Correction of human mucopolysaccharidosis type-VI fibroblasts with recombinant *N*-acetylgalactosamine-4-sulphatase

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A full-length human *N*-acetylgalactosamine-4-sulphatase (4-sulphatase) cDNA clone was constructed and expressed in CHO-DK1 cells under the transcriptional control of the Rous sarcoma virus long terminal repeat. A clonal cell line expressing high activities of human 4-sulphatase was isolated. The maturation and processing of the human enzyme in this transfected CHO cell line showed it to be identical with that seen in normal human skin fibroblasts. The high-uptake precursor form of the recombinant enzyme was purified from the medium of the transfected cells treated with NH₄Cl and was shown to be efficiently endocytosed by control fibroblasts and by fibroblasts from a mucopolysaccharidosis type-VI (MPS VI) patient. Enzyme uptake was inhibitable by mannose 6-phosphate. After uptake, the enzyme was processed normally in both normal and MPS VI fibroblasts and was shown both to correct the enzymic defect and to initiate degradation of [³⁵S]sulphated dermatan sulphate in MPS VI fibroblasts. The stabilities of the recombinant enzyme and enzyme from human fibroblasts appeared to be similar after uptake. However, endocytosed enzyme has a significantly shorter half-life than endogenous human enzyme. The purified precursor 4-sulphatase had a similar pH optimum and catalytic parameters to the mature form of 4-sulphatase isolated from human liver.

INTRODUCTION

Deficiency of the lysosomal enzyme *N*-acetylgalactosamine-4-sulphatase (4-sulphatase, arylsulphatase B, EC 3.1.6.1) is the cause of the storage disease mucopolysaccharidosis type VI (MPS VI), otherwise known as Maroteaux–Lamy syndrome. Patients with MPS VI syndrome store and excrete abnormal amounts of dermatan sulphate. Depending on the severity of the disease, a variety of clinical phenotypes is observed. Symptoms of MPS VI include growth retardation, corneal clouding, skeletal deformities and hepatosplenomegaly. However, even in the severe form of the disease, neurological development is generally normal. MPS VI is therefore a possible candidate for enzyme-replacement therapy, as there is no obvious requirement for the therapeutic agent to cross the blood–brain barrier; thus all sites of pathology are potentially accessible to circulating enzyme. The existence of an animal model for the MPS VI condition (Jezyke *et al.*, 1977) will allow evaluation of enzyme-replacement therapy to proceed. Studies with cultured fibroblasts from enzyme-deficient patients with a variety of MPS disorders have shown that active enzymes can enter the cell and are transported to the lysosome where they result in normalization of substrate turnover (Neufeld & Meunzer, 1989).

The potential for enzyme-replacement therapy of the lysosomal storage disorders has been given new meaning by a trial involving the use of purified human placental glucocerebrosidase in the treatment of a Gaucher disease patient (Barton *et al.*, 1990). The enzyme preparation used was modified to target the enzyme to macrophages, as storage in macrophages is the primary cause of pathology in Gaucher disease. Treatment was continued for a 20-week period and resulted in clinical improvement of spleen and skeletal symptoms, as well as increases in haemoglobin and platelet counts.

We plan to evaluate the possibility of such therapy for MPS VI using a feline model for this disease. This will require large

amounts (of the precursor/high-uptake form) of 4-sulphatase. As a first step towards this goal, we have expressed human 4-sulphatase in CHO-DK1 cells and shown that the recombinant enzyme is similar to endogenous human 4-sulphatase in terms of its uptake, maturation and half-life and specific catalytic activity *in vivo* and *in vitro*, and that exposure of MPS VI fibroblasts to precursor recombinant enzyme results in the correction of both the enzyme defect and storage of glycosaminoglycans.

