$ values were obtained from Lineweaver–Burk plots.

RESULTS

Purification of G6S

Extraction from human liver and application to the coupled concanavalin A–Sepharose – Blue A–agarose columns was according to the protocol already existing in this laboratory for the extraction of other lysosomal enzymes and not optimal for this enzyme (Mahuran *et al.*, 1983*a*; Clements *et al.*, 1985*a*; Freeman & Hopwood, 1986, 1989; Freeman *et al.*, 1987; Gibson *et al.*, 1987; Bielicki *et al.*, 1990). Approx. 98% of the G6S activity was bound to concanavalin A–Sepharose, and 86% in the step 1 high-speed supernatant was recovered in the CARE fraction and 5.0% was eluted from the Blue A–agarose (Table 1). CARE fractions from six separate liver extracts were combined for processing through the next steps. Binding of G6S to the chromatofocusing medium PBE94 was complete, and most of the bound enzyme was eluted with Polybuffer 74 between pH 5.4 and pH 6.0. The low recovery of enzyme activity (29%) from this step may reflect the instability of the enzyme at pH 6.0 in low-ionic-strength buffer, or aggregation at its pI in low-ionic-strength buffer. A second form of G6S was eluted at pH 5.4, and a third form that was more tightly bound required pH 4.0 and 0.25 M-NaCl for elution (Fig. 1). These two forms accounted for less than 6% of the total G6S activity loaded and were not further characterized. Although enzyme recovery was somewhat

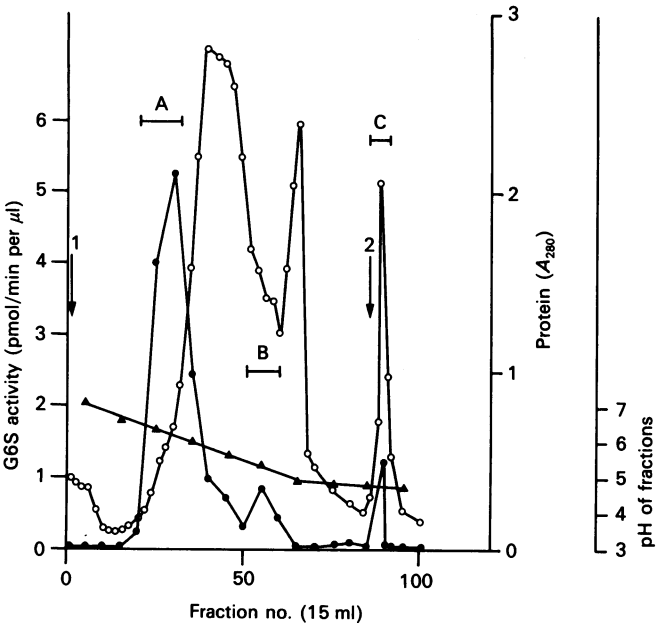

Fig. 1. Chromatofocusing chromatography of step 2 fraction (Table 1)

For experimental details see the Methods section. The arrow 1 indicates the start of Polybuffer 74 elution. Elution at arrow 2 was with Polybuffer 74 that contained an additional 0.25 M-NaCl. Fractions indicated were assayed for G6S activity (●), protein (A_{280}, ○) and pH (▲). Fractions were pooled as indicated by the horizontal bars (A, B and C).

Table 1. Purification of G6S from 5400 g of human liver

For full experimental details see the Methods section.

Step	Total activity (μmol/h)	Total protein (mg)	Specific activity (μmol/h per mg)	Purification (fold) (total)	Recovery (%)
1. Homogenate supernatant	141	274560	0.00051	1	
2. Concanavalin A–Sepharose – Blue A–agarose eluate					
CARE fraction	122	2300	0.053	104	86
Blue A–agarose eluate	7	300	0.023	45	5
3. Chromatofocusing of CARE fraction					
pI 5.7	35	632	0.055	108	24.8
pI 5.4	4	1000	0.004	7	2.8
pI < 4.0	1.8	24	0.075	147	1.2
4. TSK HW50S Fractogel chromatography					
pI 5.7	20	58	0.345	676	14
5. Cu^{2+}-chelation Sepharose chromatography	10	10	1.000	1960	7
6. Phenyl-Sepharose chromatography	7.6	6.3	1.206	2365	5.4
7. TSK G3000SW Ultrapac chromatography	5	0.28	17.857	35014	3.5
8. Cu^{2+}-chelation Sepharose chromatography	4	0.06	66.667	130719	2.8

