African Journal of Biotechnology Vol. 8 (12), pp. 2871-2877, 17 June, 2009
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2009 Academic Journals

Full Length Research Paper

Cloning and shake flask expression of hrIDS-*Like* in *Pichia pastoris*

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Accepted 8 May, 2009

The human Iduronate-2-sulfate sulfatase (hIDS-*Like*) was cloned into the methylotrophic yeast *Pichia pastoris* under the control of alcohol oxidase promoter (*AOX*1) and the α-mating factor signal peptide (α-factor). Six clones were identified by PCR. Using clone IDS28, the enzyme was secreted into the culture medium, yielding a protein with an activity of 4.213 nmol.h^{-1}.mg of total protein^{-1} at 72 h, in 0.5% v/v methanol. Several bands were revealed by western-blot, indicating that a *P. pastoris* processing was slightly different than in mammalian cells.

Key words: Iduronate-2-sulfate sulfatase, MPS II, *Pichia pastoris,* human recombinant protein, Hunter syndrome.

INTRODUCTION

The mucopolysaccharidosis (MPS) is a group of lysosomal storage disorders caused by the deficiency of the enzyme that catalyzes the degradation of glycosaminoglycans (GAGs). Lysosomal accumulation of GAGs molecules result in cellular and tissue damage and organic dysfunction (Neufeld and Muenzar, 2001). Iduronate-2-sulfate sulfatase (IDS) is one of the lysosomal enzymes (EC 3.1.6.13) involved in the degradation of mucopolysaccharides such as heparan and dermatan sulfates. Its deficiency is responsible for mucopolysaccharidosis type II (MPS II), or Hunter syndrome (MIN 309900), a rare X-linked lysosomal storage disease, characterized by a wide spectrum of clinical manifestations, ranging from mild to severe forms (Neufeld and Muenzar, 2001). The cDNA for hIDS has been cloned, the 2.3 Kb cDNA encodes a polypeptide of 550 amino acids (Wilson et al., 1990). The native IDS processing has been studied after over expression in fibroblast (Froissart et al., 1995), COS cells and lymphoblastoid cell lines (Millat et al., 1997b). This enzyme has been purified in very small quantities from urine, plasma, placenta and liver (DiNatale and Ron-sisville, 1981; Weaston and Neufeld, 1982; Lissens et al., 1984; Bielicki et al., 1990). Large quantities of enzyme are important for functional and structural studies. Natural sources cannot provide sufficient amounts of proteins for these studies.

In order to overcome this problem, it is necessary to express the enzyme in a suitable expression system. With this in mind, the methylotrophic yeast *P. pastoris* has been used to produce around 300 foreign proteins since 1984 (Cereghino and Cregg, 2000). Based on the general characteristics of this yeast, we hope to set up an efficient expression system to produce different human recombinant proteins involved in lysosomal storage disorders and several other inborn metabolic errors. This

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paper preliminarily describes the expression of a functionally active hrIDS-*Like* in *P. pastoris*.

MATERIALS AND METHODS

Materials

All reagents were purchased from Sigma®. Restriction enzymes, T4-ligase, Taq-DNA polymerase and culture media were from Gibco BRL®, plasmids DNA (pPIC9) and yeast strain GS115 (his4) and GS115/human albumin were from Invitrogen®. ECL system was from Amersham pharmacia. The hIDS-*Like* cDNA was provided by Dr. Shunji Tomatsu. The anti IDS antibody was kindly provided by Dr. Kazuko Sukegawa.

Strains and plasmids DNA

All *E. coli* transformants were mainly grown in Luria-Bertani broth (LB) (10 g L^{-1} triptone, 5.0 g L^{-1} yeast extract, 10 g L^{-1} NaCl), supplemented with 50 µg ml^{-1} of ampicillin, (LBA). The pUC13-IDS-*Like* and the *P. pastoris* expression vector pPIC9 were amplified in *E. coli* strain JM109 (*rec*A1 *sup*E44, *end*A1 *hsd*R17 *gyr*A96 *rel*A1thi Δ (*lac-pro*AB) F'[*tra*D36 *pro*AB^+ *lacI*^{q} *lacZ*ΔM15]). For *E. coli* competent cell manufacture the $CaCl_2$ procedure was used (Ausubel et al., 1994). For *E. coli* transformation was done according to Sambrook et al. (1989) was used. *P. pastoris* GS115 strain (*his*4) was used for expression of human IDS-*Like,* and GS115 albumin as expression control.