MATERIALS AND METHODS

Materials

The plasmid pRSVN.07 was a gift from Dr. Alan Robbins, Department of Biochemistry, University of Adelaide, Adelaide, SA, Australia. All enzymes for DNA manipulations, DNAase, dithiothreitol, kanamycin and streptomycin were purchased from Boehringer-Mannheim (Dulwich, SA, Australia). DNA oligonucleotides were synthesized using an Applied Biosystems 391 DNA synthesizer. L-[3,4,5-³H]Leucine (specific radioactivity 144–153 Ci/mmol), [α -³²P]dCTP (3000 Ci/mmol), Hyperfilm-MP and a mixture of [¹⁴C]methylated molecular-mass standards [(myosin (200 kDa), phosphorylase *b* (92.5 kDa), BSA (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa) and lysozyme (14.3 kDa)] were purchased from Amersham International (Fullarton, SA, Australia). Na₂³⁵SO₄ (516 mCi/mmol) was purchased from New England Nuclear (du Pont, North Ryde, N.S.W., Australia). Non-labelled molecular-mass standards were purchased from Pharmacia (North Ryde, NSW, Australia) 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). Affi-Gel 10 was purchased from Bio-Rad Laboratories (Richmond, CA, U.S.A.) and used according to the manufacturer's recommendations. Dulbecco's modified phosphate-buffered saline (PBS) was purchased from Commonwealth

Abbreviations used: BME, basal medium (Eagle's); CHO, Chinese hamster ovary; FCS, fetal calf serum; G418, G418 sulphate, Geneticin; MEM, minimal essential medium; MPS, mucopolysaccharidosis; 4-sulphatase, *N*-acetylgalactosamine-4-sulphatase; PBS, phosphate-buffered saline.

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Serum Laboratories (Melbourne, Vic., Australia). Nonidet P40, Triton X-100, mannose 6-phosphate and proteinase inhibitors (Taylor *et al.*, 1990) were purchased from Sigma (St. Louis, MO, U.S.A.). Basal medium (Eagle's) (BME), minimal essential medium (MEM), penicillin and glutamine were purchased from Flow Laboratories (Sydney, N.S.W., Australia) and fetal calf serum (FCS), Ham's F12 nutrient mixture and G418 (Geneticin) were from Gibco (Glen Waverley, Vic., Australia).

DNA manipulation

All DNA preparation, modification and cloning procedures were done using standard techniques (Ausubel *et al.*, 1989).

Scintillation counting

Samples were made up to 1 ml by addition of water and mixed with 4 ml of LKB (North Ryde, N.S.W., Australia) Optiphase Hisafe 3.

Culture and electroporation of CHO-DK1 cells

CHO-DK1 cells were kindly provided by J. Beale, Department of Biochemistry, University of Adelaide, Adelaide, SA, Australia. CHO-DK1 cells were maintained in Ham's F12 supplemented with antibiotics and 10% (v/v) FCS (F12 complete medium) at 37 °C in a 5% CO₂ atmosphere. Cells were fed with fresh medium every 2–3 days. Cells for electroporation were harvested by trypsin treatment, washed once with PBS and then resuspended in PBS at 1.2×10^7 viable cells/ml. After equilibration to 0 °C, 0.8 ml of cells was electroporated in the presence of 10–20 µg of plasmid DNA using a BRL Cell-Porator and a pulse of 275 V/330 µF. The cells were grown in non-selective medium for 48 h and then subcultured 1:5 into medium containing 0.375 mg of (active) G418/ml. Selected cells were maintained in medium containing 0.185 mg of (active) G418/ml.

Culture of fibroblasts

Human diploid fibroblasts were established from skin biopsies submitted to this hospital for diagnosis (Hopwood *et al.*, 1982). Cell lines were maintained at 37 °C in 5% CO₂ in MEM, supplemented with antibiotics, non-essential amino acids and 10% (v/v) FCS (MEM complete medium), as described previously by Taylor *et al.* (1990), or in BME supplemented with antibiotics and 10% (v/v) FCS (BME complete medium). The MPS VI cell lines, classified as clinically severe, were deficient in 4-sulphatase activity and derived from patients shown to have dermatan sulphaturia (Taylor *et al.*, 1990).