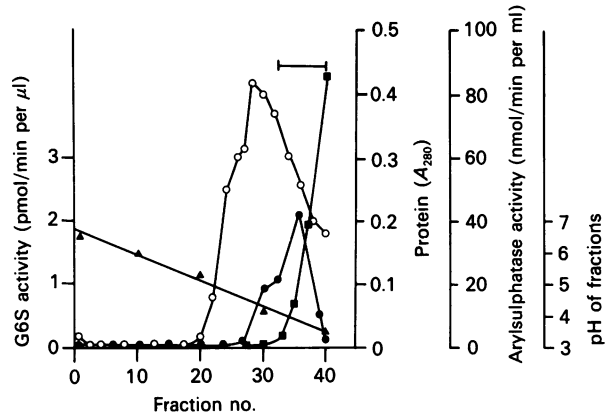

Fig. 2. Cu^{2+}-chelation Sepharose chromatography of step 4 fraction

For experimental details see the Methods section. Fractions were assayed for G6S activity (●), arylsulphatase activity (■), protein (A_{280}, ○) and pH (▲). Pooled fractions are indicated by the horizontal bar.

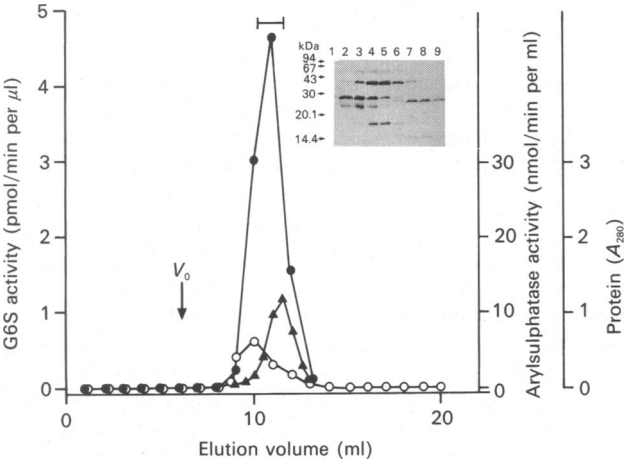

Fig. 3. TSK G3000SW chromatography of the step 6 fraction

For experimental details see the Methods section. Fractions were assayed for G6S activity (●), arylsulphatase activity (▲) and protein (A_{280}, ○) and pooled as shown by the horizontal bar. Column void volume (V_0) is arrowed. The insert shows SDS/PAGE of fractions 18–26 as shown in lanes 1–9, which correspond to the elution volumes 9.0 ml, 9.5 ml, 10.0 ml etc. to 13.0 ml. All samples were reduced with dithioerythritol and stained with Coomassie Brilliant Blue. Positions of molecular-mass standards are arrowed.

poor this step was valuable in removing a large proportion of contaminating proteins (72%), among which were the lysosomal enzymes iduronate 2-sulphatase, α-L-iduronidase and glucosamine 6-sulphatase, of which 90% or more was removed. The next step, TSK HW50S chromatography, removed 90% contaminating protein and was necessary for the removal from the enzyme solution of Polybuffer 74, which, if present, interfered with the subsequent Cu^{2+}-chelation Sepharose chromatography step by removing the bound Cu^{2+}. The apparent native molecular mass of G6S was estimated from molecular-mass calibration data to be 64 kDa. Adjusting the pH of the pooled fractions from step 4 to pH 6.5 with 0.1 M-NaOH resulted in no loss of activity. Recovery of activity from the step 5 Cu^{2+}-chelation Sepharose was approx. 50% of the enzyme loaded with 82% removal of protein (Table 1). Elution of G6S activity from this column commenced at pH 4.2, with all activity completely eluted by pH 3.5. Elution of arylsulphatase activity commenced at pH 3.8

