Recombinant α-L-iduronidase: characterization of the purified enzyme and correction of mucopolysaccharidosis type I fibroblasts

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Mucopolysaccharidosis type I (MPS I, Hurler and Scheie syndromes) is an autosomal recessive lysosomal storage disorder that results from a deficiency of the hydrolase α-L-iduronidase (IDUA) which is involved in the lysosomal degradation of both heparan sulphate (HS) and dermatan sulphate (DS). Patients with MPS I store and excrete large amounts of partially degraded HS and DS. In order to evaluate enzyme replacement therapy for MPS I patients we have expressed human IDUA cDNA in Chinese Hamster Ovary (CHO)-K1 cells utilizing a plasmid vector that places the cDNA under the transcriptional control of the human polypeptide-chain-elongation factor Iα gene promoter. A clonal cell-line that secreted recombinant IDUA in a precursor form at approximately 2.2 $\mu g/10^6$ cells per day was identified. This enzyme was shown to be endocytosed into cultured MPS I fibroblasts via mannose-6-phosphate receptors and to correct the storage phenotype of these cells by enabling the lysosomal-digestion of accumulated sulphated glycosaminoglycans. The recombinant IDUA had on SDS/PAGE a molecular mass of 85 kDa and was processed to 74 kDa and smaller forms following its uptake by fibroblasts. Milligram quantities of the recombinant IDUA were immunopurified and the enzyme was shown to have pH optimum and kinetic parameters differing from those of the mature enzyme purified from human liver. The specific activity of the recombinant enzyme was shown to increase on dilution and on incubation with reducing agents. This was in contrast to the mature IDUA form (74 kDa) which did not have its activity stimulated by reducing agents or dilution.

INTRODUCTION

α-L-Iduronidase (IDUA, α-L-iduronide iduronohydrolase, EC 3.2.1.76) is one of the hydrolases required for the lysosomal degradation of the glycosaminoglycans heparan sulphate (HS) and dermatan sulphate (DS) (Rodén, 1980; Hopwood, 1989). Deficiency of IDUA results in the lysosomal storage disorder mucopolysaccharidosis type I (MPS I) (Neufeld and Muenzer, 1989) with lysosomal accumulation and excretion of partly degraded HS and DS. MPS I patients present with a wide range of clinical symptoms ranging from the severe Hurler syndrome, with severe skeletal deformities, stiff joints, corneal opacity, mental retardation and early death, to the clinically mild Scheie syndrome, with normal intelligence and lifespan and only mild skeletal deformities, stiff joints and corneal opacity. The considerable variation of clinical presentation is thought to result from combinations of the different mutations observed in the α-L-iduronidase gene (Scott et al., 1993). IDUA has been purified from a number of human tissues where it appears to be present in varying, tissue-specific proportions of mature and precursor forms (Clements et al., 1989). Correction of the excessive storage has been demonstrated by showing that cultured MPS I fibroblasts take up exogenously-added enzyme. IDUA prepared from lung tissue, which has a high proportion of the precursor form of the enzyme, was shown to be especially efficient for correction (Clements et al., 1989). The cDNA sequence and the genomic organization for the human enzyme have been described (Scott et al., 1991, 1992a). Large amounts of stable high-uptake precursor form of IDUA will be required for replacement therapy of patients with this MPS disorder. Mildly affected patients, for example patients with Scheie syndrome or those without neurological involvement, would be the first to be considered for such treatment since sufficient enzyme transport through the blood–brain barrier has yet to be demonstrated. Large-scale production of this enzyme that is present in very low natural abundance in tissues will also allow further characterization of its tertiary structure and catalytic function.

MATERIALS AND METHODS

Materials

Kanamycin and streptomycin were purchased from Boehringer–Mannheim (Dulwich, SA, Australia). [^{3}H]Galactose (21.7 kCi/mol) and $Na_2{}^{35}SO_4$ (525 Ci/mol) was purchased from New England Nuclear (du Pont, North Ryde, NSW, Australia). Sephacryl S-200, Sephadex G-10, Blue Dextran, Percoll and molecular mass standards for SDS/PAGE were from Pharmacia (North Ryde, NSW, Australia). Affi-Gel 10 was purchased from Bio-Rad Laboratories (Richmond, CA, U.S.A.). PBS, Triton X-100 and mannose 6-phosphate were purchased from Sigma (St Louis, MO, U.S.A.). Basal medium Eagle's (BME) (modified), penicillin and glutamine were from Flow Laboratories (Sydney, NSW, Australia) and fetal calf serum (FCS) was from Gibco (Glen Waverly, Victoria, Australia). Tissue-culture and cell-factory flasks were purchased from Nunc (Roskilde, Denmark). 4-Methylumbelliferyl-α-L-iduronide was from Melford Laboratories (Ipswich, U.K.).