Determination of 4-sulphatase expression

Cell lysates were prepared by six freeze-thaw cycles in 0.5 M-NaCl/20 mM-Tris/HCl buffer, pH 7.0, and clarified by micro-centrifugation at 4 °C for 5 min. Cell extracts or media samples were assayed either directly or after dilution in assay buffer. Alternatively, enzyme was assayed after immune-capture with the monoclonal antibody 4-S 4.1 (Brooks *et al.*, 1991). Enzyme was assayed using either the fluorogenic substrate, 4-methylumbelliferyl sulphate (Gibson *et al.*, 1987) or the radio-labelled trisaccharide substrate GalNAc4S-GlcA-GalitolNAc4S (Hopwood *et al.*, 1986). Enzyme protein was quantified by e.l.i.s.a. as previously described (Brooks *et al.*, 1990). Total protein was quantified using the method of Lowry *et al.* (1951).

β-Hexosaminidase

β-Hexosaminidase activity was measured using the specific fluorogenic substrate 4-methylumbelliferyl 2-acetamido-2-deoxy-β-D-glucopyranoside (Leaback & Walker, 1961).

Analysis of 4-sulphatase processing

Materials and conditions for culturing and metabolic labelling of cells, immunoaffinity purification of 4-sulphatase, SDS/PAGE and fluorography were as described in Taylor *et al.* (1990). At confluency, cells were labelled for specified time-periods in MEM complete medium containing [³H]leucine (0.4 mCi/75 cm² flask). Cells were either harvested immediately or chased for the times specified in 5.0 ml of MEM complete medium supplemented with 53 mM-leucine. In some instances, cells were cultured in medium supplemented with 10 mM-NH₄Cl.

(NH₄)₂SO₄ was added to the cell culture medium (5.0 ml) and the precipitated material was isolated and dialysed as described by Hasilik & Neufeld (1980). Cells were harvested by trypsin digestion (Conary *et al.*, 1988) and then resuspended in 0.5 ml of 1% (v/v) Triton X-100/sodium deoxycholate (0.5 mg/ml)/SDS (0.2 mg/ml)/BSA (5 mg/ml)/1 mM-MgCl₂/1 mM-phenylmethanesulphonyl fluoride/5 mM-iodoacetamide/DNAase (0.02 mg/ml)/10 mM-EDTA/10 mM-ATP/0.25 M-NaCl/10 mM-Tris/HCl buffer, pH 7.4. Cell suspensions were then lysed by freeze-thawing six times, and nuclear material was removed by precipitation with protamine sulphate as described by Hasilik & Neufeld (1980). A solution containing a mixture of proteinase inhibitors (Taylor *et al.*, 1990) was then added to cell and medium supernatants which were then used directly for immunoaffinity purification.

Immunoaffinity purification of radiolabelled 4-sulphatase

Purified carrier 4-sulphatase isolated from human liver was added to cell extracts and cell culture medium and the (radiolabelled/carrier) 4-sulphatase was immunoaffinity-purified using monoclonal antibody 4-S 4.1 Affi-Gel as described by Taylor *et al.* (1990). The purified 4-sulphatase was then precipitated with deoxycholate/trichloroacetic acid and subjected to SDS/PAGE and fluorography (Taylor *et al.*, 1990).

Endocytosis of labelled 4-sulphatase

Endocytosis of labelled 4-sulphatase was essentially as described by Steckel *et al.* (1983) except that confluent monolayer cultures of human skin fibroblasts or CHO4S2 cells in 75 cm² flasks were incubated for 6 and 24 h with 5 ml of MEM complete medium supplemented with 10 mM-NH₄Cl and 0.4 mCi of [³H]leucine. The conditioned medium was then precipitated with 2.5 g of (NH₄)₂SO₄, the precipitate recovered, dissolved in 1.0 ml of distilled water and dialysed overnight against MEM. The volume was made up to 5 ml with MEM, adjusted to 5% (v/v) FCS and sterilized by filtration. The labelled product was added to confluent human skin fibroblast cultures (75 cm² flasks). Cells were harvested after 6 or 24 h or cultured (in BME complete medium) and harvested at intervals of up to 5 days.