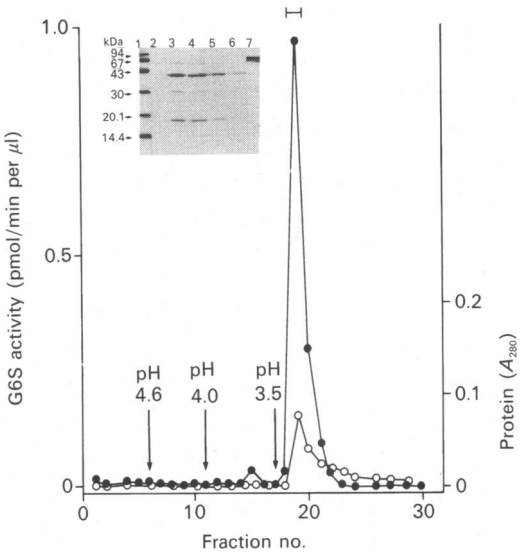

Fig. 4. Cu^{2+}-chelation Sepharose chromatography of step 7 fraction

For experimental details see the Methods section. Fractions were assayed for G6S activity (●) and protein (A_{280}, ○). Arrows indicate buffer pH changes. Fractions were pooled as indicated by the horizontal bar. The insert shows SDS/PAGE of: lane 1, molecular-mass standards; lanes 2–6, fractions 18–22 denatured in the presence of 0.1 mM-dithioerythritol; lane 7, fraction 19 under non-reducing conditions. Lanes 1–7 were stained with Coomassie Brilliant Blue.

and required a longer elution time at pH 3.5 for complete elution (Fig. 2). Under the conditions used in the hydrophobic step 6, G6S did not bind to phenyl-Sepharose CL-4B and consequently this step only removed 39% contaminants. Another estimate of native molecular mass was obtained for G6S eluted from the step 7 f.p.l.c. as being 62 kDa. SDS/PAGE of a sample from each fraction (18–26) across the f.p.l.c. profile (see Fig. 3 inset) run under reducing conditions indicated that the polypeptides most likely to be part of G6S were those at 57 kDa, 39 kDa or 19 kDa, or, in fact, all three may be involved. The few contaminants present on the SDS/PAGE were between 30 kDa and 25 kDa. The bulk of these was eliminated by selectively pooling fractions 21–23 from the f.p.l.c. step. Re-application of these fractions directly on to a small Cu^{2+}-chelation Sepharose column and use of a 'batch' method for elution rather than a linear pH gradient were only marginally successful in removing the final contaminating proteins (see Fig. 4 inset). SDS/PAGE of a sample from fractions 18–22 shows the contaminants between 30 kDa and 25 kDa. SDS/PAGE under non-reducing conditions of fraction 19, the fraction having highest activity on the second Cu^{2+}-chelation Sepharose step, resulted in the appearance of a major band at 70 kDa, a less intense band at 60 kDa and two faint bands at 50 kDa and 39 kDa (Fig. 4). The result of this eight-step purification procedure was that G6S was purified 130700-fold in 2.8% yield of activity, which is equivalent to approx. 60 μg of protein from 5.4 kg of liver. Iduronate 2-sulphatase, glucosamine 6-sulphatase, β-hexosaminidase, *N*-acetylgalactosamine 4-sulphatase, α-L-iduronidase and arylsulphatase activities were not detected in fraction 19 (Fig. 4).

Properties of G6S

Enzyme in fraction 19 from the second Cu^{2+}-chelation Sepharose column was dialysed with 0.5 mg of BSA/ml against 0.05 M-sodium acetate buffer, pH 4.0, containing 10% (v/v) glycerol and 0.1 mM-dithioerythritol. This dialysed sample was used for determination of kinetic data as well as some physical

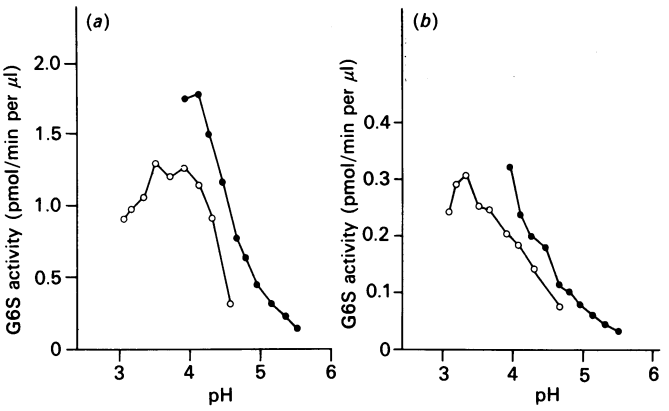

Fig. 5. Hydrolysis by liver G6S of GalNAc6S-GlcA-GalitolNAc6S and G6S-anM6S as a function of incubation pH

