Journal of Microbiology (2013) Vol. 51, No. 2, pp. 213–221 DOI 10.1007/s12275-013-2416-2
Copyright ⓒ 2013, The Microbiological Society of Korea

Low-Scale Expression and Purification of an Active Putative Iduronate 2-Sulfate Sulfatase-*Like* Enzyme from *Escherichia coli* K12

Edwin David Morales-Álvarez[1,2], Claudia Marcela Rivera-Hoyos[1,3], Angélica María Baena-Moncada[1], Patricia Landázuri[1*], Raúl A. Poutou-Piñales[3*], Homero Sáenz-Suárez[4], Luis A. Barrera[5], and Olga Y. Echeverri-Peña[5]

[1]Grupo de Investigación en Enfermedades Cardiovasculares y Metabólicas (GECAVYME), Facultad de Ciencias de la Salud, Universidad del Quindío, Armenia-Quindío, Colombia
[2]Departamento de Química, Grupo de Investigación en Genética, Biodiversidad y Manejo de Ecosistemas (GEBIOME), Facultad de Ciencias Exactas y Naturales, Universidad de Caldas, Manizales-Caldas, Colombia
[3]Laboratorio de Biotecnología Molecular. Grupo de Biotecnología Ambiental e Industrial (GBAI), Departamento de Microbiología, Facultad de Ciencias, Pontificia Universidad Javeriana, Bogotá, D.C. Colombia
[4]Unidad de Biología Celular y Microscopía, Decanato de Ciencias de la Salud, Universidad Centroccidental Lisandro Alvarado, Barquisimeto, Venezuela
[5]Instituto de Errores Innatos del Metabolismo (IEIM), Facultad de Ciencias, Pontificia Universidad Javeriana, Bogotá, D.C. Colombia

(Received August 8, 2012 / Accepted October 31, 2012)

The sulfatase family involves a group of enzymes with a large degree of similarity. Until now, sixteen human sulfatases have been identified, most of them found in lysosomes. Human deficiency of sulfatases generates various genetic disorders characterized by abnormal accumulation of sulfated intermediate compounds. Mucopolysaccharidosis type II is characterized by the deficiency of iduronate 2-sulfate sulfatase (IDS), causing the lysosomal accumulation of heparan and dermatan sulfates. Currently, there are several cases of genetic diseases treated with enzyme replacement therapy, which have generated a great interest in the development of systems for recombinant protein expression. In this work we expressed the human recombinant IDS-*Like* enzyme (hrIDS-*Like*) in *Escherichia coli* DH5α. The enzyme concentration revealed by ELISA varied from 78.13 to 94.35 ng/ml and the specific activity varied from 34.20 to 25.97 nmol/h/mg. Western blotting done after affinity chromatography purification showed a single band of approximately 40 kDa, which was recognized by an IgY polyclonal antibody that was developed against the specific peptide of the native protein. Our 100 ml-shake-flask assays allowed us to improve the enzyme activity seven fold, compared to the *E. coli* JM109/pUC13-hrIDS-*Like* system. Additionally, the results obtained in the present study were equal to those obtained with the *Pichia pastoris* GS1115/pPIC-9-hrIDS-*Like* system (3 L bioreactor scale). The system used in this work (*E. coli* DH5α/pGEX-3X-hrIDS-*Like*) emerges as a strategy for improving protein expression and purification, aimed at recombinant protein chemical characterization, future laboratory assays for enzyme replacement therapy, and as new evidence of active putative sulfatase production in *E. coli.*

***Keywords*:** iduronate 2-sulfate sulfatase, *E. coli* DH5α, recombinant protein expression, affinity chromatography

Introduction

Inborn errors of metabolism (IEM) are a group of disorders caused by defects in biochemical functioning of certain proteins. IEM are rare diseases that in general can affect 1% of newborns (Beaudet *et al*., 2001). Up to 95% of these genetic diseases are autosomal recessive and some IEM are X-linked recessive (Barrera, 1990, 1993). Currently there are over 4,000 known genetic diseases, including the IEM (Beaudet *et al*., 2001).

Many of the known IEM are associated with a deficiency or malfunction of sulfatases (Neufeld and Muenzer, 2001). The family of sulfatases involves a group of enzymes with a large degree of similarity (Parenti *et al*., 1997). Human deficiency of sulfatases generates various genetic disorders characterized by abnormal accumulation of sulfated intermediate compounds (Hopwood and Ballabio, 2001; Von Figura *et al*., 2001). To date, sixteen human sulfatases have been identified, most of them located in lysosomes, confirming that sulfatases constitute a family of evolutionarily conserved genes that share a common ancestor (Scott *et al*., 1995; Parenti *et al*., 1997). Sulfatases become active enzymes when either a serine (Ser) or cysteine (Cys) residue present in the active site is converted into formyl-glycine (FGly) (Dierks *et al*., 1998a, 1998b; Miech *et al*., 1998; von Figura *et al*., 1998; Dierks *et al*., 1999; Szameit *et al*., 1999; Waldow *et al*., 1999).

Hunter syndrome or Mucopolysaccharidosis type II (MPSII), (OMIM: 309900) is characterized by the deficiency of iduronate 2-sulfate sulfatase (IDS), (EC 3.1.6.13), causing the lysosomal accumulation of heparan sulfate and dermatan sulfate, with consequent increased urinary excretion. Deficiency of this enzyme results in severe or moderate disease manifestations. The most frequent clinical signs are: coarse features, dysostosis multiplex, skeletal deformities, hepatosplenomegaly, small stature, pulmonary hypertension, alterations in

*For correspondence. (P. Landázuri) E-mail: plandazu@uniquindio.edu.co; Fax: (57-6) 746 01 58; (R.A. Poutou-Piñales) E-mail: rpoutou@javeriana.edu.co; Fax: (57-1) 320 83 20, ext: 4021

the myocardium, mental retardation and premature death (Neufeld and Muenzer, 2001).

Currently there are several cases of genetic diseases treated with enzyme replacement therapy (ERT), such as bovine adenosine deaminase (EC 1.4.3.4) used in the treatment of a severe form of combined immunodeficiency (Hilman and Sorensen, 1994), and glucocerebrosidase (EC 3.2.1.45) for treatment of type I Gaucher disease (Niederau *et al.*, 1998; Friedman *et al.*, 1999), which prevents progressive manifestations of the disease (Weinreb *et al.*, 2002). It is known that ERT for Hurler syndrome (MPSI) through the supply of α-L-iduronidase (EC 3.2.1.76) has allowed the recovery of normal liver size in 8/10 patients after 26 weeks of treatment and has decreased down to 61% the apnea and hypopnea episodes during sleep time (Kakkis *et al.*, 2001). Additionally, Pompe disease and the Niemann-Pick type B disease have been studied, where α-glucosidase (EC 3.2.1.3) and sphingomyelinase (EC 3.1.4.12) are the respective deficient lysosomal enzymes involved. Currently, studies are conducted in relation with the pathology of hereditary angioedema, a condition in which there is a deficiency of C1 inhibitor of esterase, and *Pemphigus vulgaris* in which there is an autoimmune response to desmoglein-3. In relation to the MPSII, it has been possible to correct the deficiency in Hunter fibroblasts by supplying recombinant IDS expressed in CHO cells (Bond *et al.*, 1997; Kakkis *et al.*, 2001); additionally, the FDA and European commission have approved the drug Elaprase™, a recombinant form of IDS produced in a continuous line of human cells (Muenzer *et al.*, 2006, 2007; García *et al.*, 2007). All these studies have generated great interest in the development of recombinant protein expression systems towards the ERT for MPSII and other diseases.