Glycosaminoglycan turnover

Medium from CHO4S2 cells cultured in the presence of 10 mM-NH₄Cl was concentrated 7-fold by ultrafiltration (4-sulphatase stock solution) and shown to contain recombinant 4-sulphatase with an activity of 6.1×10^6 pmol/min per ml (6.5 µg of 4-sulphatase/ml). Fibroblasts from both normal individuals and severely affected MPS VI patients were grown to confluency. At 2 h before addition of radiolabel, the culture medium was changed to Ham's F12 complete medium supplemented with 100 µM-Na₂SO₄, but without antibiotics. Cells were labelled by culturing for 24 h in the presence of 20 µCi of Na₂³⁵SO₄/ml. The cells were then treated with trypsin and washed by centrifugation/resuspension in PBS to remove extracellular ³⁵S-labelled glycoconjugates (Yanagishita & Hascall, 1984) and replated in MEM complete medium supplemented with 2.6×10^6 ,

8.7×10^3 or 2.6×10^4 pmol/min per ml of recombinant 4-sulphatase. The cells were cultured for up to 92 h. The medium was then removed, the cells were rinsed with PBS, harvested by trypsin treatment and washed with PBS by centrifugation. The resultant cell pellets were solubilized in 0.25 M-sucrose/0.2 M-KCl/0.5% (v/v) Triton X-100 containing a standard mixture of proteinase inhibitors (Taylor *et al.*, 1990). The solubilized cell pellets were analysed for 4-sulphatase activity, protein content and radioactivity.

Subcellular fractionation

Fibroblasts from two different severely affected MPS VI patients were grown, labelled and stripped of extracellular labelled glycoconjugate as described above, except that a 16 h chase in BME complete medium between labelling and removal of extracellular glycoconjugate was added to allow efflux of unincorporated label. After 1 h to allow the labelled cells to re-attach, the culture medium was changed to BME complete medium supplemented with 2.44×10^4 pmol/min per ml of recombinant 4-sulphatase. The cells were incubated for 3 h and then harvested by trypsin treatment, washed by resuspension in 0.25 M-sucrose, pH 7.0 (by addition of Tris base). The cells were then repelleted and finally resuspended in 1 ml of the same buffer and lysed by repeated (15–20 times) hypobaric shock (Singh *et al.*, 1987). The homogenate was centrifuged at 200 *g* for 10 min to pellet nuclear debris and unbroken cells. The postnuclear supernatant was adjusted to a final volume of 1.5 ml with 0.25 M-sucrose/10 mM-Hepes/NaOH, pH 7.0, and loaded on to 17 ml of 25% Percoll made iso-osmotic with 0.25 M-sucrose/10 mM-Hepes/NaOH, pH 7.0, and centrifuged at 30000 *g*, 4 °C for 60 min. The resulting gradient was collected in 1 ml fractions and fractions were analysed for ^{35}S radioactivity, β -hexosaminidase and 4-sulphatase activity.

