

Characterization of recombinant human lysosomal beta-hexosaminidases produced in the methylotrophic yeast *Pichia pastoris*

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Abstract

β -hexosaminidases (Hex) are dimeric enzymes involved in the lysosomal degradation of glycolipids and glycans. They are formed by α - and/or β -subunits encoded by *HEXA* and *HEXB* genes, respectively. Mutations in these genes lead to Tay Sachs or Sandhoff diseases, which are neurodegenerative disorders caused by the accumulation of non-degraded glycolipids. Although tissue-derived Hex have been widely characterized, limited information is available for recombinant β -hexosaminidases. In this study, human lysosomal recombinant Hex (rhHex-A, rhHex-B, and rhHex-S) were produced in the methylotrophic yeast *Pichia pastoris* GS115. The highest specific enzyme activities were 13,124 for rhHexA; 12,779 for rhHex-B; and 14,606 U .mg⁻¹ for rhHex-S. These results were 25- to 50-fold higher than those obtained from normal human leukocytes. Proteins were purified and characterized at different pH and temperature conditions. All proteins were stable at acidic pH, and at 4 °C and 37 °C. At 45 °C rhHex-S was completely inactivated, while rhHex-A and rhHex-B showed high stability. This study demonstrates *P. pastoris* GS115 potential for polymeric lysosomal enzyme production, and describes the characterization of recombinant β -hexosaminidases produced within the same host.

Keywords: β -N-acetylhexosaminidases; characterization; *Pichia pastoris*; recombinant hexosaminidases; Sandhoff disease; Tay Sachs disease.

Introduction

Tay Sachs (TSD, OMIM #272800) and Sandhoff (SD, OMIM #268800) diseases are lysosomal storage disorders (LSD) known as GM2 Gangliosidosis. They are autosomal recessive disorders caused by mutations in *HEXA* or *HEXB* genes, which encode for the α - or β -subunits, respectively, of the human lysosomal β -N-acetylhexosaminidases (Hex, EC 3.2.1.52) A, B, or S [1, 2]. TSD and SD are clinically indistinguishable, except that in SD central nervous system (CNS) and visceral organs are affected, whereas in TSD only the CNS is impaired. Clinically, these disorders are characterized by apathy, hyperacusis, motor weakness, hypotonia, poor head control, decreasing attentiveness,

usual appearance of a macular cherry-red spot in the retina, seizures, and progressive mental deterioration followed by blindness, deafness, and spasticity, leading to a state of decerebrate rigidity. Patients usually die around 3 years of age [3].

Currently, specific therapy for TSD and SD is not available. Nevertheless, some experimental therapies include enzyme replacement therapy (ERT), bone marrow or neural progenitor cell transplantation, gene therapy, and substrate deprivation therapy [4]. Pre-clinical studies of ERT for GM2 gangliosidoses have been conducted using a recombinant modified Hex-B produced in Chinese Hamster Ovary (CHO) cells, which allowed *in-vitro* GM2 ganglioside storage reduction and in the parenchyma of intracerebroventricularly injected mice [5]. Furthermore, the use of a highly mannosylated recombinant Hex-A enzyme produced in *Ogataea minuta* yeast resulted in GM2 storage reduction and 7.8 % lifespan increase in the SD mouse model [6].

Human native β -hexosaminidases have been purified from liver [7-11], kidney [12], brain [10], and placenta [13-15]. On the other hand, human β -hexosaminidases have been recombinantly produced in CHO [5, 16], HeLa [17], and NG141 and NG125 cell lines [18], as well as in insect cells [17, 19, 20] and yeast (*O. minuta* and *Pichia pastoris* KM71H) [21-23]. Native enzymes have been used for structural and biochemical characterization, and *in vivo* and *in vitro* GM2 degradation assays [7, 12, 24, 25]. However, recombinant hexosaminidases have not been completely characterized under different pH or temperature conditions.