Prokaryotic systems such as *Escherichia coli* have been the most widely used for recombinant protein production at both industrial and laboratory scales. *E. coli* is a Gram-negative bacterium and was the first organism to have its genome sequenced and annotated. Therefore, several methods have been developed to improve gene cloning and protein expression in *E. coli*. This is mainly due to its genetic simplicity, fast growth rate, easy handling, high cell density production and the availability of an increasingly large number of vectors and host strains. Also, it needs minimal and inexpensive substances for propagation (Baneyx and Mujacic, 2004; Majidzadeh-A *et al.*, 2010; Nabavinia *et al.*, 2011).

In previous work, we expressed hrIDS-*Like* in *Pichia pastoris* (Córdoba-Ruiz *et al.*, 2009; Landázuri *et al.*, 2009). Recently, we demonstrated the transient expression of hrIDS by using *E. coli* K12 (JM109) (Poutou-Piñales *et al.*, 2010). Another human sulfatase, the *N*-acetylgalactosamine-6-sulfate sulfatase (GALNS), has been successfully expressed in the active form in *E. coli* (BL21) (Poutou, 2006; Rodríguez *et al.*, 2010). Considering the importance of these findings and some common characteristics between GALNS and IDS (Neufeld and Muenzer, 2001; Poutou, 2006), in our present work we expressed hrIDS-*Like* in *E. coli* DH5α (K12) as a strategy for (i) improving expression of this protein, and (ii) facilitating its purification. This strategy is aimed at the chemical characterization of the recombinant protein and future ERT laboratory assays. Our results provide new evidence of active sulfatase production in *E. coli*.

Materials and Methods

Escherichia coli K12 strain

DH5α (F^- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17(r_K^- m_K^+), λ–) was used for transformation and expression of the designed genetic construction (Sambrook and Russell, 2001).

Obtaining the hrIDS-cDNA

The plasmid pUC13-hrIDS was digested with *Eco*RI (Invitrogen, USA) according to the manufacturer's instructions. For the prediction of the expected size of the restriction fragments, we carried out a sequence analysis of pUC13-hrIDS using the NEBcutter program (Vincze *et al.*, 2003).

Cloning hrIDS-cDNA into pGEX-3X

The *Eco*RI restriction product (hrIDS-cDNA) was cloned into pGEX-3× (GE Healthcare, USA) previously linearized with the same enzyme, to obtain the pGEX-3X-hrIDS-*Like* plasmid. The ligation reaction was performed using 0.1 μg and 0.05 μg of insert and vector respectively, using the LigaFast kit (Promega, USA) and employing 3 units of T4-DNA ligase in 5 μl of 2× Rapid Ligation Buffer [1×: 30 mM Tris-HCl (pH 7.8±0.2), 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP and 5% (w/v) PEG] in a final volume of 10 μl, and incubated for 3 h at room temperature (25°C). The reaction was stored at -20°C until the transformation process (Sambrook and Russell, 2001).

Transformation of *E. coli* DH5α with pGEX-3X-hrIDS-*Like*

Recombinant vector pGEX-3X-hrIDS-*Like* was introduced into competent *E. coli* DH5α cells using the $CaCl_2$ method and transformed by heat shock at 42°C (Sambrook and Russell, 2001; Zhao *et al.*, 2009). As transformation controls *E. coli* DH5α and DH5α/pGEX-3X were used. Recombinant colonies of *E. coli* DH5α/pGEX-3X-hrIDS-*Like* were plated on both Luria-Bertani (LB) (10 g/L Triptone, 5 g/L Yeast extract and 10 g/L NaCl) and LBA (LB supplemented with 100 μg/ml ampicillin) (Sambrook and Russell, 2001) and were then incubated at 37°C overnight. Recombinant colonies were grown in LBA broth and mixed with LBA fresh media supplemented with 60% (v/v) glycerol in 1:1 (v/v) proportion for preparing a Master Cell Bank, and conserved at -70°C as stocks until screening and preliminary expression assays (Meza *et al.*, 2004).

Molecular screening

Twenty colonies of *E. coli* DH5α/pGEX-3X-hrIDS-*Like* were selected for screening of cDNA in frame orientation through restriction enzyme analysis with *Eco*RI and *Pvu*II (Invitrogen, USA) in order to demonstrate the presence, in frame, of the gene cloned. For the prediction of the fragments that would result from the restriction enzyme analysis mentioned above, we carried out a sequence analysis of pGEX-3X-hrIDS-*Like* using NEBcutter (Sambrook and Russell, 2001; Vincze *et al.*, 2003).

DNA electrophoresis analysis

Agarose gels were prepared at 1% (w/v) in 1× TBE [10.8 g/L Tris-base, 5.5 g/L boric acid and 4 ml of 0.5 M EDTA (pH 8.0±0.2)] containing 0.5 μg/ml ethidium bromide and were run at 80 V for 45 min and visualized under UV light. As molecular size markers, Lambda DNA/*Hin*dIII (Invitrogen, USA) and Lambda DNA/*Pst*I (Fermentas INC, Canada) were used.

hrIDS-cDNA sequencing

The construct pGEX-3X-hrIDS-*Like* was used to sequence the hrIDS-cDNA at the Instituto Nacional de Salud (INS, Bogotá D.C.) using the primers IDS-Forward (5′-GGAATT CTCCGAAACGCAGGCCAACTCG-3′) and IDS-Reverse (5′-GGCGGCCGCAGAATTCCACTGAGGGATGTCTG-3′). The reaction was carried out in an ABI PRISM 377 automatic sequencer using the BigDye® Terminator reaction v1.1 Cycle Sequencing (Applied Biosystems, USA). In order to compare the obtained sequence with the sequences reported for human IDS (hIDS), BLAST and ClustalW2 programs were used (Altschul *et al*., 1997; Larkin *et al*., 2007). Furthermore, the conceptual translation was performed using the translate tool for comparison with the native protein hIDS (Gasteiger *et al*., 2005).

Bioinformatic analysis

Considering the homology found after sequencing the cloned cDNA, and to compare the 3D structure of hrIDS-*Like* with the model proposed for the native hIDS, multiple alignments were performed with the sequence of the mature hrIDS-*Like* protein [starting from threonine (Thr) 34, using HHpred] (Söding, 2005) in order to find homologies with proteins whose tertiary structure have been experimentally determined and reported in the Protein Data Bank (www.pdb.org). For the construction and visualization of the 3D model we used Swiss-PdbViewer v.4.02 (Guez and Peitisch, 1997), and additionally we performed the 3D visualization of the structure using POV 3.5.

Protein expression assays

In this assay 1 ml from a stock of *E. coli* DH5α/pGEX-3X-hrIDS-*Like* was inoculated into 9 ml of LBA and cultured overnight at 30°C, 200 rpm and OD_{600} between 0.8-1. Then, 10 ml of each culture were used to inoculate different 500 ml shake flasks containing 90 ml of LBA, which were incubated at 30°C and 200 rpm and OD_{600} between 0.8–1.0, for 18 h. As negative controls, *E. coli* DH5α and DH5α/pGEX-3X were used. Culture samples (3.5 ml) were taken every 2 h, in order to assay optical density (OD_{600}), pellet disruption, protein concentration in culture media and cell lysates, SDS-PAGE and detection of hrIDS-*Like* enzyme activity as previously described (Maleki *et al*., 2010; Dakterzada *et al*., 2012).