Construction of yeast expression plasmid for IDS

Plasmid construction, DNA isolation, enzyme restriction assay, agarose gel electrophoresis and cloning procedures were performed as described by Ausubel et al. (1994) and Sambrook et al. (1989). Plasmid pUC13-IDS-*Like* was digested with *Eco*RI, The fragment encoding hIDS-*Like* was excised as a 1.5 Kb band and inserted into *Eco*RI site of pPIC9. A *P. pastoris* expression vector carrying *AOX*1 promoter, *AOX* transcription terminator and the α-mating factor signal peptide sequence (SP) and the auxotrophic complementation gene His4 was used (Invitrogen, 1996). The resulting construct was denominated pPIC9-IDS-*Like* and was characterized by PCR, restriction analysis and nucleotide sequence. PCR reactions were performed in Bio-Rad thermocycler Gene.Cycler™; the inserted DNA sequence of the construct was determined using Taq cycle automated sequencing with big dye deoxy terminators in a ABI prism 310 Perkin Elmer equipment, with the α-factor primer. For plasmid amplification, the pPIC9-hIDS-*Like* was transformed into JM109 competent cells, which were plated on LBA. The positive colonies were then grown in LBA to prepare DNA for transformation in *P. pastoris.*

Transformation of pPIC9-IDS-*Like* in *P. pastoris*

P. pastoris GS115 was grown in YPD and prepared for transformation according to the manufacturer's instruction manual. The plasmid pPIC9-IDS-*Like* was linearized by digestion with *Sal* I, purified and transformed (10 µg of DNA) in *P. pastoris* by electroporation in a BIO-RAD electroporator using 1500 V, 25 µF and 200 Ω. The resulting transformants were selected on a minimal dextrose (MD) agar plates (1.34% w/v yeast nitrogen base, $4x10^{-5}$% w/v biotin and 2% w/v dextrose) after incubation of the electroporation mixture for 3 h at 30ºC in 1.0 M sorbitol, without shaking (Invitrogen, 1996).

PCR amplification of hIDS-*Like* fragment, from genomic DNA of *P. pastoris* transformants

In order to test the integration of pPIC9-IDS-*Like* into *P. pastoris* genome, the DNA isolation from transformants was carry out as described in the *Pichia* expression manual version I (Invitrogen, 1996). The primers, α-factor (TAC TAT TGC CAG CAT TGC TGC) and IDSp2 (ATG CCG CCA CCC CGG ACC GGC CGA), were used for amplification of 826pb fragment length. Plasmids pPIC9 and pPIC9-IDS-*Like* served as negative and positive controls respectively. PCR procedure was performed as described previously.

Growth curves and expression

Selected clones were grown in 100 ml YPD at 30ºC, 250 r.p.m., between 14 to 20 h depending on the carbon source studies results (Poutou et al., 2005). The cells were further centrifuged and induced by changing the culture media to 100 ml of BMMY (1% w/v yeast extract, 2% w/v peptone, 100 mM potassium phosphate, pH 6 ± 0.2, 1.34% w/v yeast nitrogen base, 4 x 10^{-5}% w/v biotin, 0.5% v/v methanol). The induction was maintained for 120 h, methanol was added at a final concentration of 0.5% v/v every 24 h. The cell density, X (g DCW L^{-1}) (DCW, Dry cell weight), was determined by a calibration curve (X vs. OD_{620nm}) represented by X = 1.1726 OD_{620nm} (R^2 = 0.987). Protein concentration was measured by Bradford method. Several clones were used as control for the experiments (GS115/pPIC9-hAlbumine (PALB), GS115 (PP) and GS115/pPIC9 (PPNEG)). Each experiment was run at least 3 times, with a coefficient of variation (VC) similar or lower than 20%.