Human β -hexosaminidases extracted from human liver have a molecular weight between 100 and 103 kDa [7]. However, Hex-S extracted from brain and liver displayed a molecular weight of 152 and 154 kDa, respectively [10]. The estimated molecular weight of Hex-A and Hex-B produced in CHO and HeLa cells was between 63 and 69 kDa [5, 17]. Last, mutated Hex-A produced in NG141 and NG125 cell lines showed a molecular weight of 49 kDa [18]. Furthermore, reported isoelectric point (*pI*) for human β -hexosaminidases are 5.4 and 4.2 for Hex-A and Hex-S, respectively [7, 26], while for Hex-B it may vary between 7.1 and 7.9 [12, 26]. These Hex-B *pI* values contrast with those reported for the Hex-B sub-subunits (i.e β a and β b), which may vary from 4.5 to 5.0, and from 5.3 to 6.0 for β a and β b, respectively [13, 25].

A growing number of studies have revealed the possibility to produce active and therapeutic forms of lysosomal proteins in microorganisms [27]. Previously, we reported the production of active human lysosomal enzymes in *P. pastoris* and *Escherichia coli* [28-31]. In this work, we carried out the production of the recombinant human lysosomal β -hexosaminidases (rhHex-A, rhHex-B, rhHex-S) in the methylotrophic yeast *P. pastoris* GS115, and the effect of temperature and pH on the stability of the recombinant enzymes was also evaluated. These results represent valuable information towards the use of *P. pastoris* in the development of an ERT for GM2 Gangliosidoses.

Materials and methods

Pichia pastoris GS115 transformation

cDNA of α - (GenBank AAH84537) and β - (GenBank AAH17378) subunits of human β -hexosaminidases were codon-optimized for *Pichia sp.* expression. Optimized cDNA of α - and β -subunits were designed to encode recombinant β -hexosaminidases

with a 6x-His tag at the N-terminal, and synthesized by GeneArt[®] (Life Technologies Corporation). Optimized cDNAs were inserted downstream of the *Saccharomyces cerevisiae* α -factor secretion signal present in the pPIC9K vector (Life Technologies Corporation) to produce pPIC9k-alpha and pPIC9k-beta expression vectors. These vectors were confirmed by restriction analysis. To transform competent *P. pastoris* GS115 cells (Life Technologies Corporation) vectors were linearized with *Sa*I and *Pme*I, respectively and electroporated using a Gene Pulser Xcell[™] Electroporation System (Bio-Rad Laboratories) at 1400 V y 200 Ω . *P. pastoris* GS115 was transformed independently with each subunit (pPIC9k-alpha or pPIC9k-beta) or co-transformed with both subunits. As a control, *P. pastoris* GS115 was transformed with a linearized empty pPIC9K vector. Transformed clones were selected using minimal dextrose medium (MD: 1.34 % yeast nitrogen base (YNB), 4 x 10⁻⁵ % biotin, 2 % D-glucose). All procedures were carried out using standard molecular biology protocols [32].

Gene insertion for α -subunit (241 bp amplicon) was confirmed by PCR using the primers 5'-gtttcctctgctgctcaacc-3' and 5'-cggacaacaacaacactgg-3', and 5'-tcatcgactcccaagattc-3' and 5'-tggagtgtaaacgtgggaca-3' for β -subunit (155 bp amplicon). Phenotype of *P. pastoris* clones was confirmed by PCR by using the *AOX1* primers FW 5'-gactggtccaattgacaagc-3' and RW 5'-gcaaatggcattctgacatcc-3' (Life Technologies Corporation), as reported by Ayra *et. al.* [33]. All PCR reactions were carried out as follows: 94 °C 1 min denaturation, 55 °C 1 min annealing, and 72 °C 1 min/kb extension, for 30 to 35 cycles.