IDUA expression construct

The IDUA expression construct, pRSVN.ID21 (Scott et al., 1991), was modified by replacing the sequence between the

Abbreviations used: BME, basal medium Eagle's; BSA, bovine serum albumin; CHO, Chinese Hamster Ovary; DMEM, Dulbecco's modified Eagle's medium; DS, dermatan sulphate; FCS, fetal calf serum; GAG, glycosaminoglycan; HS, heparan sulphate; IDUA, α-L-iduronide iduronohydrolase; MPS I, mucopolysaccharidosis type I; DTE, dithioerythritol; CS, chondroitinase sulphate; DMG, dimethylglutaric acid.

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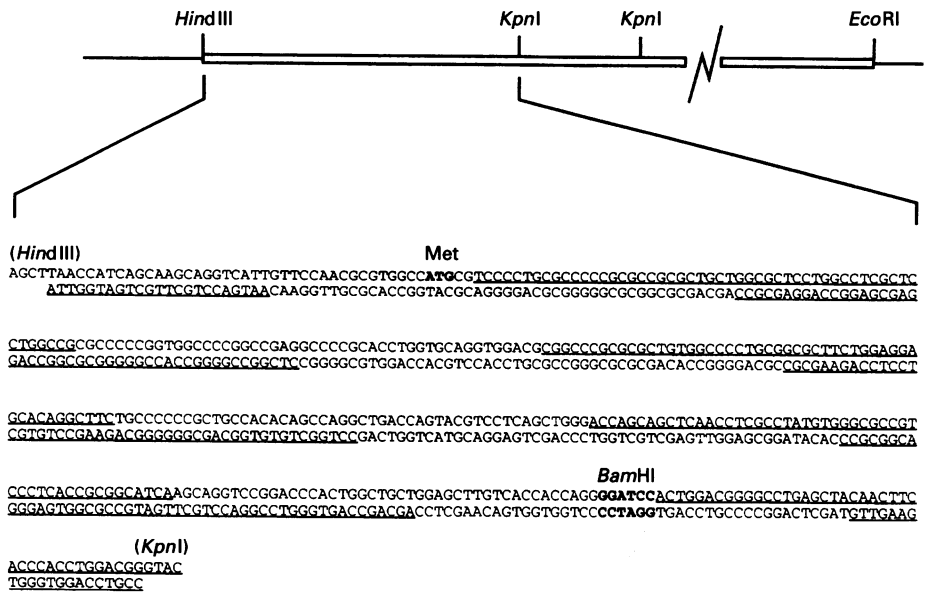

Figure 1 Construction of IDUA cDNA

pRSVNID21 is shown with vector sequences indicated by the solid line and IDUA cDNA sequences by the open bar. Relevant restriction enzyme sites are also shown. The sequence cloned between the (5′) *Hin*dIII site and the polymorphic *Kpn*I site as described under Materials and methods is shown below, alternate oligonucleotides being indicated by underlining. The IDUA initiation codon is indicated by Met, the *Bam*HI site introduced by a wobble codon is also indicated.

*Hin*dIII site, which delineates the 5′-end of the IDUA cDNA sequence, and the polymorphic *Kpn*I site at position 440 of the published cDNA sequence (Scott et al., 1991) with the 394 bp sequence shown in Figure 1. This sequence was synthesized as 17 overlapping oligonucleotides as indicated in the Figure. Each oligo was individually 5′-phosphorylated using T4 polynucleotide kinase and then mixed in equimolar amounts. The oligonucleotide mixture was then heated to 95 °C and annealed by slow cooling. pRSVN.ID21 was restricted to completion with *Hin*dIII, then partially restricted with *Kpn*I and finally dephosphorylated with alkaline phosphatase. The partial digestion product corresponding to cutting at the *Kpn*I site at position 440 was then gel-purified and used as a vector to clone the annealed oligonucleotide mixture. Recombinants containing the correct sequence were identified by restriction-enzyme mapping and one such recombinant, designated pRSVN.ID, was chosen and used for all subsequent experiments.

pRSVN.ID was then modified by replacing the rous sarcoma virus long terminal repeat promoter with the human elongation factor 1α gene promoter fragment from pEFBOS in an exactly analogous manner as described in Bielicki et al. (1993) to give pEFN.ID.

IDUA expression

pEFN.ID was electroporated into Chinese Hamster Ovary (CHO)-K1 cells as described previously (Anson et al., 1992). Individual clones were isolated and assayed for secretion of IDUA activity into the culture medium. The cell line secreting the highest levels of activity, CHOID.D6, was chosen and used for all subsequent experiments.