Pellet disruption

Samples (0 and 18 h) were treated as follows: cells were collected by centrifugation at 5,000×g and 4°C for 5 min. The supernatant was stored at -20°C until use. The pellet was suspended in lysis buffer [50 mM Tris-HCl (pH 7.5±0.2), 200 mM NaCl, 5% glycerol (v/v), 1 mM dithiothreitol and 1 mM PMSF] with 300 μg/ml of lysozyme, and incubated for 1 h at 4°C. After incubation, cells were treated with 4 freeze-thaw cycles (5 min in liquid N_2 followed by 5 min at 37°C) and centrifuged at 4,000×g for 10 min. Both cell lysate and culture supernatant were assayed for hrIDS activity.

Detection of hrIDS-Like enzyme specific activity

The disruption supernatants of each sample (10 μl) were mixed with 20 μl of substrate solution containing 1.25 nM of 4-methylumbelliferyl-α-iduronate 2-sulfate (MU-αIdoA-2S) dissolved in 0.1 M CH_3COONa/CH_3COO (pH 5.0±0.2) and 10 mM $Pb(CH_3COO)_2{\cdot}3H_2O$, followed by incubation at 37°C for 4 h. Then, 40 μl of Pi/Ci buffer [0.4 M NaH_2PO_4, 0.2 M $C_6H_5Na_3O_7{\cdot}2H_2O$ (pH 4.5±0.2), 0.02% NaN_3 (w/v)] and 10 μl of LEBT were added. The solutions were incubated at 37°C for 37 h. The reaction was stopped with 650 μl of stop buffer [0.5 M $NaHCO_3/NaCO_3$ (pH 10.7± 0.2), with 1.7 mM glycine]. Fluorescence was determined in a Turner 450 fluorometer, with excitation and emission wavelengths of 360 and 415 nm, respectively (Voznyi *et al*., 2001). Concentration of hrIDS was determined by ELISA following the methodology previously described by Sosa *et al*. (2011). Ninety six well microplates were coated with rabbit anti-IDS IgG (Peña *et al*., 2005) and incubated at 37°C for 3 h, and then incubated overnight at 4°C. The wells were blocked with non-fat dry milk in PBS and with PBS 0.05% Tween 20, the antigen was added in PBS and incubated 1 h at 37°C. Biotinylated IgY anti-IDS antibodies (Sosa *et al*., 2011) were applied and incubated at 37°C for 3 h, and then incubated overnight at 4°C followed by washes with PBS 0.05% Tween 20. Peroxidase-labelled streptavidin (Promega, USA) was used according to the manufacturer's instructions. The bound streptavidin was detected with TMB as a substrate; absorbance was read at 450 nm. Total enzyme activity was expressed as U/mg (nmol/h/mg) of specific protein.

Quantification of proteins in the culture medium and cell lysate

The total protein concentrations in culture media and cell lysates of *E. coli* DH5α/pGEX-3X-IDS-*Like* and DH5α/pGEX-3X were determined by using the bicinchoninic acid kit for protein determination (Sigma-Aldrich, USA).

SDS-PAGE

Samples of disrupted cells were separated electrophoretically in polyacrylamide gels 12% (w/v) using Tris-Glycine 1× buffer [3.02 g/L Tris-base, 18.8 g/L Glycine, 10 g/L SDS 2% (w/v)]. A 20 μl volume of sample at a concentration of 1 μg/μl was used with 1× of loading buffer [50 mM Tris-HCl (pH 6.8±0.2), 2% SDS (w/v), 0.1% (w/v) bromophenol blue, 10% (v/v) glycerol, 100 mM β-mercaptoethanol] in a 3:1 (v/v) ratio. The pre-stained BenchMark™ Protein Ladder (Invitrogen) was used as molecular weight marker. Gels were stained with Coomassie brilliant blue [0.25 g Coomassie Brilliant Blue R-250 in 90 ml of methanol:H_2O (1:1 (v/v)) and 10 ml of glacial acetic acid] (Laemmli, 1970).

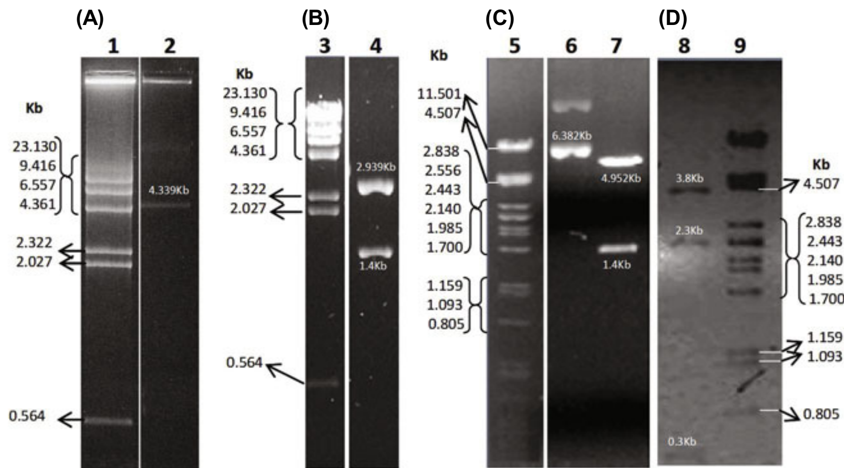

Fig. 1. **Agarose gel electrophoreses [1% (w/v) in 1×-TBE]** (A) 1, Molecular size marker λ*Hin*dIII; 2, pUC13-hrIDS. (B) 3, Molecular size marker λ*Hin*dIII; 4, pUC13-hrIDS digested with *Eco*RI. (C) 5, Molecular size marker λ*Pst*I; 6, pGEX-3X-hrIDS-*Like*; 7, pGEX-3X-hrIDS-*Like* digested with *Eco*RI. (D) 8, pGEX-3X-hrIDS-*Like* digested with *Pvu*II; 9, Molecular size marker λ*Pst*I. DNA photographs were contrasted by using Quantity One from BioRad (V4.6.9).

Purification of hrIDS-*Like*

Another 500 ml shake flask culture was prepared and the crude extract of the disrupted pellet was used in the purification assay. Purification was performed by affinity chromatography on a glutathione-agarose matrix (Sigma-Aldrich) at 4°C. The column was equilibrated with PBS (8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, 0.24 g/L KH_2PO_4, pH 7.4± 0.2). Triton X-100 was added to a final concentration of 1% (v/v) to the product of cell lysis; the mixture was passed through the affinity column (2 ml/min); once the protein was attached, the column was washed with PBS-T buffer [PBS containing 1% Triton X-100 (v/v)].

For the recovery of the GST-hrIDS fusion protein (GST, glutathione-S-transferase, EC 2.5.1.18), elution buffer was used (10 mM reduced glutathione in 50 mM Tris-HCl, pH 9.5±0.2 at 4°C). For the release of the fusion protein eluate, the collected fraction was treated with Factor X Activation Peptide (Xa) (La Vallie and McCoy, 1994) (Sigma-Aldrich) (1 mM $CaCl_2$, 100 mM NaCl, and 50 mM Tris-HCl, pH 8.0± 0.2 at 22°C for 16 h) (Nagai and Thogersen, 1987). Factor Xa removal was performed with the Factor Xa Removal kit (Nagai and Thøgersen, 1984) (Sigma-Aldrich). A culture of *E. coli* DH5α/pGEX-3X was used as negative control.

Western blot analysis of the hrIDS-*Like*

Pure hrIDS-*Like* was analyzed by western blot following the methodology previously described by Sosa *et al.* (2011). After separation in SDS-PAGE, protein bands were transferred to a 0.45 μm nitrocellulose membrane previously soaked in transfer buffer (3.03 g/L Tris, 14.04 g/L glycine, 1 g/L SDS, 200 ml methanol, pH 8.3±0.2).The membrane was blocked with non-fat dry milk in PBS buffer with 0.2% (v/v) Tween 20 solution. After washing, the membrane was cut into strips and incubated with polyclonal IgY anti-IDS (Sosa *et al.*, 2011) for 1 h at room temperature. The membrane was washed and incubated with anti IgY-HRP (conjugate) and revealed using the DAB substrate (Sigma-Aldrich).