Shake flask cultures

P. pastoris clone screening was carried-out in 10 and 100 mL. *P. pastoris* GS115/pPIC9k-alpha (rhHex-S), *P. pastoris* GS115/pPIC9k-beta (rhHex-B), and *P. pastoris* GS115/pPIC9k-alpha/beta (rhHex-A) clones were grown in YPD (1 % w/v yeast extract, 2 % w/v peptone, 2 % dextrose w/v) for 48 h at 28 °C and 250 r.p.m. Cells were harvested by centrifugation and the pellet was resuspended in BMG medium (100 mM potassium phosphate pH 6.0, 1.34 % YNB, 4 x 10⁻⁵ % biotin, 1 % glycerol) and cultured for 24 h at 28 °C and 250 r.p.m. Last, cells were recovered as previously described and resuspended in BMM (100 mM potassium phosphate pH 6.0, 1.34 %; YNB, 4 x 10⁻⁵ %; biotin 1 % glycerol, 0.5 % methanol) and cultured for 144 h at 28 °C and 250 r.p.m. Methanol was added every 24 h to maintain a final concentration of 0.5 %. *P. pastoris* transformed with an empty pPIC9K vector was used as negative expression control, and was cultured under the conditions described above. Aliquots were taken every 24 h and stored at -20 °C until their use. The cell density was determined by using a previously described calibration curve ($\text{g} \cdot \text{L}^{-1} = 0.528 \times \text{OD}_{600\text{nm}} \times 0.619, r^2 = 0.98$) [28].

Bioreactor culture

Clones with the highest activity at shake flask scale were evaluated at 1.65 L scale in a 3.7 L Bioengineering reactor. Cultures were prepared in a modified FM22 saline medium (composition per liter: 25.74 g KH₂PO₄, 3 g (NH₄)₂SO₄, 8.58 g K₂SO₄, 0.6 g CaSO₄ 2H₂O, 40 g glycerol, 7.02 g MgSO₄ 7H₂O, 4 x 10⁻⁵ % biotin, 1 mL mineral traces, and 5 mL silicone) [34, 35]. Protein production was performed in three phases: i) a batch culture with glycerol to achieve 40 g $\cdot \text{L}^{-1}$ biomass, ii) a fed-batch culture

with glycerol to achieve 60 g .L⁻¹ biomass, and iii) a fed-batch induction phase with methanol. Methanol concentration was maintained at 0.5 ± 0.005 % by using an ALCOSENS probe (Heinrich Frings GmbH & Co. KG), with an automatic feed control. Cultures were carried out at pH of 5.0 (maintained with 7 % ammonium hydroxide) 28 °C, and under limited oxygen conditions.

Crude protein extraction and enzyme purification

Recombinant proteins were purified from culture medium thanks to α -factor secretion signal presence. Culture medium (crude extract) was filtered sequentially through paper Whatman No. 1 and 42, Pellicon XL Filter Module Durapore 0.45 μ m - 50 cm² (Merck/Millipore), and 0.22 μ m polyether sulphone membrane (Merck/Millipore). Permeate was ultrafiltered in a Stirred Ultrafiltration Cell through a 100 kDa cut-off Ultracel[®] ultrafiltration disc (Merck/Millipore). Subsequently, samples were dialyzed under previously established conditions: i) 25 mM acetate buffer pH 5.5 for rhHex-S, ii) 25 mM acetate buffer pH 5.0 for rhHex-B, or iii) 25 mM or 10 mM phosphate buffer pH 6.0 for rhHex-A. Dialyzed samples were purified by using a low-pressure chromatography system (BioLogic[™] LP System, Bio-Rad Laboratories), using Q-Sepharose Fast Flow column (Amersham Biosciences) for rhHex-S, Q-Sepharose Fast Flow or Macro-Prep DEAE Support (Bio-Rad Laboratories) for rhHex-A, while rhHex-B was purified by using Macro-Prep High S Support. Protein elution was performed by a 0 to 0.5 M NaCl linear gradient. Fractions with enzyme activity were concentrated by Amicon ultracentrifugal filters Ultracel[®] 50 K (Merck/Millipore) and stored at -20 °C until their use. All procedures were carried out at 4 °C. Protein purification was monitored by SDS-PAGE under reducing conditions and hexosaminidase enzyme activity.

Amino acid sequencing

Recombinant proteins were separated by SDS-PAGE [36], electro blotted to PVDF membranes (Millipore) [37], and visualized with Coomassie blue stain. The proteins of interest were excised from the membrane and subjected to automated Edman sequencing on a Procise 492 protein sequencer controlled by Procise software (version 5.1) [38] at the Protein Chemistry Laboratory, Texas A&M University.