Large-scale production of recombinant 4-sulphatase

For large-scale production of recombinant 4-sulphatase from CHO4S2 cells, 40 100 mm dishes of cells were grown to confluency and used to inoculate 10 g of neutral density glass microcarrier beads (90–150 μm , density 1.03, Sigma catalogue no. G2517) according to the manufacturer's recommendations. The cells were then cultured in 1 litre of Ham's F12 complete medium, supplemented with 20 mM-Hepes/NaOH, pH 7.3, in two siliconized 1-litre stirrer culture vessels in air atmosphere. After 3 days the medium was changed with fresh medium supplemented with 20 mM-Hepes buffer (pH 7.3)/10 mM- NH_4Cl . Subsequently, the medium was changed every 2–3 days. After every 7 days the culture was grown in medium without NH_4Cl for 2 days, as continuous long-term culture in the presence of NH_4Cl appeared to have a detrimental effect on cell viability. The collected medium was clarified by centrifugation at 7000 rev./min for 10 min at 4 °C. 4-Sulphatase was purified directly from the collected medium using a 20 ml monoclonal antibody 4-S 4.1 Affi-Gel essentially as described by Gibson *et al.* (1987) except that the medium was applied directly to a column equilibrated with 20 mM-Tris/HCl buffer (pH 7.0)/0.15 M-NaCl/10% (v/v) glycerol/4 mM-EDTA/0.02% (w/v) NaN_3 . The column was washed with 5 column vol. of the equilibration buffer before being eluted with 0.1 M-sodium citrate buffer (pH 4.0)/2 M-NaCl/10% (v/v) glycerol/0.02% (w/v) NaN_3 . Fractions containing 4-sulphatase activity were pooled and concentrated by Amicon ultrafiltration using a YM10 Diaflo membrane pretreated by washing for 30 min in 0.1% (v/v) Triton X-100 and rinsing with water. The enzyme was stored in 0.05 M-sodium citrate buffer (pH 4.0)/1.0 M-NaCl/10% (v/v) glycerol/0.02% (w/v) NaN_3 . Purified enzyme was precipitated

and analysed by SDS/PAGE as described in Bielicki *et al.* (1990).

RESULTS AND DISCUSSION

Construction of the 4-sulphatase expression plasmid, pRSVN.4S

An *EcoRI/XbaI* fragment (bp 39–1809; Fig. 2 of Peters *et al.*, 1990) of the 4-sulphatase cDNA ASB2-5 was excised, gel-purified and subcloned into *EcoRI/XbaI* cut pUC19. The resulting clone was modified by the insertion of four overlapping oligonucleotides between the *EcoRI* site and the *AvaI* site (bp 108; Fig. 2 of Peters *et al.*, 1990), as shown in Fig. 1, to give p4SFL. The insertion of these oligonucleotides completed the coding sequence of 4-sulphatase (Litjens *et al.*, 1991) and also added 30 bp of 5'-non-coding sequence derived from the rat prepro-insulin gene sequence (Cullen, 1988). This sequence was added in an attempt to ensure efficient translation (Cullen, 1988) of the mRNA transcribed from the recombinant 4-sulphatase cDNA clone. An *EcoRI/StuI* (bp 1650; Fig. 2 of Peters *et al.*, 1990) fragment was excised from p4SFL, made blunt-ended using the Klenow fragment of DNA polI and gel-purified. This fragment was then cloned into the *BamHI* site (also made blunt-ended) of the expression vector pRSVN.07, placing the cDNA under the transcriptional control of the Rous-sarcoma-virus long terminal repeat. Recombinants were identified by colony hybridization and the orientation of the insert was determined by restriction enzyme analysis.

Expression of human 4-sulphatase by CHO cell transformants

Twelve individual G418-resistant clones containing the pRSVN.07/4S expression construct were assayed for enzyme activity. Cell lysates were prepared from confluent 12-well dishes and equalized by protein concentration. These extracts were assayed for 4-sulphatase activity using the fluorimetric assay. Enzyme activity ranged from background (116) to 9100 pmol/min per ml, the average being 3420. Immune-capture assay of enzyme activity confirmed that this enzyme activity was due to expression of human 4-sulphatase (results not shown). The recombinant enzyme showed identical activities (normalized to 4-sulphatase in control human fibroblast) in both the fluorogenic and the radiolabelled trisaccharide substrate enzyme assays. The highest expressing clone, CHO4S2, was then analysed in more detail.

CHO-DK1 and CHO4S2 cells were seeded into duplicate (ten) 100 mm dishes in Ham's F12 complete medium and grown until approx. 80% confluent. The medium was replaced with 10 ml of fresh medium; in addition, the medium in five of the duplicate plates was made 10 mM- NH_4Cl to promote secretion of precursor 4-sulphatase. Samples of medium were collected at 24 h intervals for 5 days. All samples were assayed for sulphatase activity using the fluorimetric assay. More than 95% of this activity was 4-sulphatase. The results (Fig. 2) show that the presence of NH_4Cl stimulates the secretion of 4-sulphatase activity by CHO4S2 cells, resulting in a level of 96×10^3 pmol/min per ml (approx. $5 \mu\text{g/ml}$) after 5 days.