(*a*) GalNAc6S-GlcA-GalitolNAc6S in 50 mM-sodium acetate buffer (●) and 50 mM-sodium formate buffer (○). (*b*) G6S-anM6S in 50 mM-sodium acetate buffer (●) and 50 mM-sodium formate buffer (○). For experimental details see the Methods section.

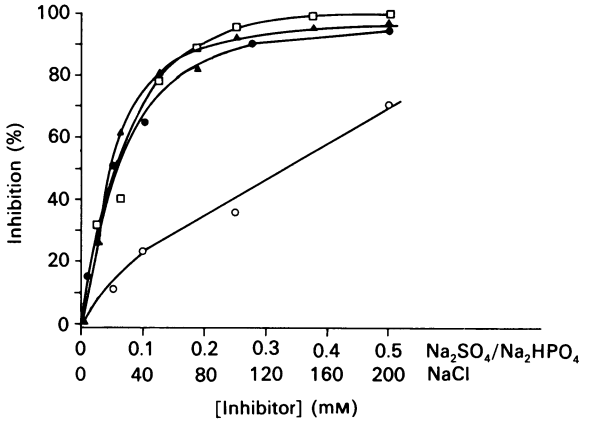

Fig. 6. Hydrolysis of GalNAc6S-GlcA-GalitolNAc6S by liver G6S as a function of NaCl (▲), Na_2HPO_4 (○) and Na_2SO_4 (●) concentration and of G6S-anM6S by G6S as a function of NaCl (□) concentration at pH 4.0 in 50 mM-sodium acetate buffer containing 500 μg of BSA/ml

For experimental details see the Methods section.

properties of the enzyme. The pH optimum for G6S activity towards C6S and KS oligosaccharide substrates was between pH 3.5 and pH 4.0 (Fig. 5). The K_m with the KS-derived disaccharide substrate at the pH optimum was 50 μM and the $V_{max.}$ 0.0889 nmol/min per mg, and with the C6S-derived trisaccharide they were 12 μM and 1.454 nmol/min per mg respectively, indicative of a 4-fold stronger binding of the enzyme to the trisaccharide substrate and a 16-fold greater turnover rate.

Effect of increasing concentrations of NaCl on G6S activity with either the disaccharide or the trisaccharide substrate showed 50% inhibition at 20 mM- and 24 mM-NaCl respectively (Fig. 6). Sulphate was found to be a potent inhibitor of G6S activity towards trisaccharide substrate, with 50 μM giving 50% inhibition. Phosphate was a less potent inhibitor, with 50% inhibition of G6S activity at a concentration of 325 μM.

DISCUSSION

We have purified, by an eight-step column procedure, G6S from human liver by more than 130000-fold with a recovery of 2.8% activity of one major form. The procedure has the advantage that other lysosomal enzymes involved in glycosaminoglycan degradation can be conveniently obtained from the same liver (Mahuran *et al.*, 1983*a*; Clements *et al.*, 1985*a*; Freeman & Hopwood, 1986, 1989; Freeman *et al.*, 1987; Gibson *et al.*, 1987; Bielicki *et al.*, 1990). An important purification step in the procedure was that of chromatography on TSK G3000SW, which led to a 15-fold purification of G6S with a native molecular mass of 62 kDa. This preparation contained 57 kDa, 39 kDa and 19 kDa polypeptide species on SDS/PAGE under reducing conditions. The *N*-terminal amino acid sequences obtained from the 39 kDa and 57 kDa polypeptides (results not shown) were the same and showed considerable similarity to the *N*-terminal amino acid sequences of other sulphatases (Bielicki *et al.*, 1990; Wilson *et al.*, 1990; Peters *et al.*, 1990). The *N*-terminal amino acid sequence of the 19 kDa polypeptide (results not shown) showed some similarity to amino acid sequence found towards the *C*-terminal in the sulphatase family (Wilson *et al.*, 1990; Peters *et al.*, 1990). As judged from yields of amino acids obtained during *N*-terminal sequencing, the 39 kDa and 19 kDa polypeptides were present in approximately equal amounts in the purified G6S from liver. These findings, together with the observation that the native molecular mass of unreduced G6S obtained by SDS/PAGE was 70 kDa (see discussion below)