Tryptic fragment digestion analysis of purified proteins by MALDI-TOF

Recombinant proteins were analyzed by SDS-PAGE [36], and the 100 kDa bands from rhHex-A and rhHex-S, and the 200 kDa band from rhHex-B were de-stained and treated as previously described [39]. Tryptic digested peptides were processed by MALDI-TOF Mass Spectrometer (Shimadzu/Kratos). The results were analyzed through Peptide Mapping (Thermo Fisher) and ProFound (<http://prowl.rockefeller.edu/prowl-cgi/profound.exe>). Tryptic fragment digestion analysis was done at the Protein Chemistry Laboratory, Texas A&M University.

Hexosaminidase activity assay

Hexosaminidase activity was assayed by using 4-methylumbelliferyl- β -D-acetylglucosaminide (MUG, Sigma-Aldrich) or 4-methylumbelliferyl- β -D-acetylglucosaminide sulfate (MUGS, Calbiochem) substrates, as previously described

[40, 41]. Human leukocyte β -hexosaminidase activity was assayed in samples from healthy individuals following the protocol described by Shapira *et al.* (1989) [40]. A 2.5 mM MUG substrate was used dissolved in citrate-phosphate at pH 4.4. 10 mM MUGS substrate was dissolved in deionized water. Reaction was stopped by 0.2 M glycine/0.2 M Na_2CO_3 solution pH 9.8 addition. Released 4-methylumbelliferone (MU) fluorescence was quantified in a Turner Fluorometer at 450/350 nm emission/excitation. One unit (U) was defined as the amount of enzyme hydrolyzing 1 nmol of substrate per hour. Specific hexosaminidase activity was expressed as U .mg⁻¹ of total protein determined by Lowry assay, and volumetric activity as U .mL⁻¹.

Characterization of recombinant hexosaminidases

pH effect: The effect of pH on the activity of recombinant hexosaminidases was evaluated at 3.5, 4.0, 5.0, 5.5, 6.0, 7.0, and 7.5. Each purified enzyme was incubated with 25 mM sodium citrate, or 25 mM phosphate buffer for acidic and basic pH values respectively. Samples were incubated at 37 °C for 1 h. After incubation enzyme activity was measured as described above, using MUGS for rhHex-A, and MUG for rhHex-B and rhHex-S.

Temperature effect: The effect of temperature on enzyme activity was carried out at the pH of maximum activity for each enzyme (i.e. pH 5.0 for rhHex-A; pH 4.0 for rhHex-S, and pH 4.5 for rhHex-B). Recombinant enzymes were incubated at (4, 37, and 45) °C during 2, 4, 6, 8, 10, 12, 24, 48, and 72 h. After incubation enzyme activity was measured as previously described, using MUGS for rhHex-A, and MUG for rhHex-B and rhHex-S.

Stability in human serum: To assess the stability of the recombinant hexosaminidases in human serum 1 μL of each purified enzyme was incubated with 49 μL of pooled human serum from healthy individuals. The mixture was incubated for 1, 3, 6, and 24 h at 37 °C. Human serum not spiked with recombinant enzyme was used as control and incubated under the described conditions. After incubation enzyme activity was measured as described above, using MUG substrate. Serum samples from healthy individuals were obtained after signed consent, and approved by the Research and Ethics Committee of the School of Science at Pontificia Universidad Javeriana.

Results and discussion

Pichia pastoris GS115 recombinant hexosaminidase production

Five *P. pastoris* GS115 clones for each enzyme rhHex-A, rhHex-B, and rhHex-S, were randomly selected and screened at 10 mL. At this scale, the highest enzyme activity levels determined with MUG substrate were 208.7, 145.4, 1,717.5 U .mL⁻¹, for rhHex-A, rhHex-B, and rhHex-S, respectively. Three *P. pastoris* methanol phenotypes related to non-deletions (Mut⁺), one deletion (Mut^S), or two deletions (Mut⁻) in AOX genes promoters have been reported. Mut^S mutants grow in methanol at a slow rate and need much less methanol during the induction phase compared with Mut⁺ mutants [42]. As expected, all β -hexosaminidase clones evaluated displayed a genotype compatible with Mut⁺ phenotype, as well as the presence of α -, β -, or α/β -subunit cDNA. Clones with the highest volumetric activity levels were scaled-up to 100 mL and 1.65 L scales.