SDS-PAGE and western-blot analysis

For electrophoretic analysis, 20 µl of concentrated culture supernatant at different hours of induction were mixed with a similar volume of 1 X SDS loading buffer (50 mM Tris-HCl pH 6.8 ± 0.2; 100 mM dithiotreitol; 2% w/v SDS; 0.1% w/v bromophenol blue and 10% v/v glycerol). The proteins were separated by 8% w/v SDS/PAGE gel and stained with Coomassie brilliant blue R-250. For western-blot analysis resultant electrophoresis was transferred to a nitrocellulose membrane by transverse electrophoresis 30 V and 4°C overnight. The nitrocellulose membrane was blocked overnight at 4°C in 5% w/v skim milk dissolved in Tris-buffer saline TBS (10 mM Tris-HCl, pH 8.0 ± 0.2, 150 mM NaCl) containing 0.05% v/v Tween 20. Then the membrane was incubated overnight with a monoclonal antibody anti-IDS (1:250 in 1% w/v BSA in TBS). Immune complexes were detected with horseradish peroxidase-labeled antibodies (1:2000 in 1% w/v BSA in TBS) using the ECL system.

Enzyme activity (fluorometric assay)

The supernatant (10 µl) of fermentation was mixed every 24 h with 20 µl of substrate solution containing 1.25 nM of 4-methylumbelliferyl-α-iduronate 2-sulphate (MU-µIdoA-2S) dissolved in 0.1 M CH_3COONa/CH_3COO pH 5.0 ± 0.2 and 10 mM $Pb(CH_3COO)_2.3H_2O$. It incubated to 37ºC for 4 h, after which were added 40 µl of Pi/Ci buffer (NaH_2PO_4 0.4 M, $C_6H_5Na_3O_7.2H_2O$ 0.2 M, pH 4.5 and NaN_3 0.02% w/v) and 10 µl de LEBT. The solution was incubated to 37ºC for 37 h. The reaction was stopped with 650 µl of stop buffer ($NaHCO_3/NaCO_3$ 0.5 M, pH 10.7 ± 0.2, with glycine 1.7 mM). The fluorescence was determined in a fluorometer Turner 450, with wave lengths of excitation and emission of 360 and 415 nm, respectively. The control was leukocytes or human plasma

(Voznyi et al., 2001). Enzyme activity was expressed as nmol of substrate converted h^{-1} mg^{-1} of total protein.

RESULTS

Construction of recombinant pPIC9-hIDS-*Like* expression plasmid

The cDNA encoding the hIDS-*Like* was inserted into the pPIC9 (Figure 1). IDS-*Like* cDNA insertion and orientation into the pPIC9 vector was confirmed by PCR, enzyme restriction analysis and sequencing. The expected size band of 826 bp, and the characteristic restriction fragments obtained from the pPIC9-hIDS-*Like* when *Sac* I and *EcoR* I were used (2300 and 1500 bp), confirmed that the plasmid contained of the IDS-*Like* cDNA (1.5 Kb). Sequencing also showed that the fragment was in frame with the signal peptide region in pPIC9 vector and was under transcriptional control of *AOX*I promoter. Using BLAST program (Altschul et al., 1997), this fragment revealed a 97% homology with the hIDS sequence reported in Gene bank under the access number GI13544076 (data not shown).

IDS-*Like* cDNA direction of integration into the *P. pastoris* genome

Integration of the recombinant vector into the *P. pastoris* genome was confirmed by PCR. Some of genomic DNA extracted from different yeast clones showed an expected band of 826 bp and some others did not. *Sal* I digestion of the recombinant vector pPIC9-hIDS-*Like* prior to transformation, led to gene insertion events at the HIS4 locus, therefore all HIS^+ transformants had the same methanol utilization phenotype as the parent strains (Mut^+). 7 clones (IDS10, IDS28, IDS92, IDS94, IDS144, IDS149 and IDS153) were identified by PCR analysis in which the integration of IDS-*Like* cDNA into pPIC9 had taken place in frame with *AOX*1 promoter (Figure 2).