Synthesis and maturation of 4-sulphatase by CHO4S2 cells

CHO4S2 cells were radiolabelled for 24 h and the 4-sulphatase present both in cell lysates and in medium was then assayed by immunopurification and SDS/PAGE. Under non-reducing conditions, 4-sulphatase from cell lysates migrated as a 57 kDa polypeptide. Under reducing conditions, this was shown to be composed of 43 kDa and (a faint) 8 kDa polypeptides. Enzyme purified from medium contained an additional 66 kDa polypeptide which became the only detectable enzyme species in the

Fig. 3. SDS/PAGE and fluorography of 4-sulphatase immunopurified from fibroblasts cultured in the presence of radiolabelled 4-sulphatase isolated from CHO4S2 cells cultured in 10 mM-NH₄Cl

For details, see the Materials and methods section. The positions of the molecular-mass standards (in kDa) are indicated by arrows. (a) Normal control fibroblasts were incubated for 6 or 24 h in medium containing radiolabelled (precursor) 4-sulphatase from CHO4S2 cells, or 24 h in medium containing identical enzyme and 5 mM-mannose 6-phosphate (Man6P). (b) Fibroblasts from a severely affected MPS VI patient were cultured in the presence of radiolabelled (precursor) 4-sulphatase from CHO4S2 cells for 24 h, 24 h in the presence of 5 mM-mannose 6-phosphate, or for 24 h and then chased in fresh medium for 72 h.

Demonstration of correction of the glycosaminoglycan turnover defect

Fibroblasts from MPS patients rapidly accumulate ³⁵S radioactivity intracellularly owing to the lysosomal storage of incompletely degraded glycosaminoglycans (Fratantoni *et al.*, 1968). Turnover of these glycosaminoglycans in normal fibroblasts proceeds as an apparently first-order process that is markedly slower in MPS fibroblasts (Fratantoni *et al.*, 1968). We have measured the intracellular turnover of ³⁵S-labelled glycoconjugates to demonstrate that endocytosis of recombinant 4-sulphatase results in the correction of glycosaminoglycan turnover in MPS VI fibroblasts.

Normal and MPS VI fibroblasts were metabolically labelled with Na₂³⁵SO₄ and cultured in the presence or absence of CHO4S2-derived precursor 4-sulphatase for up to 92 h (Fig. 4). The cells were then harvested by trypsin treatment and incorporated ³⁵S was counted. The data showed a marked difference in the levels of intracellular ³⁵S accumulating in fibroblasts from MPS VI patients and normal controls. This difference is abolished by cell culture in the presence of 4-sulphatase at each of three concentrations evaluated (Fig. 4). This suggests that with even the lowest amount of added enzyme, 4-sulphatase activity is no longer the limiting factor in the lysosomal turnover of the ³⁵S-labelled glycoconjugates present. Other similar experiments

Fig. 4. Effect of recombinant 4-sulphatase on intracellular ³⁵S-labelled glycoconjugates after 92 h

Normal and MPS VI fibroblasts were labelled with Na₂³⁵SO₄ in medium with decreased sulphate. At zero time, the medium was exchanged for MEM complete medium, supplemented with (precursor) 4-sulphatase from CHO4S2 cells at three concentrations: A, no enzyme; B, 2.8 µg/ml; C, 0.93 µg/ml; D, 0.28 µg/ml. The cells were incubated as described in the Materials and methods section for 92 h, harvested by trypsin treatment and washed with PBS by centrifugation. Pellets were extracted in 0.25 M-sucrose/0.2 M-KCl/0.5% (v/v) Triton X-100 containing a cocktail of proteinase inhibitors. ³⁵S radioactivity and total protein concentration were measured for control (stippled) and MPS VI (hatched) fibroblasts.