IDUA production and purification

CHOID.D6 cells were grown in a two-layer cell factory (Nunc; 1200 cm^2) and fed fresh medium every 3–4 days. The Ham's F12 medium was alternatively supplemented with 10% (v/v) FCS or 10 mM NH_4Cl in order to stimulate release of lysosomal enzymes to the culture medium (Anson et al., 1992). The conditioned serum-free medium supplemented with NH_4Cl was collected, clarified by centrifugation (200 *g* for 10 min at 4 °C) and stored at 4 °C. IDUA was purified from the collected medium using a 6 ml monoclonal antibody Id1A-Affi-Gel, essentially as described by Clements et al. (1989). Briefly, the medium was applied directly to the column equilibrated with 0.20 M NaCl/20 mM Tris/HCl, pH 7.0/5% (v/v) glycerol/5% (v/v) ethanol/0.02% (w/v) NaN_3. The column was washed with at least five volumes of the same buffer before being eluted with 1.5 M NaCl/50 mM Na citrate, pH 4.0/5% (v/v) glycerol/5% (v/v) ethanol/0.02% (w/v) Na azide. Fractions of ~ 3 ml were collected in tubes with 0.5 ml 0.5 M Tris/HCl, pH 7.0. Fractions containing IDUA activity were pooled and concentrated and the buffer composition was changed to 20 mM dimethylglutaric acid (DMG), pH 6.0/0.2 M NaCl/5% (v/v) glycerol/5% (v/v) ethanol/0.02% (w/v) NaN_3, in a 50 ml Amicon concentrator. The samples were then stored at 4 °C.

Concentration of the pure enzyme was determined by the Markwell et al. (1978) modification of the Lowry method. Purified enzyme was precipitated and analysed by SDS/PAGE as described by Bielicki et al. (1990).

Culture of fibroblasts

Human diploid fibroblasts were established from skin biopsies submitted to this Department for diagnosis (Hopwood et al., 1982). These cells were grown in BME supplemented with antibiotics and 10% (v/v) FCS. Fibroblasts were maintained at 37 °C in 5% CO_2 and were not used beyond 15 passages. Most studies of MPS I cells were done with one cell line, SF 3568. This cell line is homozygous for $W_{402}X$, a mutation resulting in premature termination of translation and no detectable IDUA activity (Scott et al., 1992b).

Enzyme assays

IDUA activity was measured by a fluorogenic assay using the specific substrate 4-methylumbelliferyl-α-L-iduronide (Clements et al., 1985a).

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

Radioactive labelling

$^{35}SO_4$-labelling

MPS I and normal fibroblasts were incubated for 2 h in Ham's F12 without FCS or PSK (penicillin/streptomycin/kanamycin) in order to reduce the free SO_4^{2-} pools before labelling, and then labelled with 12 μCi $^{35}SO_4^{2-}$/ml in the same medium for 4 days followed by chase in BME with 10% (v/v) FCS and PSK for 2 weeks.

^{3}H-Galactose-labelling

Fibroblasts were incubated overnight in Dulbecco's modified Eagle's Medium (DMEM) without glucose, but supplemented with 1 mM of non-essential amino acids, 6 mM Na-pyruvate and PSK, and with 10% (v/v) calf serum dialysed against the same medium. The cells were labelled with 1 μCi [^{3}H]galactose/ml in a fresh lot of the same medium for 4 days followed by chase in BME with 10% (v/v) FCS and PSK for 2 weeks.

Uptake of IDUA in cells

MPS I fibroblasts were incubated with varying amounts of IDUA (0.8–100 ng IDUA/ml) in the BME cell-culture medium for different periods of time (1 h–3 days) and then harvested. IDUA uptake via the mannose-6-phosphate receptor pathway was shown by incubation of cells in medium containing different concentrations of mannose-6-phosphate (0–4 mM) for 2 h before adding IDUA (at 0.8 or 8 ng/ml of the same medium) and incubation for a further 48 h before harvesting of medium and cells.

Subcellular fractionation

Cells were harvested following treatment with trypsin, washed by centrifugation and resuspended in 0.25 M sucrose/0.05 M Hepes, pH 7.0, pelleted, suspended in 1 ml of the same buffer and then lysed by repeated (10 times) hypobaric shock (Singh et al., 1987). The cell homogenate was centrifuged at 200 *g* for 10 min to pellet nuclear debris and unbroken cells, the pellet was again resuspended in 1 ml of the same buffer, subjected to hypobaric shock (10 times) and centrifuged as above. The two supernatants were combined and adjusted to a final volume of 2 ml with 0.25 M sucrose/10 mM Hepes/NaOH, pH 7.0, and loaded on to 17 ml of 22.5% (v/v) Percoll made iso-osmotic with 0.25 M sucrose/10 mM Hepes/NaOH, pH 7.0, and centrifuged at 50000 *g* for 60 min at 4 °C. The resulting gradient was collected in 1 ml fractions.

Cell lysates

Cell lysates were prepared by six freeze–thaw cycles of trypsinized and pelleted cells in 0.5 ml of 0.5 M NaCl/20 mM Tris/HCl buffer, pH 7.0, and clarified by centrifugation at ~ 13000 *g*, 4° C for 3 min. The supernatant was used directly, with added markers, in gel filtration.

Gel filtration

Gel filtration using a Sephacryl S-200 (1.5 × 94 cm) column in 0.5 M NaCl/50 mM Tris/HCl, pH 7.0/0.02% (w/v) NaN_3. The column was run at 26 ml/h and ~ 3 ml fractions were collected.