Expression of hrIDS-*Like*

The hrIDS-*Like* was expressed in *P. pastoris* using the recombinant vector pPIC9-hIDS-*Like* (9.5 Kb). Growth curves in YPD-BMMY, growth-induction media and the IDS activities are shown in Figure 3 (IDS activity controls in Table 1). Higher IDS-*Like* activity values were obtained with the strain IDS28 at 72 h of induction (4.213 nmol h^{-1} mg^{-1} of total protein). The strain with lower IDS activity was IDS153 (0.643 nmol h^{-1} mg^{-1} of total protein) (Figure 3). 24 h after the beginning of induction, aliquots of this culture were collected, to examine the hrIDS-*Like* by western-blot (Figure 4). Several putative hrIDS-*Like* peptides of approximately 109, 92, 89, 82, 67, 49 and 40 kDa were found in the culture medium obtained from GS115/pPIC9-hIDS-*Like* (Figure 4; lanes 4 and 5). This peptidic profile was not detected in culture media from GS115 (Figure 4 lane 1).

DISCUSSION

The SDS-PAGE analysis of supernatant revealed that hrIDS-*Like* had been processed into several peptides of different molecular weight, one of them, 92 kDa, produced a more intense band than the others did. Western blot analysis revealed bands of 109, 92, 89, 82, 67, 49 and 40 kDa, approximately. Human IDS processsing in mammalian cells in over-expressing transfected fibroblast, COS cells and lymphoblastoid cell line (Froissart et al., 1995; Millat et al., 1997b) has been studied. In these cells hIDS is synthesized as 72 - 76 kDa precursor, later converted into a phosphorylated 90 kDa form and processed by proteolytic cleavage through several intermediates of 55 and 45 kDa mature polypeptides, containing hybrid and complex oligosaccharide chains (Froissart et al., 1995; Millat et al., 1997b). It has been proposed that IDS maturation into a functionally active sulfatase requires, in addition to the glycosylation step, the modification of a cysteine 84 residue to Formil-glycine (FGly) (Froissart et al., 1995; Millat et al., 1997b; Millat et al., 1997a).

The peptidic profile of hrIDS-*Like* expressed in *P. pastoris* was size related to those produced during the processing of the IDS in mammalian cells. However, the molecular weight of the peptides of hrIDS-*Like* expressed in yeast were slightly bigger that the ones observed in mammalian cells, which may be due to what are up to the moment as yet unknown factors one of which could be the glycosylation pattern differences between *Homo sapiens* and *P. pastoris*. It has been demonstrated that a higher glycosylation pattern occurred in *P. pastoris* (Kukuruzinska et al., 1987; Grinna and Tschopp, 1989; Trimble et al., 1991; Montesino et al., 1998). Along these lines, Montesino et al. (1998, 1999) reported variations in the carbohydrate size in proteins expressed in *P. pastoris*, in which the frequent oligosaccharides structures found were $Mn_8GlcNAc_2$ and $Mn_9GlcNac_2$.

Perhaps the 109, 92, 89, 82, 67 bands could be related to the 90, 76, 66, 45 - 55 bands of the IDS expressed in mammalian cells. The smallest bands, 49 and 40 kDa, could be others glycosylated forms or degradation products of the bigger ones. These remain to be proved in future works.

The results described here suggest that the hrIDS-*Like* expressed in *P. pastoris* has an electrophoretic pattern slightly different in size ranging to that reported for mammalian cells. However, the biological activity found in this work and the possibility of modification of the side chains, are promising for the potential use of this protein in enzyme replacement therapy (ERT).