Results

Obtaining the hrIDS-cDNA

Once pUC13-hrIDS (Fig. 1A) was digested with *Eco*RI, a band corresponding to 1.4 kb-cDNA-hrIDS was obtained (Fig. 1B).

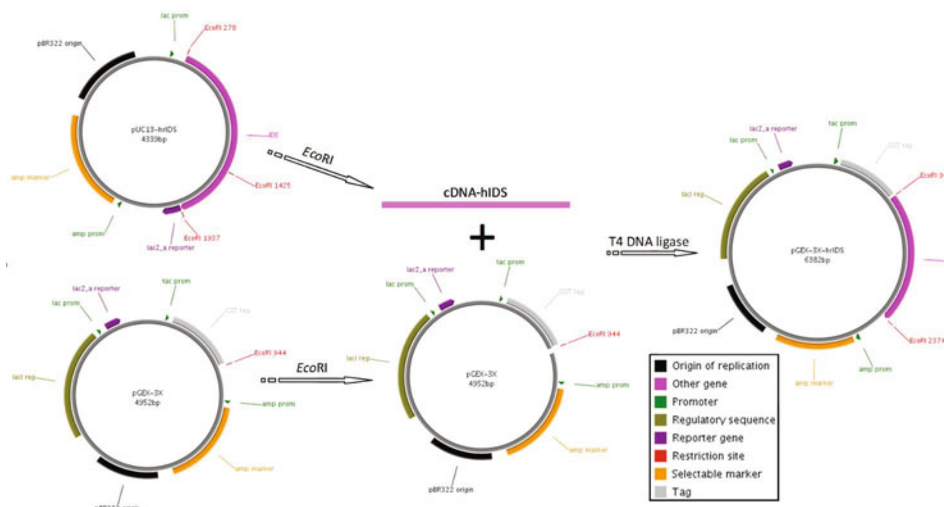

Fig. 2. **Diagram of the cloning strategy and plasmid vectors.** Plasmid map design was performed using PlasMapper (V2.0) (Dong *et al.*, 2004).

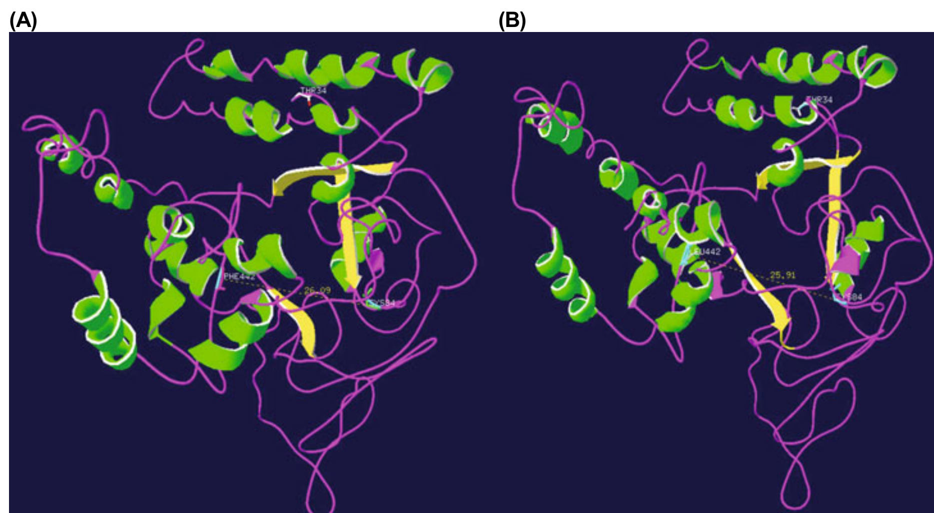

Fig. 3. **3D models of the native hIDS and hrIDS-*Like*.** In both cases, Thr 34 highlighted at the amino terminus, proves that the model represents the mature protein because of the lack of the signal peptide (25 residues) and of the pro-peptide (8 residues). (A) model of the hIDS showing a distance of 26.09Å between Phe 442 and Cys 84. (B) hrIDS-*Like* model showing a distance of 25.91Å between Leu 442 and Cys 84. For the construction and visualization of the models we used Swiss-Pdb-Viewer v.4.02 and POV 3.5.

Cloning hrIDS-cDNA in pGEX-3X

The cloning strategy, the maps of the plasmid vectors and the final constructs are shown in Fig. 2. The construct pGEX-3X-hrIDS had a size of 6.4 kb (Fig. 1C), and the cutting with *Eco*RI released the fragment of the recombinant IDS (1.4 kb).

Molecular screening

Figure 1C shows the plasmid pGEX-3X-hrIDS-*Like* and its respective restriction analysis using *Eco*RI revealed a band of 5 kb (vector) and another band of 1.4 kb confirming the presence of the hrIDS in the expression construct. Cloning in-frame with the *tac* promoter was demonstrated by digestion with *Pvu*II, which should generate fragments with sizes of 3766, 2255, 268, and 93 bp as reported by the virtual restriction analysis. Figure 1C shows 3 bands with approximate sizes of 3.8, 2.3, and 0.3 kb that are consistent with those expected; however, the band below 0.1 kb could not be observed due to the gel concentration used.

hrIDS-cDNA sequencing

The sequence obtained from the hrIDS was compared with the sequence corresponding to the coding region of *Homo sapiens* IDS, transcript variant 1, mRNA (NCBI Reference Sequence: NM_000202.5) and there was an identity of 99%. There were two transition-type substitutions: C1269T and T1324C. Analyzing the results of the conceptual translation, the first substitution was silent for the amino acid proline (Pro) and the second one resulted in a change of phenylalanine (Phe) for leucine (Leu).

Bioinformatic analysis

Multiple alignments of the mature protein showed homology with fragments of 40 proteins with experimentally determined tertiary structure. These molecules showed identities between 14 and 25%, similarities between 0.010 and 0.424, and occurrence probabilities between 33.38 and 99.99%. They also showed a high homology with the tertiary structure of arylsulfatase (1HDH, EC 3.1.6.1) from *Pseudomonas aureginosa*, arylsulfatase A (1AUK, EC 3.1.6.8) from *Homo sapiens*, Ectonucleotide pyrophosphatase (3NKQ, EC 3.1.4.39) from *Mus musculus*, and N-Acetylgalactosamine-4-sulfatase (1FSU, EC 3.1.6.12) from *Homo sapiens*. To obtain a 3D model of the hrIDS-*Like,* homologous fragments of the four previously mentioned proteins were linked, and only 12 amino acids with no 3D prediction were added manually. These amino acids were added one by one based on the secondary structure prediction, to finally expose the structure to several energy minimization cycles. The resulting model includes the mutation where Phe 442 changes by Leu. The 3D models proposed for the native hIDS (Sáenz *et al*., 2007) and hrIDS-*Like* are shown in Fig. 3. Using these models the distance between the Phe or Leu and Cys 84 (key amino acid in the active center of the protein) was measured; additionally the Root Mean Square (RMS) reported a value of 0.012 (Guez and Peitisch, 1997).