Ion-exchange of GAG

Medium from the pulse–chase labelling of cultured fibroblasts was collected and applied to a 2 ml DEAE-Sepharose column equilibrated in 0.35 M NaCl/50 mM Tris/HCl, pH 8.0. After extensive washing with the equilibrium buffer, the column was washed with 0.35 M NaCl/50 mM Na-acetate buffer, pH 4.0, before elution of glycosaminoglycan (GAG) fraction with 1.5 M NaCl/50 mM Na-acetate, pH 4.0.

Maturation of precursor recombinant IDUA

Precursor IDUA purified from the culture medium of CHOID.D6 cells was cultured with MPS I fibroblasts under standard conditions for 2 days–2 weeks. Cells were harvested with trypsin and cell lysate prepared as described above. This cell lysate was diluted so that the NaCl concentration was reduced to below 0.2 M and then applied to the ID1A Affi-Gel for purification of IDUA as described above.

IDUA from normal control fibroblasts was also purified by affinity chromatography over ID1A-Affi-Gel.

Scintillation counting

Samples with mixed with a minimum of four volumes LKB (North Ryde, NSW, Australia) Optiphase Hisafe 3, to a total volume of at least 2 ml and measured in an LKB Wallack 1216 Rackbeta II liquid scintillation counter.

RESULTS AND DISCUSSION

Yield of recombinant IDUA from CHO-cell media

No difference in IDUA concentration from CHOID.D6 cells was found in cell-factory media supplemented with FCS or NH_4Cl.

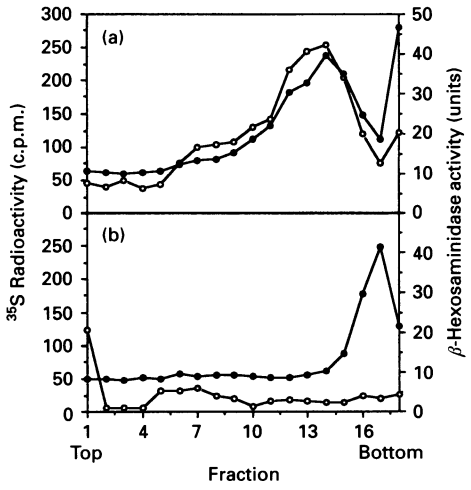

Figure 2 Subcellular fractionation of storage product

Percoll gradient fractionation of ^{35}S-sulphate radiolabelled MPS I (**a**) and normal control (**b**) fibroblasts. Cells were cultured, pulse–chase-labelled and fractionated as described under Materials and methods. ^{35}S-Radioactivity (○); β-hexosamidase activity (●).

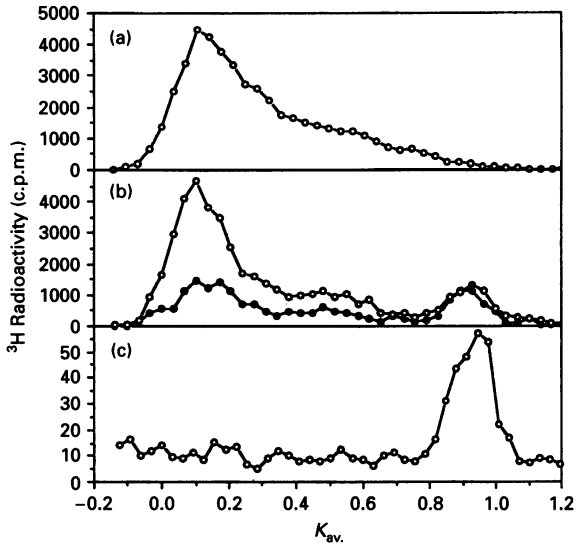

Figure 3 IDUA degradation of storage product *in vivo*

S-200 Gel filtration of cell-lysates (**a**, **b**) and media (**c**) from MPS I cells pulse–chase-labelled with [^{3}H]galactose. Untreated cells (**a**); cells incubated for 4 h (○) and 8 h (●) with IDUA (**b**); medium from cells incubated with IDUA for 8 h (**c**).

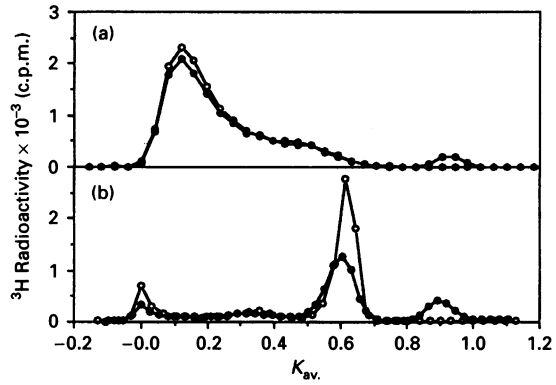

Figure 4 IDUA degradation of storage product *in vitro*

S-200 Gel filtration of GAG from [^{3}H]galactose-labelled MPS I cell media concentrated on DEAE-Sepharose and incubated, 24 h, 37 °C, prior to gel filtration without (○) and with (●) IDUA (**a**). (**b**) As (**a**) but GAG samples were incubated overnight at 37 °C with chondroitinase ABC prior to incubation without (○) and with (●) IDUA.