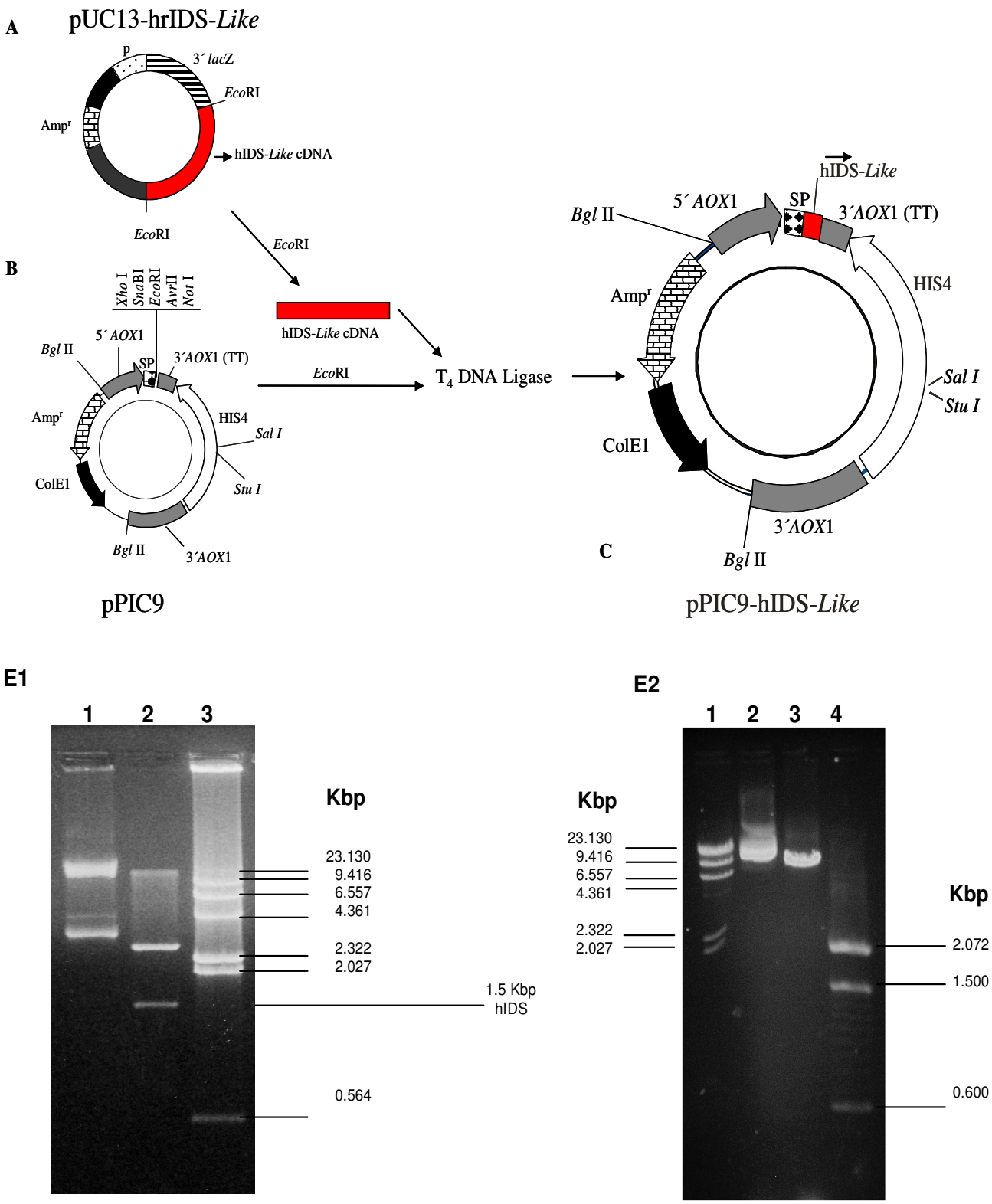

Figure 1. Genetic construction for the expression of hIDS-*Like* in *P. pastoris* and restriction analysis. The hIDS-*Like* cDNA was excised with *Eco*R I from pUC13-hIDS-*Like* (A) and cloned at *Eco*R I site in pPIC9 (B); the recombinant vector was termed pPIC9-hIDS-*Like* (C). Electrophoresis E1 and E2 show a restriction analysis of parental pUC13-hIDS-*Like* and pPIC9 vector in 1% (w/v) agarose gel stained with 0.025% (w/v) of ethidium bromide. E1, lane 1: pUC13-IDS-*Like* (4.4 kb), lane 2: pUC13-IDS-*Like* digest with *EcoR* I (pUC13 2.7 and IDS-*Like* fragment 1.5kb), lane 3: λ *Hin*d III marker. E2, lane 1: λ *Hin*d III marker, lane 2: pPIC9 (8.0kb), lane 3: pPIC9 digest with *EcoR* I (8.0kb) and lane 4: 100pb ladder marker.

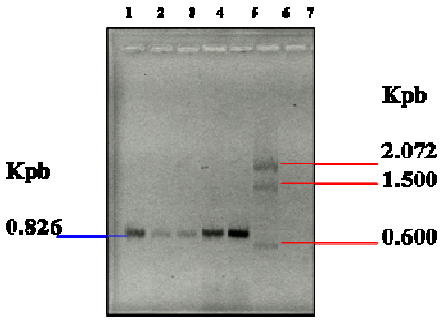

Figure 2. 1% (w/v) agarose gel electrophoresis of the PCR analysis of 5 clones of GS115/pPIC9-hIDS-*Like*. Lane 1: IDS92, Lane 2: IDS94, Lane 3: IDS144, Lane 4: IDS149, Lane 5: IDS153, Lane 6: 100 bp Ladder (Gibco BRL), Lane 7: Negative control of PCR. IDS10 and IDS28 are not included.