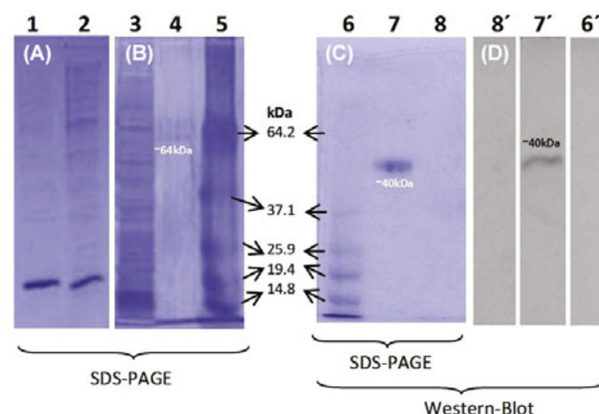

Fig. 4. **SDS-PAGE (12% w/v) and Western blot analysis.** (A) 1, Cell lysate of *E. coli* DH5α/pGEX-3X; 2, Cell lysate of *E. coli* DH5α/pGEX-3X-IDS-*Like*. (B) 3, Cell lysate of *E. coli* DH5α/pGEX-3X-IDS-*Like*. 4, Purification product GST-hrIDS-*Like*; 5, Molecular weight marker (Benchmark). (C) 6, Molecular weight marker (Benchmark); 7, Product of the cutting of hrIDS-*Like* with Factor Xa and Factor Xa Removal kit ; 8, Negative control. (D) 8′, Negative control; 7′, Product of the cutting with Factor Xa; 6′. Molecular weight marker (Benchmark).

Detection of hrIDS-Like specific enzyme activity

We conducted two IDS activity assays at 18 h of culture, using the cell lysate of *E. coli* DH5α/pGEX-3X-hrIDS, and DH5α/pGEX-3X as negative control. The concentrations obtained by ELISA for *E. coli* DH5α/pGEX-3X-hrIDS-Like were 78.13 and 94.35 ng/ml and the specific IDS activities were 34.20 and 25.97 nmol/h/mg, for the first and second ELISA and activity assays, respectively. No activity was found in *E. coli* DH5α/pGEX-3X. The total protein concentrations for *E. coli* DH5α/pGEX-3X and DH5α/pGEX-3X-hrIDS-*Like* in the cell lysate (18 h) were 3480 and 3441 μg/ml respectively and lysates were then analyzed by SDS-PAGE (Fig. 4A).

Purification of hrIDS

Figure 4B shows the preliminary results of the purification of the GST-hrIDS fusion protein. A band with molecular weight of 64 kDa was observed, part of which belongs to GST (Kaplan *et al*., 1997). After treating the pure fusion protein with Factor Xa and Factor Xa Removal kit (where the GST was removed by Factor Xa and retained by the column), a band corresponding to the hrIDS was observed by SDS-PAGE (Fig. 4C). Figure 4D shows the results of the western blot, in which a band of approximately 40 kDa was identified as hrIDS using polyclonal antibody.

Discussion

The human IDS is a lysosomal enzyme with 550 amino acids, of which 25 at the N-terminal position comprise the signal peptide and the next 8 are eliminated during protein processing and the mature protein isoforms vary from 42 to 170 kDa (Nyhan and Ozand, 1998; Neufeld and Muenzer, 2001; Sáenz *et al*., 2007). The main objective of this study was to achieve the expression of the active hrIDS-*Like* in *E. coli* K12 (DH5α) in order to improve the enzyme activity and to use a fusion protein to facilitate purification by affinity chromatography.

The conceptual translation corresponding to the hrIDS-*Like* obtained in this work revealed a large degree of identity when compared with the native protein (NCBI Reference Sequence: NP_000193.1) despite the two substitutions found. Nucleotide substitution at position 1269 resulted in a silent mutation of Pro. The substitution at nucleotide 1324 resulted in a change of Phe by Leu, where the functionality of the protein was not compromised. There are no mutation reports for this position (UniProt P22304) (Sáenz, 2005; Sáenz *et al*., 2007). The 3D model of the native hIDS shows a distance of 26.09 *Å* between the amino acid residue Phe 442 and Cys 84 (active site). This distance is decreased in the hrIDS-*Like* model to 25.91 *Å* due to the substitution of Phe 442 by Leu (which is the reason why we call it hrIDS-*Like*). This shift did not affect the activity and, as seen in Fig. 3, there is no easily observable change in the previously proposed 3D structure (Sáenz *et al*., 2007); which was supported by the RMS measure (Guez and Peitisch, 1997).

Our IDS activity results were at least seven times higher than the reported values of 2.5 nmol/h/mg for IDS expressed in *E. coli* JM109/pUC13-hrIDS-*Like* at a 400 ml-shake-flask scale (Poutou-Piñales *et al*., 2010) and 4.2 nmol/h/mg for IDS-*Like* expressed in *P. pastoris* GS115/pPIC9-hrIDS-*Like* at a 100 ml-shake-flask scale (Landázuri *et al*., 2009). Our IDS activity proved to be very similar to the reported values ranging from 24.4 to 29.5 nmol/h/mg for IDS expressed in *P. pastoris* GS115/pPIC9-hrIDS-*Like* at 1 L of effective working volume at a 3 L bioreactor scale (Poutou *et al*., 2005; Córdoba-Ruiz *et al*., 2009).

GALNS (EC 3.1.6.4), another human sulfatase with similar characteristics, (Neufeld and Muenzer, 2001) has been expressed using two different expression systems: *E. coli* BL21/pGEX-3X-GALNS (Rodríguez *et al*., 2010) and *E. coli* GI724/pLEX-GALNS (unpublished data) (Poutou, 2006). The activity obtained with BL21/pGEX-3X-GALNS was 0.078 nmol/h/mg in 3 L of effective working volume at a bioreactor scale, and the activity found with GI724/pLEX-GALNS was 7 nmol/h/mg (Poutou, 2006). By using the expression system *E. coli* DH5α/pGEX-3X-hrIDS-*Like* we improved the IDS-*Like* activity, even when compared with the expression of GALNS that had been produced using similar and different expression systems. These results are further evidence of the possibility of expressing human active sulfatases in *E. coli*, in particular using the strain DH5α, which also stands out as a far more efficient strain for the production of recombinant proteins (Lili *et al*., 2006) and it does not require expression inducers (IPTG, Isopropyl β-D-1-thiogalactopyranoside) because the plasmid copy number that the strain can retain exceeds the *lac* repressor action (Invitrogen, 2001).

Native *E. coli* DH5α does not express active sulfatases; however, the active expression of hrIDS obtained in this work, once *E. coli* DH5α was transformed with the construct pGEX-3X-hrIDS, allows us to think that the FGly generation system was able to transform the Cys 84 (Millat *et al*., 1997; Sáenz *et al*., 2007) at the active site of the enzyme (Dierks *et al*., 1998b; Szameit *et al*., 1999; Benjdia *et al*., 2007). This

Table 1. **Chronologic comparative results of different systems employed for the production of hrIDS**

Systems	*E. coli* JM109	*P. pastoris* GS115		*E. coli* BL21	*E. coli* DH5α
	pUC13-hrIDS	pPIC9-hrIDS		pGEX-5X-1-hrIDS	pGEX-3X-hrIDS
hrIDS, ng/ml (ELISA)	nd	nd		nd	78-94
Western-Blot, Bands, *kDa*	40, 41, 43, 49, 52, 62 and 97	40, 49, 67, 82, 89, 92 and 109		37 and 42	40
Specific activity, nmol/h/mg	2.82	4.21	29.5	nde	**25.97-34.20**
Assay scale, ml	400	100	1000	50	100
References	Landázuri (2002), Poutou (2006), Poutou-Piñales *et al*. (2010)	Poutou *et al*. (2005), Poutou (2006)	Sáenz (2005), Córdoba-Ruiz *et al*. (2009)	Unpublished	This work

nd, not determined
nde, not determined even

fact, combined with the absence of sulfatase activity in *E. coli* DH5α/pGEX-3X (negative control), confirms that the activity detected in this work is caused by the cloned hrIDS-cDNA.