The average secretion of IDUA to the media was found to be approximately 2.2 μg/10^6 cells over 24 h.

MPS I storage and excretion products

[^{3}H]Galactose or ^{35}S-sulphate labelling of MPS I fibroblasts resulted in storage (Figures 2a and 3a) and excretion of high molecular mass labelled GAG (Figure 4). In contrast, ^{35}S-labelled lysosomal storage product could not be demonstrated (Figure 2b) in cell lysates from normal control fibroblasts. In Figure 2a it is also apparent that in spite of a wide distribution of size/density of lysosomes in the Percoll gradient the ratio of lysosomal marker (β-hexosaminidase) and marked for storage product (^{35}S) is virtually constant for most of the gradient (fractions 7–15). It is only the bottom fractions (16–18), the newly synthesized high-density lysosomes, that the ratio changes drastically with an increasing proportion of β-hexosaminidase to ^{35}S (similar results in four different experiments). These ratio differences may indicate redistribution of radiolabelled material and/or enzyme between new and old lysosomes.

Culture medium from radiolabelled MPS I cells produced high-molecular mass radiolabelled GAG that could be concentrated by DEAE-Sepharose chromatography. The isolated radiolabelled GAG could be partly degraded *in vitro* by incubation with recombinant IDUA for 24 h at 37 °C (Figure 4a). Degradation of the radiolabelled GAG with chondroitinase ABC, demonstrated that it contained ~ 70% chondroitin sulphate/dermatan sulphate (CS/DS) (Figure 4b), while deamination at pH 1.5 (Shively and Conrad, 1976) demonstrated that it contained ~ 25% HS (data not shown). Interestingly, it was found that while the direct incubation of the radiolabelled GAG with IDUA resulted in ~4% degradation to radiolabelled monosaccharides (Figure 4a), incubation with IDUA after chondroitinase ABC digestion of the radiolabelled GAG resulted in ~ 20% of the radioactivity eluting with the V_t on S-200 (Figure 4b). This may be consistent with IDUA being able to hydrolyse Δ4,5-uronic acid residue from the chondroitinase-produced disaccharides. Also, assuming an equal distribution of label into all monosaccharide residues and that all CS/DS and HS chains have non-reducing-end iduronic acid residues that are accessible to cleavage, 4% degradation following incubation with IDUA implies an average of 25 monosaccharide residues in these GAGs. This, depending on the extent of sulphation, corresponds to average GAG molecular mass in the range 5–7.5 kDa. However, another explanation is that all of the non-reducing-end iduronic acid residues on the CS/DS chains are not fully susceptible to hydrolysis by IDUA until after digestion of the GAG chains with chondroitinase to produce a trisaccharide substrate with iduronic acid residues at their non-reducing ends. Using this amount of monosaccharide, as released from the CS/DS fraction, to calculate the molecular mass of the CS/DS chains gives an average chain length of approximately 2–3 kDa.

Uptake of IDUA by MPS I cells

Endocytosis, via a mannose-6-phosphate receptor-mediated pathway, leads to the uptake of IDUA and correction of GAG storage in MPS I cells. Incubation of ^{35}S-labelled MPS I cells with recombinant IDUA resulted in hydrolysis of high molecular mass species (Figure 3) and, provided the dose was high enough (Figure 5a), release of more than 90% of the label to the culture medium within 24 h (Figure 5b). A near maximal effect for 24 h incubation was reached at the approximate level of 3.5×10^{-12} M or 0.3 ng IDUA/ml culture medium. This IDUA concentration corresponds to approximately 1000 molecules/cell, which is much lower than the receptor capacity for the cell [cf. maximal insulin-like growth factor (IGF) II effect at ~ 10^{-9} M via the mannose-6-phosphate receptor; Kovacina et al., 1989]. After an initial level of ~ 15%/h for release of stored ^{35}S-radiolabelled material, a steady state was reached at approximately 5%/h (% relating to initially stored material) (Figure 5b). Studies of [^{3}H]galactose-labelled material in cell lysates also indicated a steady-state process since the V_t material absent at time 0 (Figure 3a) is at approximately the same level at 4 h and 8 h after addition of IDUA (at 100 mg/ml). This is in contrast to the total level of labelled storage material in cells, the level at 8 h being ~ 45% of that at 4 h (Figure 3b). These two sets of data suggest