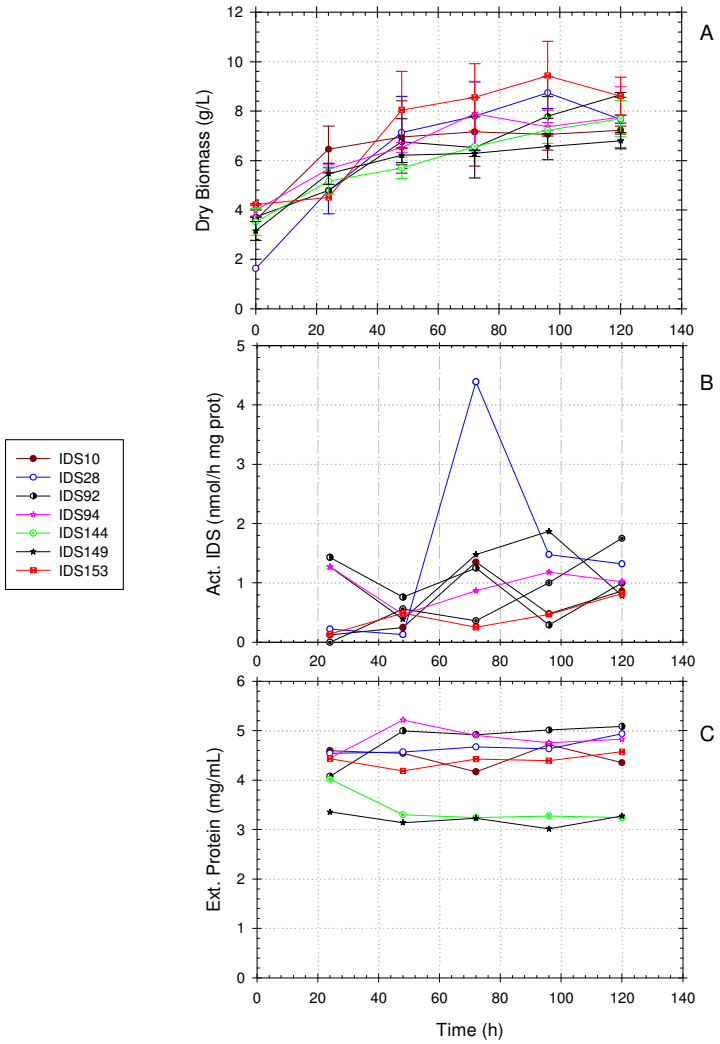

Figure 3. Growth curve of *P. pastoris* strains after methanol induction. Clones and control strains of *P. pastoris* were grown in 500 ml shake flask fermentation with 100 ml of BMGY culture media during 15 h and then induced by changing the culture media to BMMY at 30ºC, 250 rpm, 120 h by addition of 0.5% (v/v) of methanol each 24 h. **A:** $LN(X/X_0)$ (h). **B:** IDS Act. (nmol. h^{-1}. mg $^{-1}$ total protein). **C:** mg ml^{-1} of extracellular proteins.

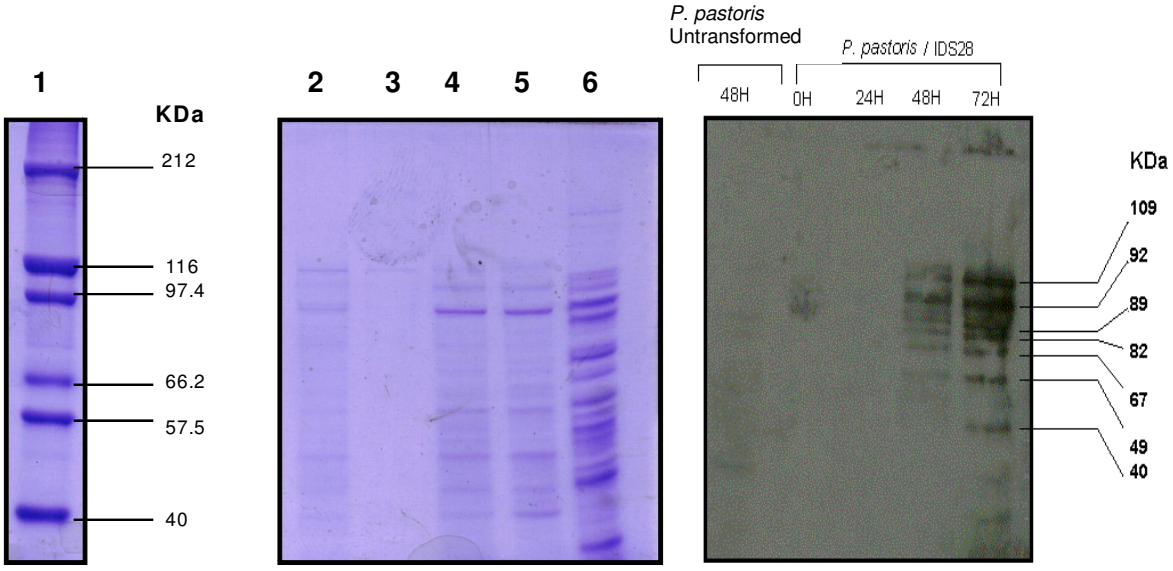

Figure 4. SDS-PAGE 8%w/v and Western-blot of proteins prepared from supernatant of GS115/pPIC9-hrIDS-*Like*. Immobilized samples of crude extract of proteins from culture medium were revealed with monoclonal antibody anti IDS, and an anti mouse horseradish peroxidase conjugated secondary antibody. Each lane was loaded with 20 µl of different concentrations of culture supernatant. Lane 1: Molecular weight marker. Lane 2: supernatant 48 h GS115 (His^-), Lanes 3 - 6, supernatants of 0, 24, 48, and 72 h of GS115/pPIC9-hrIDS-*Like* (IDS28). Cultured in YPG-BMMY.

Table 1. Controls employed for hrIDS-*Like* activity detection, during all experiment.

Controls	Replics	hIDS-*Like* activity (nmol h^{-1} mg^{-1} Tot. prot.) ± SD
Serum	36	13.43 ± 2.72
Leukocytes	10	6.01 ± 1.08

ACKNOWLEDGMENTS

This study was supported by instituto Colombiano Para la Ciencia y la Tecnología Francisco José de Caldas, COLCIENCIAS (grant No. 1203-12-16811).

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