The fusion protein GST was not secreted into the culture medium. For this reason, the purification of GST-hrIDS was conducted from the cell lysate using only the soluble form of the protein. In a previous work we did the purification of hrIDS by ultrafiltration and three fractions were separated (>100 kDa, 30–100 kDa and <30 kDa). hrIDS activity was detected in the three fractions (Poutou-Piñales *et al*., 2010). These results suggested that hrIDS was proteolytically processed by *E. coli*, producing bands of different molecular weights which were identified by western blot (Poutou-Piñales *et al*., 2010). In the present work, we have selected pGEX-3X to facilitate purification by using affinity chromatography, based on the fact that pGEX-3X contains the GST acting as the fusion protein affinity tag (Bader and Leisinger, 1994; Kaplan *et al*., 1997; Saluta and Bell, 2005).

Contrary to the methodology of the work of Rodríguez *et al.* (2010) in which the inclusion bodies retained the major part of GALNS activity, we maintained all the shake flask cultures at 30°C instead of 37°C to minimize the formation of inclusion bodies and to decrease the hrIDS degradation due to temperature.

Figures 4C and 4D show that after the treatment of GST-hrIDS with Factor Xa and Factor Xa Removal kit, a 40 kDa protein was released and recognized by the IgY polyclonal antibody. Contrary to results in which the hrIDS expression was achieved using different systems (Landázuri *et al*., 2009; Poutou-Piñales *et al*., 2010), we obtained a single pure protein band with a molecular weight near to 42 kDa, a very similar size to that of the hIDS isoform most frequently described in literature (Di Natale and Ronsisville, 1981; Archer *et al*., 1982; Weaston and Neufeld, 1982; Lissens *et al*., 1984; Bielicki *et al*., 1990, 1993; Froissart *et al*., 1995). We still need to: *i.* demonstrate whether our 40 kDa protein is the same as the 42 kDa isoform *ii*. demonstrate recombinant protein activity after the purification step and *iii*. confirm the purification results by ELISA, in order to remove some possible problems regarding protein solubility and activity after GST elimination. Table 1 shows the comparative results of different systems employed for the production of hrIDS supporting the improvement reported in this paper.

The biological activity found in this work, and the lack of a clear influence caused by the change of Phe to Leu on the active site of the protein, suggest the possibility of modification of the side chains, in order to direct them to the target organ, based on imposed glycosylation by humanized yeast (Vervecken *et al*., 2004; Jacobs *et al*., 2008) or chemically added glycosylation to the product obtained in *E. coli*; which is promising for the potential use of this protein in enzyme replacement therapy (ERT).

Hunter Syndrome is considered an orphan disease and in most cases supportive therapy is applied. Many of the Latin American health systems do not cover the costs of such therapies. For example, for Fabry disease and Gaucher, the ERT cost varies from $100,000 to 200,000/year/patient (Poutou, 2006). In this sense, our purpose in the long term is to produce recombinant proteins that can be used in the treatment of such diseases. For this reason, a consistent production of complex proteins like hrIDS, glycosylated (in *P. pastoris*) or not glycosylated (in *E. coli*) is very important.

Conclusion

In summary, we have improved seven-fold the hrIDS activity at a 100 ml scale compared with previous work even at higher scales (Landázuri *et al*., 2009; Poutou-Piñales *et al*., 2010). By using a lower culture temperature and a strain (DH5α) capable of retaining a high copy number of pGEX-3X-rIDS-*Like*, the purification of a single pure protein band was facilitated. The protein was then recognized by a polyclonal antibody. *E. coli* DH5α/pGEX-3X-hrIDS-*Like* was a suitable system for low-scale production of the recombinant IDS.

Acknowledgements

This research was supported and funded by the Genetics and Biochemistry Laboratory and the Master's Program in Biomedical Sciences of the Universidad del Quindío (Armenia, Colombia), and the Institute for Inborn Errors of Metabolism at the Pontificia Universidad Javeriana (Bogotá, Colombia). We thank Dr. Shunji Tomatsu (Saint Louis University, USA) for providing the IDS cDNA and Dr. John Carlos Castaño (Universidad del Quindío, Colombia) for donating the pGEX-3X vector. Finally, we thank Sandra Constantino for copy-editing the English manuscript.

References

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W.J., and Lipman, D. 1997. Gapped BLAST and PSI-LAST: A new generation of protein database search programs. *Nucleic Acid Res.* **25**, 3389–3402.

Archer, I.M., Harper, P.S., and Wusteman, F.S. 1982. Multiple forms of Iduronate 2-sulphate sulphatase in human tissues and body fluids. *Biochem. Biophys. Acta.* **708**, 134–140.

Bader, R. and Leisinger, T. 1994. Isolation and characterization of the *Methylophilus* sp. strain DMll gene encoding dichloromethane dehalogenase/glutathione S-transferase. *J. Bacteriol.* **176**, 3466–3473.

Baneyx, F. and Mujacic, M. 2004. Recombinant protein folding and misfolding in *Escherichia coli*. *Nat. Biotechnol.* **22**, 1399–1408.

Barrera, L.A. 1990. Enfermedades genéticas de origen metabólico. *Pediatría* **25**, 64–68.

Barrera, L.A. 1993. Errores innatos del metabolismo. *Acta Méd. Colomb.* **18**, 31–40.

Beaudet, A.L., Scriver, C.R., Sly, W.S., and Valle, D. 2001. The metabolic and molecular bases of inherited disease. McGraw-Hill.

Benjdia, A., Deho, G., Rabot, S., and Berteau, O. 2007. First evidences for a third sulfatase maturation system in prokaryotes from *E. coli asl*B and *yde*M deletion mutants. *FEBS Lett.* **581**, 1009–1014.

Bielicki, J., Freeman, C., Clements, P.R., and Hopwood, J.J. 1990. Human liver iduronate 2-sulfatase. Purification, characterization and catalytic properties. *Biochem. J.* **271**, 75–86.

Bielicki, J., Hopwood, J., Wilson, P.J., and Anson, D.S. 1993. Recombinant human iduronate -2- sulphatase: correction of mucopolysaccharidosis - type II fibroblasts and characterization of the purified enzyme. *J. Biochem.* **289**, 241–246.

Bond, C.S., Clements, P.R., Ashby, S.J., Collyer, C.A., Harrop, S., Hopwood, J.J., and Guss, M. 1997. Structure of human lysosomal sulfatase. *Struct.* **5**, 277–289.

Córdoba-Ruiz, H.A., Poutou-Piñales, R.A., Echeverri-Peña, O.Y., Algecira-Enciso, N.A., Landázuri, P., Sáenz, H., and Barrera-Avellaneda, L.A. 2009. Laboratory scale production of the human recombinant iduronate 2-sulfate sulfatase-Like from *Pichia pastoris*. *Afr. J. Biotechnol.* **8**, 1786–1792.

Dakterzada, F., Mobarez, A.M., Roudkenar, M.H., and Forouzandeh, M. 2012. Production of pentameric cholera toxin B subunit in *Escherichia coli*. *Avicenna J. Med. Biotechnol.* **4**, 89–94.

Di Natale, P. and Ronsisville, L. 1981. Identification and partial characterization of two enzymes forms of iduronate sulfatase from human placenta. *Biochem. Biophys. Acta*. **661**, 106–111.