Table 2. Physical properties of polypeptides of human sulphatases purified from liver

Sulphatase	Polypeptide molecular mass by SDS/PAGE (kDa) +Dithioerythritol	Polypeptide molecular mass by SDS/PAGE (kDa) −Dithioerythritol	Native molecular mass (kDa)	Reference
N-Acetylgalactosamine 4-sulphatase	43/8	57	43	Gibson *et al.* (1987); Taylor *et al.* (1990)
N-Acetylgalactosamine 6-sulphatase	39/19	70	62	Present paper
Iduronate 2-sulphatase Form A	42/14	42/14	42–65	Bielicki *et al.* (1990)
Glucuronate 2-sulphatase	47/19.5	47/19.5	63	Freeman & Hopwood (1989)
Glucosamine *N*-sulphatase	55	55	110	Freeman & Hopwood (1986)
Glucosamine 6-sulphatase Form A	78	78	75	Freeman *et al.* (1987)
Form B	43/32	48/32	75	

suggested that the 19 kDa and 39 kDa polypeptides are disulphide-linked to produce the 70 kDa component.

Consistent but different multi-polypeptide band patterns are also observed for other lysosomal enzymes, namely α-L-iduronidase (Clements *et al.*, 1989) and the sulphatases iduronate 2-sulphatase (Bielicki *et al.*, 1990), glucosamine 6-sulphatase (Freeman *et al.*, 1987), glucuronate 2-sulphatase (Freeman & Hopwood, 1989) and *N*-acetylgalactosamine 4-sulphatase (Gibson *et al.*, 1987), purified to homogeneity from these same livers (Table 2). Of these, *N*-acetylgalactosamine 4-sulphatase and G6S are the only enzymes that have polypeptides linked by disulphide bonding. *N*-Acetylgalactosamine 4-sulphatase has 43 kDa and 8 kDa polypeptides that under non-reducing conditions yielded a single protein species with a molecular mass of 57 kDa on SDS/PAGE, whereas the molecular mass of the native protein assessed by gel-permeation chromatography was 43 kDa (Table 2). It appears that G6S has a similar pattern and relationship between reduced and non-reduced polypeptide molecular masses on SDS/PAGE and a smaller native molecular mass on gel-permeation chromatography. That is, the 19 kDa and 39 kDa polypeptides required to form active G6S in human liver appear to yield a protein with a native molecular mass determined by SDS/PAGE run under non-reducing conditions that is larger than the sum of the two polypeptides and that observed by gel-permeation chromatography.

Other investigators have reported purification of G6S from human placenta. Glössl *et al.* (1979) described the isolation (20000-fold) of the enzyme in a 11% yield with a native molecular mass of 100 kDa containing a single polypeptide of 78 kDa. However, the preparation was considered not to be pure since β-*N*-acetylhexosaminidase activity was detected and different polypeptide bands were observed on SDS/PAGE. Lim & Horwitz (1981) reported that G6S purified (3000-fold) from human placenta with a 6% recovery had a native molecular mass of 90 kDa and contained a single polypeptide of 85 kDa. It would therefore seem likely that both groups reporting purification of G6S from human placenta have not achieved purification to homogeneity.