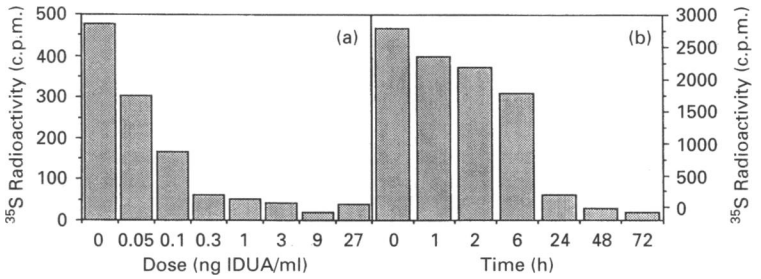

Figure 5 Correction of MPS I cells following incubation with recombinant IDUA

^{35}S-Radioactivity in cell lysates; 24 h incubation of radiolabelled cells with different amounts of IDUA (**a**); cells harvested at different times after incubation with ~ 9 ng of IDUA/ml of culture medium (**b**).

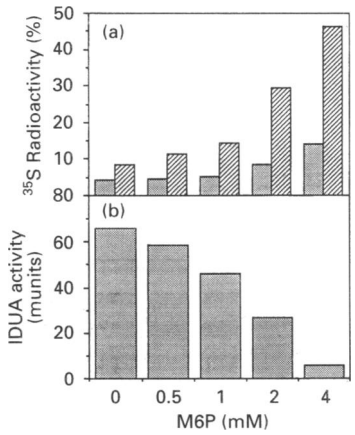

Figure 6 Mannose 6-phosphate inhibition of IDUA correction of MPS I cells

Residual ^{35}S-radioactivity in pulse–chase-labelled cells after 24 h incubation with ~ 8 ng IDUA/ml (stripped bars) or 0.8 ng IDUA/ml culture medium (hatched bars). The radioactivity is expressed as % of the radioactivity in parallel cell cultures that were not incubated with IDUA (**a**). IDUA activity in cell extracts from the 8 ng IDUA/mf medium experiment (**b**). The cells were cultured for 2 h in the range of mannose 6-phosphate concentrations indicated before addition of IDUA.

the presence of a regulatory mechanism to control the rate of GAG hydrolysis. A build-up of monosaccharides or sulphate may result from a limited capacity of the lysosomal membrane transporters involved in the removal of these monomers from the lysosome. The presence of sulphate and/or monosaccharides in the lysosome may inhibit further hydrolysis of GAG. This proposal should be compared with the studies by Rome and Hill (1986) reporting that there is no accumulation of either sulphate or hexosamine monomers from GAG-degradation in fibroblast lysosomes.

A concentration-dependent inhibition of the IDUA correction of MPS I cells could be demonstrated with increasing amounts of mannose-6-phosphate in the cell culture medium (Figures 6a and b). Inhibition of hydrolysis was dependent on both IDUA and mannose-6-phosphate concentrations present in the culture medium. At ~ 0.8 ng IDUA/ml the release of storage product by IDUA was inhibited by ~ 40% of initially stored material at 4 mM mannose-6-phosphate, whereas at a 10-times higher IDUA concentration (~ 8 ng/ml) the inhibition at 4 mM mannose-6-phosphate was only ~ 10% (Figure 6a). These observations

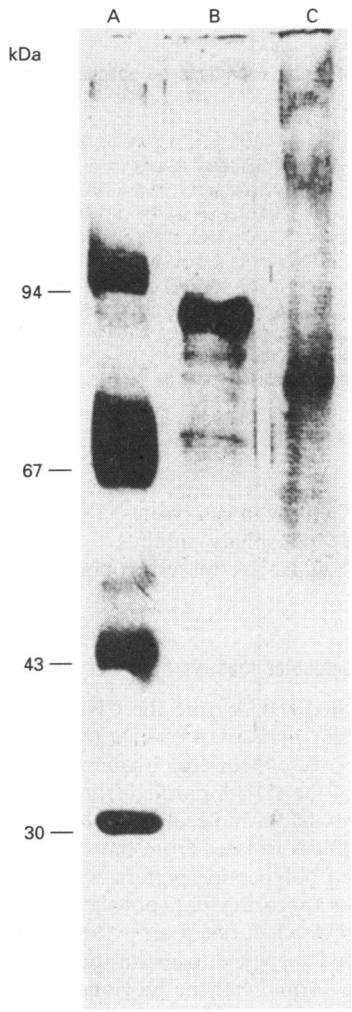

Figure 7 Silver stained SDS/PAGE of IDUA immunopurified from various sources

The positions of the molecular-mass standards (in kDa) are labelled in Lane **A**. Lane **B**, affinity-purified recombinant IDUA from the CHO-cell factory. Lane **C**, IDUA affinity-purified from lysates of MPS I cells cultured in the presence of the IDUA shown in Lane B. For details see the Materials and methods section.

demonstrate the high-efficiency of clearance of stored GAG by minute amounts of IDUA. If, instead of release of storage