Dierks, T., Lecca, M.R., Schlotterhose, P., Schmidt, B., and von Figura, K. 1999. Sequence determinants directing conversion of cysteine to formylglycine in eukariotic sulfatases. *Embo. J.* **18**, 2084–2091.

Dierks, T., Lecca, M.R., Schmidt, B., and von Figura, K. 1998a. Conversion of cysteine to formylglycine in eukaryotic sulfatases occurs by a common mechanism in the endoplasmic reticulum. *FEBS Lett*. **423**, 61–65.

Dierks, T., Miech, C., Hummerjohann, J., Schmidt, B., Kertesz, M.A., and von Figura, K. 1998b. Posttranslational formation of formylglycine in prokaryotic sulfatases by modification of either cysteine or serine. *J. Biol. Chem*. **273**, 25560–25564.

Dong, X., Stothard, P., Forsythe, I., and Wishart, D. 2004. PlasMapper: a web server for drawing and auto-annotating plasmid maps. *Nucleic Acids Res*. **32**, W660-664.

Friedman, B., **Vaddi, K**., **Preston, C**., **Mahon, E**., **Cataldo, J.R., and Macpherson, J.M.** 1999. A comparison of pharmacological properties of carbohydrate remodeled recombinant and placental-derived β-glucocerebrosidase: Implications for clinical efficacy in treatment of Gaucher disease. *Blood* **93**, 2807–2816.

Froissart, R., Millat, G., Mathiu, M., Bozon, D., and Maire, I. 1995. Processing of iduronate 2-sulfatase in human fibroblasts. *Biochem. J.* **309**, 425–430.

García, A.R., DaCosta, J.M., Pan, J., Muenzer, J., and Lamsa, J.C. 2007. Preclinical dose ranging studies for enzyme replacement therapy with idursulfase in a knock-out mouse model of MPS II. *Mol. Gen. Metabol*. **91**, 183–190.

Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S., Wilkins, M.R., Appel, R.D., and Bairoch, A. 2005. Protein identification and analysis tools on the ExPASy server, pp. 571–660. Humana Press, New York, USA.

Guez, N. and Peitisch, M.C. 1997. SWISS-MODEL and the Swiss-PdbViewer: An environment for comparative protein modeling. *Electroph*. **18**, 2714–2723.

Hilman, B.C. and Sorensen, R.U. 1994. Management options: SCIDS with adenosine deaminase deficiency. *Ann. Aller.* **72**, 395–402.

Hopwood, J.J. and Ballabio, A. 2001. Multiple sulfatase deficiency and the nature of sulfatae family. *In* Scriver, C.R., Beaudet, A., Sly, W., and Valle, D. (eds.), pp. 3725–3732. The metabolic and molecular bases of inherited disease. Mc Graw-Hill, New York, N.Y., USA.

Invitrogen. 2001. Subcloning efficiency DH5α. Catalog no. 18265-017.

Jacobs, P.P., Geysens, S., Vervecken, W., Contreras, R., and Callewaert, N. 2008. Engineering complex-type N-glycosylation in *Pichia pastoris* using GlycoSwitch technology. *Nat. Protocols* **4**, 58–70.

Kakkis, E.D., Muenzer, J., Tiller, G.E., Waber, L., Belmont, J., Passage, M., Izykowki, B., Philips, J., Doroshow, R., Walot, I., and *et al.* 2001. Enzyme-replacement therapy in mucopolysaccharidosis I. *New Eng. J. Med*. **344**, 182–188.

Kaplan, W., Husler, P., Klump, H., Erhardt, J., Sluis-Cremer, N., and Dirr, H. 1997. Conformational stability of pGEX-expressed *Schistosoma japonicum* glutathione S-transferase: A detoxification enzyme and fusion-protein affinity tag. *Prot. Sci*. **6**, 399–406.

La Vallie, E.R. and McCoy, J.M. 1994. Enzymatic cleavage of fusion proteins with factor Xa. John Wiley & Sons, pp. 16.14.16–16.14.17. New York, N.Y., USA.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Landázuri, P. 2002. Clonación Del cDNA de la Iduronato 2-Sulfato Sulfatasa Humana. Expresión de la Enzima en *Escherichia coli* y *Pichia pastoris*, p. 134. Tesis Doctoral Thesis, Pontificia Universidad Javeriana, Bogotá, Columbia.

Landázuri, P., Poutou-Piñales, R.A., Acero-Godoy, J., Córdoba-Ruiz, H.A., Echeverri-Peña, O.Y., Sáenz, H., Delgado-Boada, J.M., and Barrera-Avellaneda, L.A. 2009. Cloning and shake flask expression of hrIDS-*Like* in *Pichia pastoris*. *Afr. J. Biotechnol.* **8**, 2871–2877.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., López, R., Thompson, J.D., Gibson, T.J., and Higgins, D.G. 2007. Clustal W and Clustal X version 2.0. *Bioinformatics* **23**, 2947–2948.

Lili, W., Chaozhan, W., and Xindu, G. 2006. Expression, renaturation and simultaneous purification of recombinant human stem cell factor in *Escherichia coli*. *Biotechnol. Lett*. **28**, 993–997.

Lissens, W., Zenati, A., and Liebaers, I. 1984. Polyclonal antibodies against iduronate 2-sulfatase from human urine. *Biochem. Biophys. Acta*. **801**, 365–371.

Majidzadeh-A, K., Mahboudi, F., Hemayatkar, M., Davami, F., Barkhordary, F., Adeli, A., Soleimani, M., Davoudi, N., and Khalaj, V. 2010. Human tissue plasminogen activator expression in *Escherichia coli* using cytoplasmic and periplasmic cumulative power. *Avicenna J. Med. Biotechnol.* **2**, 131–136.

Maleki, A., Roohvand, F., Tajerzadeh, H., Khanahmad, H., Nobari, M.B., Beiruti, A., and Najafabadi, A.R. 2010. High expression of methylotrophic yeast-derived recombinant human erythropoietin in a pH-controlled batch system. *Avicenna J. Med. Biotechnol.* **2**, 197–206.

Meza, R.A., Monroy, A.F., Mercado, M., Poutou, R.A., Rodríguez, P., and Pedroza, A.M. 2004. Study of the stability in real time of cryopreserved strain banks. *Univ. Sci.* **9**, 35–42.

Miech, C., Dierks, T., Selmer, T., von Figura, K., and Schmidt, B. 1998. Arysulfatase from *Klebsiella pneumoniae* carries a formylglycine generated from serine. *J. Biol. Chem.* **273**, 4835–4837.

Millat, G., Froissart, R., Maire, I. and Bozon, D. 1997. Characterization of iduronate sulfate sulfatase mutants affecting N-glycosilations sites and cysteine-84 residue. *Biochem. J.* **326**, 243–247.

Muenzer, J., Gucsavas-Calikoglu, M., McCandless, S.E., Schuetz, T.J., and Kimura, A. 2007. A phase I/II clinical trial of enzyme replacement therapy in mucopolysaccharidosis II (Hunter syndrome). *Mol. Genet. Metab*. **90**, 329–337.

Muenzer, J., Wraith, J.E., Beck, M., Giugliani, R., Harmatz, P., Eng, C.M., Vellodi, A., Martin, R., Ramaswami, U., Gucsavas-Calikoglu, M., and *et al.* 2006. A phase II/III clinical study of enzyme replacement therapy with idursulfase in mucopolysaccharidosis II (Hunter syndrome). *Gen. Med*. **8**, 599.

Nabavinia, M.S., Nasab, M.N., Meshkat, Z., Derakhshan, M., and Khaje-Karamadini, M. 2011. Construction and evaluation of an expression vector containing Mtb32C (Rv0125) of *Mycobacterium tuberculosis*. *Avicenna J. Med. Biotechnol.* **3**, 207–210.