Substrate structure has an important influence on the specificity of action and catalytic efficiency of sulphatases involved in the desulphation of glycosaminoglycan substrates in the lysosome (Hopwood, 1989). Purified G6S is able to desulphate *N*-acetylgalactosamine 6-sulphate or galactose 6-sulphate residues derived from C6S and KS respectively. G6S is unable to desulphate glucosamine 6-sulphate residues in KS or heparan sulphate (results not shown). Thus G6S is specific for sulphate esters linked C-6 to hexose or *N*-acetylhexosamine with the galactose configuration. It also appears that purified G6S is approx. 60–70-fold more catalytically efficient at desulphating the C6S-derived trisaccharide than the disaccharide derived from KS. This difference in activity towards KS and C6S substrates was also observed for G6S present in crude fibroblast homogenates (Hopwood & Elliott, 1983*a*), and is not likely to be explained by simply having decreased activity towards the disaccharide, since a tetrasaccharide substrate derived from KS is desulphated with similar activity to the disaccharide (Hopwood & Elliott, 1983*b*).

This work was supported by grants from the National Health and Medical Research Council of Australia and the Adelaide Children's Hospital Research Foundation. We thank Larissa Cox for expert technical assistance and Peter Clements, Craig Freeman and Gary Gibson for helpful discussion, and Viv Muller for preparation of substrates and discussion.

REFERENCES

Bielicki, J., Freeman, C., Clements, P. R. & Hopwood, J. J. (1990) Biochem. J. **271**, 75–86
Chang, P., Rosa, N. E. & Davidson, R. G. (1981) Anal. Biochem. **117**, 382–389
Clements, P. R., Mahuran, D. & Hopwood, J. J. (1983) J. Chromatogr. **261**, 77–82
Clements, P. R., Brooks, D. A., Saccone, G. T. P. & Hopwood, J. J. (1985*a*) Eur. J. Biochem. **152**, 21–28
Clements, P. R., Muller, V. & Hopwood, J. J. (1985*b*) Eur. J. Biochem. **152**, 29–34
Clements, P. R., Brooks, D. A., McCourt, P. A. G. & Hopwood, J. J. (1989) Biochem. J. **259**, 199–208
Freeman, C. & Hopwood, J. J. (1986) Biochem. J. **234**, 83–92
Freeman, C. & Hopwood, J. J. (1989) Biochem. J. **259**, 209–216
Freeman, C., Clements, P. R. & Hopwood, J. J. (1987) Biochem. J. **246**, 347–354
Gibson, G. J., Saccone, G. T. P., Brooks, D. A., Clements, P. R. & Hopwood, J. J. (1987) Biochem. J. **248**, 755–764
Glössl, J., Truppe, W. & Kresse, H. (1979) Biochem. J. **181**, 37–46
Hopwood, J. J. (1989) in Heparin: Chemical and Biological Properties, Clinical Applications (Lane, D. & Lindahl, U., eds.), pp. 191–227, Edward Arnold, London
Hopwood, J. J. & Elliott, H. (1983*a*) Clin. Sci. **65**, 325–331
Hopwood, J. J. & Elliott, H. (1983*b*) Carbohydr. Res. **117**, 263–274
Hopwood, J. J., Elliott, H., Muller, V. & Saccone, G. T. P. (1986) Biochem. J. **234**, 507–514
Laemmli, U. K. (1970) Nature (London) **227**, 680–685
Lim, C. T. & Horwitz, A. L. (1981) Biochim. Biophys. Acta **657**, 344–355
Mahuran, D., Clements, P. & Hopwood, J. J. (1983*a*) Biochim. Biophys. Acta. **757**, 359–365
Mahuran, D., Clements, P. R., Carella, M. & Strasberg, P. M. (1983*b*) Anal. Biochem. **129**, 513–516
Neufeld, E. F. & Muenzer, J. (1989) in The Metabolic Basis of Inherited Disease (Scriver, C. R., Beaudet, A. L., Sly, W. S. & Valle, D., eds.), 6th edn., pp. 1565–1587, McGraw-Hill, New York
Peters, C., Schmidt, B., Rommerskirch, W., Rupp, K., Zühlsdorf, M., Vingron, M., Meyer, H. E., Pohlmann, R. & von Figura, K. (1990) J. Biol. Chem. **265**, 3374–3381
Taylor, J. A., Gibson, G. J., Brooks, D. A. & Hopwood, J. J. (1990) Biochem. J. **268**, 379–386
Wilson, P. J., Morris, C. P., Anson, D. S., Occhiodoro, T., Bielicki, J., Clements, P. R. & Hopwood, J. J. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 8531–8535

Received 4 February 1991/30 April 1991; accepted 8 May 1991