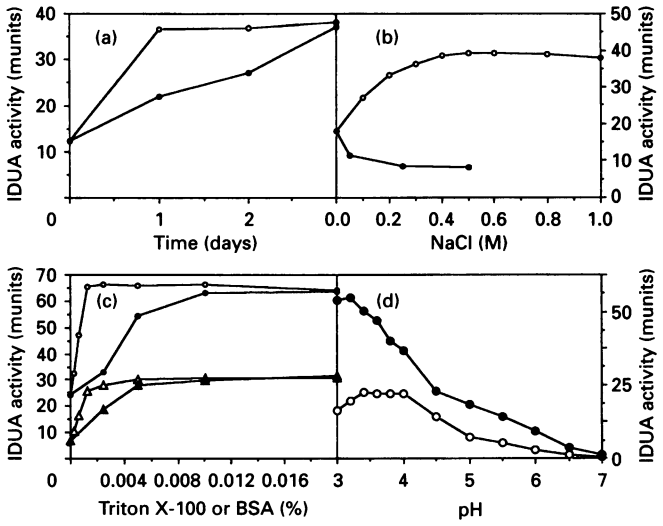

Figure 8 Effect of buffer conditions on specific activity of reduced and unreduced IDUA

(**a**) IDUA incubated at 4 °C (●) or 37 °C (○) in the presence of DTE (5 mg/ml) in 0.1 M DMG buffer, pH 4.5/0.35% (w/v) BSA prior to assay at 1:100 dilution in the same buffer without DTE. (**b**) IDUA was preincubated with (○) or without (●) 5 mg DTE/ml for 24 h at 37 °C in 50 mM DMG buffer, pH 4.5, before dilution (1:160) into IDUA activity assay buffer containing increasing amounts of NaCl. (Mean of two experiments, the difference between the two was less than 7%). (**c**) Reduced IDUA, from (**b**), assayed in 50 mM DMG, pH 3.6, in the presence of increasing amounts of BSA (●, ▲) or Triton X-100 (○, △). Incubations (●, ○) contain 0.3 M NaCl and (▲, △) are free of added NaCl. (Single determinations, the plateau-levels were constant up to 0.08% of Triton X-100 or BSA.) (**d**) IDUA reduced (●) or not reduced (○), as described in (**b**), was assayed in 0.1% (w/v) BSA and 50 mM DMG buffer at different pHs. (Results were within 17% of the concentration-corrected values for a 1:4 dilution of the enzyme.)

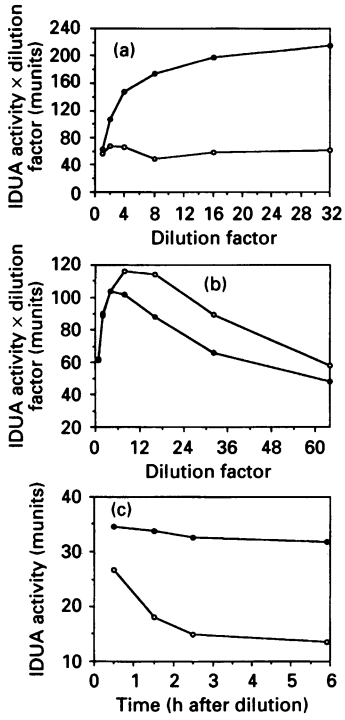

Figure 9 Effect of IDUA concentration and conditions on enzyme activity

(**a**) IDUA was reduced by incubating with 5 mg DTE/ml for 24 h at 37 °C in 50 mM DMG buffer, pH 4.5, before dilution and assay in the same buffer (without DTE) with 0.1% (w/v) BSA (●) or free of BSA (○). (Less than 5% difference between two experiments.) (**b**) Dilution and assay of reduced [as in (**a**)] IDUA in 0.1% (w/v) BSA (○), or 0.01% (v/v) Triton X-100 (●) with buffer not containing BSA or detergent. (Less then 10% difference between two experiments.) (**c**) Time, at 24 °C, after dilution of reduced [as in (**a**)] IDUA in buffer with (●) or without (○) 0.1% (w/v) BSA, before assay in the same buffer. (Similar results for two other dilution series.)

product, IDUA activity in cell lysates is measured, we find that 4 mM mannose-6-phosphate inhibits ~95% of the IDUA uptake in the ~8 ng IDUA/ml series of incubations (Figure 6b).

Apparent IDUA molecular masses

The affinity-purified IDUA from the CHO-cell medium had an apparent molecular mass of ~85 kDa (Figure 7), whereas the major IDUA, recovered from the lysate of MPS I cells cultured in the presence of the IDUA purified from the CHO expression system for 2 days, had a molecular mass of ~75 kDa. After a further 5 days IDUA isolated from the same cell system had a more complicated polypeptide pattern with several lower molecular mass bands appearing (data not shown). Thus the secreted recombinant IDUA when taken up by fibroblasts has a similar molecular size and response to maturation, presumably in the lysosome, to the normal enzyme previously studied in cultured fibroblasts (Myerowitz and Neufeld, 1981; Taylor et al., 1991).