Nagai, K. and Thøgersen, H.C. 1984. Generation of beta-globin by sequence-specific proteolysis of a hybrid protein produced in *Escherichia coli*. *Nature* **309**, 810–812.

Nagai, K. and Thøgersen, H.C. 1987. Synthesis and sequence-specific proteolysis of hybrid proteins produced in *Escherichia coli*. *Meth. Enzymol.* **153**, 461–481.

Neufeld, E.F. and Muenzer, J. 2001. The mucopolysaccharidoses. *In* Scriver, C.R., Beaudet, A.L., Sly, W., and Valle, D. (eds.), The metabolic and molecular bases of inherited disease, pp. 3421–

3452. McGraw-Hill, New York, N.Y., USA.

Niederau, C., Vom Dahl, S., and Haussinger, D. 1998. First long-term results of imiglucerase therapy of type 1 Gaucher disease. *Eur. J. Med. Res.* **3**, 25–30.

Nyhan, W.L. and Ozand, P.T. 1998. Hunter disease/mucopolysaccharidoses type II (MPSII) iduronate sulfatase deficiency, Atlas of Metabolic Diseases, pp. 455–461. Chapman and Hall Medical, New York, USA.

Parenti, G., Meroni, G. and Ballabio, A. 1997. The sulfatase gene family. *Curr. Opin. Gen. Devel.* **7**, 386–391.

Peña, O., Sosa, A., Echeverri, O., Sáenz, H., and Barrera, L.A. 2005. Producción de anticuerpos policlonales IgG contra la proteína Iduronato-2-sulfato sulfatasa y desarrollo de un sistema de detección para IDS humana recombinante. *Biomédica.* **25**, 181–188.

Poutou, R.A. 2006. Expresión de sulfatasas humanas en *Escherichia coli* y *Pichia pastoris*, p. 162. Tesis Doctoral Thesis, Pontificia Universidad Javeriana, Bogotá, D.C., Columbia.

Poutou, R.A., Córdoba, H., Quevedo, B.E., Landázuri, P., Echeverri, O.Y., Sáenz, H., Vanegas, A., Acero, J., Gónzalez, A., Herrera, J., and *et al.* 2005. Expresión de iduronato 2-sulfato sulfatasa humana recombinante (IDShr) en *Pichia pastoris*. *Univ. Sci.* **10**, 75–96.

Poutou-Piñales, R.A., Vanegas Niño, A., Landázuri, P., Sáenz, H., Lareo, L., Echeverri, O.Y., and Barrera Avellaneda, L.A. 2010. Human sulfatase transiently and functionally active expressed in *E. coli* K12. *Elect. J. Biotechnol.* **13**, article 8.

Rodríguez, A., Espejo, Á.J., Hernández, A., Velásquez, O.L., Lizaraso, L.M., Cordoba, H.A., Alméciga-Díaz, C., Barrera, L.A., and Sanchez, O. 2010. Enzyme replacement therapy for Morquio A: an active recombinant N-acetylgalactosamine-6-sulfate sulfatase produced in *Escherichia coli* BL21. *J. Ind. Microbiol. Biotechnol.* **37**, 1193–1201.

Sáenz, H. 2005. Expresión, purificación parcial y estudios computacionales de la IDShr producida en *Pichia pastoris*, p. 180. Doctoral Thesis, Pontificia Universidad Javeriana, Bogotá, D.C., Colombia.

Sáenz, H., Lareo, L., Poutou, R.A., Sosa, C., and Barrera, L.A. 2007. Predicción computacional de la estructura terciaria de la iduronato 2-sulfato sulfatasa humana. *Biomédica.* **27**, 7–20.

Saluta, M. and Bell, P. 2005. Troubleshooting GST fusion protein expression in *E. coli*. Amersham Biosciences, Piscataway, NJ, USA.

Sambrook, J. and Russell, D.W. 2001. Molecular cloning: A laboratory manual, p. 2100. Cold Spring Harbor Laboratory Press, New York, N.Y., USA.

Scott, H.S., Blanch, L., Guo, X.-H., Freeman, C., Orsborn, A., Baker, C., Sutherland, G.R., Morris, C.P. and Hopwood, J.J. 1995. Cloning of the sulphamidase gene and identification of mutations in Sanfilippo A syndrome. *Nat. Genet.* **11**, 465–467.

Söding, J. 2005. Protein homology detection by HMM-HMM comparison. *Bioinformatics* **21**, 951–960.

Sosa, A.C., Espejo, A.J., Rodriguez, E.A., Lizaraso, L.M., Rojas, A., Guevara, J., Echeverri, O.Y., and Barrera, L.A. 2011. Development of a sandwich enzyme linked immunosorbent assay (ELISA) for the quantification of iduronate-2-sulfate sulfatase. *J. Immunol. Methods* **368**, 64–70.

Szameit, C., Miech, C., Balleinninger, M., Bernhard., S., von Figura, K., and Dierks, T. 1999. The iron sulfur protein *Ats*B is required for posttranslational formation of formylglycine in the *Klebsiella* sulfatase. *J. Biol. Chem.* **274**, 15375–15381.

Vervecken, W., Kaigorodov, V., Callewaert, N., Geysens, S., De Vusser, K., and Contreras, R. 2004. *In vivo* synthesis of mammalian-like, hybrid-type N-glycans in *Pichia pastoris*. *Appl. Environ. Microbiol.* **70**, 2639–2646.

Vincze, T., Posfai, J., and Roberts, R.J. 2003. NEBcutter: a program to cleave DNA with restriction enzymes. *Nucleic Acid Res.* **30**, 3688–3691.

von Figura, K., Bemhard, S., Thorsten, S., and Dierks, T. 1998. A novel protein modification generating an aldehyde group in sulfatases: Its role in catalysis and disease. *BioEssay* **20**, 505–510.

von Figura, K., Gieselman, V., and Jaeken, J. 2001. Metachromatic leukodystrophy. *In* Scriver, C.R., Beaudet, A., Sly, W., and Valle, D. (eds.), The metabolic and molecular bases of inherited disease, pp. 3695–3724. Mc Graw-Hill, New York, N.Y., USA.

Voznyi, Y.V., Keulemans, L.M., and van Diggelen, O.P. 2001. A fluorogenic enzyme assay for the diagnosis of MPS II (Hunter disease). *J. Inherit. Metabol. Dis.* **24**, 675–680.

Waldow, A., Schmidt, B., Dierks, T., von Bülow, R., and von Figura, K. 1999. Amino acids residues forming the active site of arylsulfatase A. *J. Biol. Chem.* **274**, 12284–12288.

Weaston, A. and Neufeld, E.F. 1982. Iduronate of human plasma. *Meth. Enzymol.* **83**, 573–578.

Weinreb, N., Charrow, J., Anderson, H.C., Kaplan, P., Kolodny, E.H., Mistry, P., Pastores, G., Rosenbloom, B.E., Ronald, C., Wappner, R., and *et al.* 2002. Eficacia de la terapia de reemplazo enzimático en 1.028 pacientes con enfermedad de Gaucher tipo 1 después de 2 a 5 años de tratamiento: Comunicación del Gaucher registry. *Amer. J. Med.* **113**, 1–8.

Zhao, Y., Liu, Z., Yu, S., Wen, S., Hammarstrom, L., and Rabbani, H. 2009. Construction of a high efficiency PCR products cloning T vector using pGEM-5zf (+). *Avicenna J. Med. Biotechnol.* **1**, 37–39.