Fig. 5. Percoll gradient subcellular fractionation of MPS VI fibroblasts exposed to recombinant 4-sulphatase

Radiolabelled MPS VI fibroblasts were exposed to a pulse of recombinant 4-sulphatase and cell lysates were then fractionated on Percoll gradients as described in the Materials and methods section. ■, ³⁵S radioactivity; ▲, β-hexosaminidase activity (nmol/3 h per ml); ●, 4-sulphatase activity (pmol/min per ml × 10).

demonstrated that this effect was reproducible, the average decrease in accumulation of intracellular ³⁵S after correction of the defect with the recombinant enzyme and a 30 h chase period being 7.9-fold (± 1.1 , $n = 5$).

Localization of endocytosed recombinant 4-sulphatase

To confirm the lysosomal localization of the endocytosed recombinant 4-sulphatase that is inferred from the initiation of glycosaminoglycan turnover, postnuclear supernatants from both corrected and control MPS VI fibroblasts, which had been labelled with Na₂³⁵SO₄, were fractionated on Percoll gradients. Analysis of these gradients showed that in the corrected MPS VI cells, 4-sulphatase activity fractionated with both the lysosomal enzyme β-hexosaminidase, and with ³⁵S-labelled material, in the dense fractions of the gradient (Fig. 5). Control MPS VI

Fig. 6. SDS/PAGE of 4-sulphatase immunopurified from media of CHO4S2 cells grown in the presence of 10 mM-NH₄Cl

Lane 1 shows purified 4-sulphatase, and lane 2 molecular-mass markers. The sizes of the molecular-mass standards are indicated on the right-hand side and the calculated molecular masses of the 4-sulphatase bands are indicated on the left (in kDa).

fibroblasts contained no significant levels of 4-sulphatase activity. This analysis therefore confirmed the localization of the endocytosed enzyme to the lysosome and also showed that this occurs within 3 h of exposure of skin fibroblasts to recombinant enzyme.

Large-scale production of 4-sulphatase from CHO4S2 cells

We have used high-density carrier bead/stirrer cultures grown in the presence of 10 mM-NH₄Cl to produce 3 mg of 4-sulphatase/l of culture media. This 4-sulphatase consists mostly of the precursor 66 kDa polypeptide (Fig. 6). Trace amounts of the 43 kDa and 8 kDa polypeptides are also present (Fig. 6). About 1 mg of 4-sulphatase/l of culture medium was obtained after purification. This represented a recovery of approx. 35% of the total 4-sulphatase present in the culture medium. The pH optimum of the purified 4-sulphatase toward the fluorogenic substrate in sodium dimethylglutarate buffer was 5.3. The K_m , V_{max} , and specific activity for this preparation of 4-sulphatase were 4.8 mM, 17 μ mol/min per mg and 19 μ mol/min per mg for the fluorogenic substrate. The corresponding values for the trisaccharide substrate were 6.0 μ M, 0.238 μ mol/min per mg and 0.362 μ mol/min per mg. These values suggest that this preparation of 4-sulphatase, containing mostly the 66 kDa polypeptide, has similar catalytic parameters to those reported (Gibson *et al.*, 1987) for the mature form isolated from human liver, which mostly consists of the 43 kDa and 8 kDa polypeptides, with the possible exception of the V_{max} and K_m values for the trisaccharide substrate. However, remeasurement of these values with purified liver 4-sulphatase gave values of 0.188 μ mol/min per mg and 5.7 μ M, suggesting that the previously

published values of 2 μ mol/min per mg and 60 μ M may be incorrect.

In summary, the CHO4S2 cell line expressed human 4-sulphatase in significant amounts. Exogenous enzyme taken up by mannose 6-phosphate receptor-dependent endocytosis appeared to be processed through similar steps to those observed for the endogenous enzyme (Taylor *et al.*, 1990). Endocytosis of the recombinant enzyme leads to its incorporation into functional lysosomes and clearance of intracellular stored glycosaminoglycans. The recombinant enzyme would therefore appear to be suitable for initial enzyme-replacement therapy experiments in the feline MPS VI model described by Jezyke *et al.* (1977).

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