Activity of IDUA

The specific activity of IDUA purified from the CHO expression system (but not the mature forms of IDUA) increased during incubation with the reducing agent dithioerythritol (DTE) (Figure 8a). The observed level of activity increase upon reduction varied from 2–7-fold for different IDUA preparations. The enzyme activity of the reduced precursor form of IDUA increased with increasing NaCl concentration in the buffer to a maximum at ~0.5 M NaCl (Figure 8b). This was not observed with the unreduced precursor IDUA (Figure 8b) or mature IDUA (data not shown). IDUA activity was further enhanced by the presence of BSA or non-ionic detergent Triton X-100 in the assay buffer. The effect for both BSA and detergent on enzyme activity was concentration-dependent and reached a maximum level of ~3–4 times the zero addition value. The maximum effect was reached at ~0.001% for Triton X-100 and at ~0.01% for BSA (Figure 8c). The effects on activity or Triton X-100 and NaCl were additives for the reduced IDUA (Figure 8c). Whereas Triton X-100 or BSA stimulated the unreduced precursor IDUA (data not shown), NaCl acted as an inhibitor (Figure 8b).

The pH optimum of the enzyme was also found to be dependent of the state of reduction of the enzyme (Figure 8d).

An unexpected finding was that the specific activity of recombinant IDUA increased several-fold upon dilution (Figure 9a). This increase in activity was dependent on the composition of the diluting buffer, Triton X-100 or BSA being a requisite (Figures 9a and b), with greater increases occurring with increasing NaCl concentrations (data not shown). This property of the enzyme may reflect an inhibition by self-aggregation at higher concentrations, dilution resulting in a shift in equilibrium towards monomers and lower oligomeric forms analogous with the inactivation by dilution studied by Attwood et al. (1993). However, it may be that not even in this system are the most active species the monomers, since activity declines with time

following a 20-fold dilution into a buffer without BSA or Triton X-100 (Figure 9c). Alternatively, IDUA monomers may be active but unstable without the presence of detergent or BSA as protective agents. That the interaction between IDUA and BSA/Triton X-100 is of a low affinity type is demonstrated in Figure 9b where dilution into buffer without protective agents results in loss of the activity increase as the concentration of BSA or Triton X-100 is reduced in the presence of a constant amount of IDUA.

Cu^{2+} was found to be inhibitory to the unreduced enzyme, with half maximal inhibition at ~ 0.2 mM. A series of other divalent cations (Mg, Ca, Zn, Mn, Fe) were tested but none had measurable inhibitory or stimulatory effects in the concentration range up to 5 mM. Also, dialysis of IDUA against EDTA followed by additions of these cations did not result in increased IDUA activity (cf. Clements et al., 1985a,b).

The kinetic parameters K_m and $V_{max.}$ of IDUA were also dependent on the above-mentioned conditions. For the reduced precursor form of IDUA at a concentration of 7.6 nM the K_m was ~ 1.51 mM and V_{max} ~ 71 μM/min which gives a $k_{cat.}$ of ~9400 mol product/mol enzyme per min. Whereas at an IDUA concentration of 0.34 nM, a K_m of ~ 1.78 mM, $V_{max.}$ of 18.4 μM/min and $k_{cat.}$ ~ 54000 mol product/mol enzyme per min was obtained toward the fluorogenic substrate in 0.05 M DMG buffer at pH 3.3 containing 0.15 M NaCl and 0.05% (v/v) Triton X-100.

These kinetic parameters can be compared with values for mature IDUA, purified from human liver, of K_m 110 μM and $k_{cat.}$ 2132 mol product/mol enzyme per min at pH 2.4 (Freeman and Hopwood, 1992). Two K_m values of 37 μM and 1.92 mM, with corresponding $k_{cat.}$ values of 299 and 650 mol of product formed/mol of enzyme per minute were observed at pH 4.8 for the mature IDUA from liver, whereas the purified recombinant precursor form of IDUA had one K_m which was dependent on the concentration of IDUA present. Both $k_{cat.}$ values obtained for the recombinant IDUA were 4.4- and 25-times higher than the maximum $k_{cat.}$ reported for the mature IDUA isolated from human liver (Freeman and Hopwood, 1992). These differences may simply represent contributions to IDUA activity observed from reduction, dilution and composition of the diluting buffer which was not observed to the same degree for mature IDUA.

CONCLUSION

We have demonstrated high-level expression of IDUA in CHO-K1 cells using a recombinant expression-construct and affinity-purification of the secreted precursor form of the enzyme. Although this form of the enzyme shows considerable differences in its kinetic parameters as compared with the mature form of the enzyme, it is effectively endocytosed by the mannose-6-phosphate receptor pathway, is able to correct the storage phenotype of MPS I fibroblasts and undergoes 'maturation' after uptake and localization to the lysosomes of these cells. The purified recombinant could therefore be used for enzyme replacement therapy of MPS I. The availability of large amounts of enzyme will also make possible the design of studies to define the tertiary structure of IDUA.

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