Recombinant β -hexosaminidase shake flask level production was carried-out in 100 mL with 0.5 % methanol during 144 h induction. For rhHex-A, the highest volumetric and specific activities, determined with MUG substrate were observed at 72 h of induction with values of 182.2 U.mL⁻¹ and 1,207.6 U .mg⁻¹, respectively. The highest levels of volumetric and specific activities of rhHex-B were observed at 72 h of induction, with values of 19,257.3 U .mL⁻¹ and 11563.3 U .mg⁻¹. Last, for rhHex-S the highest volumetric and specific activities were observed at 96 h of induction with values of 8,173.7 U .mL⁻¹ and 36,292.06 U .mg⁻¹, respectively. *P. pastoris* transformed with the empty pPIC9K vector (negative control) showed a maximum total β -hexosaminidase activity of 3.2 U .mg⁻¹, which was notably lower compared with clones expressing recombinant human β -hexosaminidases. Furthermore, Uniprot and GenBank search did not identify β -hexosaminidase-like proteins in *P. pastoris* CBS 7435 (Taxonomy ID 981350), DMSZ 70382 (Taxonomy ID 638632), and GS115 (Taxonomy ID 644223). Taken together, these results demonstrate β -hexosaminidase activity detected in clones expressing rhHex-A, rhHex-B, and rhHex-S was due the result of human Hex cDNAs insertion and not to endogenous *P. pastoris* β -hexosaminidases-like enzymes presence. Furthermore, these results show substrate specificity used to follow-up recombinant human β -hexosaminidases production.

As expected, at 1.65 L specific activities values were increased, since growth and induction conditions (pH, temperature, aeration, and feed rate) were carefully controlled, improving heterologous protein production [43]. We evaluated different concentrations of glycerol and methanol during batch and fed-batch stages, respectively. The results showed that 40 g .L⁻¹ glycerol and 0.5 % v/v methanol, yielded highest biomass concentration and enzyme activity levels for the recombinant β -hexosaminidases. Thus, all bioreactor experiments were performed with 0.5 % v/v methanol, resulting in final biomass between 120 to 190 g L⁻¹, and total protein concentration of 1.6, 1.4, and 2.5 mg .mL⁻¹ for clones expressing rhHex-A, rhHex-B, and rhHex-S, respectively. Dissolved oxygen (DO) was maintained below 10 % according to microorganism requirements. Limited oxygen conditions have been previously described to contribute in cell lysis reduction and heterologous enzyme yield production increase [44]. Furthermore, it has been demonstrated that cultures under limited oxygen conditions lead to higher yields of protein production than those with high oxygen supply [45, 46]. As a case in point, production of recombinant human iduronate-2-sulfatase in *P. pastoris* GS115, a recombinant human lysosomal enzyme was increased under oxygen-limited conditions [47].

The highest specific and volumetric enzyme activities at 1.65 L were 13,124.1 U .mg⁻¹ and 25,762.6 U .mL⁻¹ for rhHex-A, respectively. For rhHex-B at this scale, specific and volumetric activities were 12,779.4 U .mg⁻¹ and 17,330.1 U .mL⁻¹, respectively. Last, rhHex-S at 24 h of induction had a specific activity of 21,025.9 U .mg⁻¹. Nevertheless, this result was not consistent with low protein concentration and volumetric activity observed at this time. Therefore, 14,606.0 U .mg⁻¹ and 15,556.1 U .mL⁻¹ at 96 h were considered the highest specific and volumetric activities for rhHex-S, respectively (**Figure 1**). These enzyme activity results were determined with non-sulfated substrate (MUG) and were between 25- and 50-fold higher compared with those obtained from leukocyte samples from healthy individuals processed in our laboratory (total β -hexosaminidase specific activity

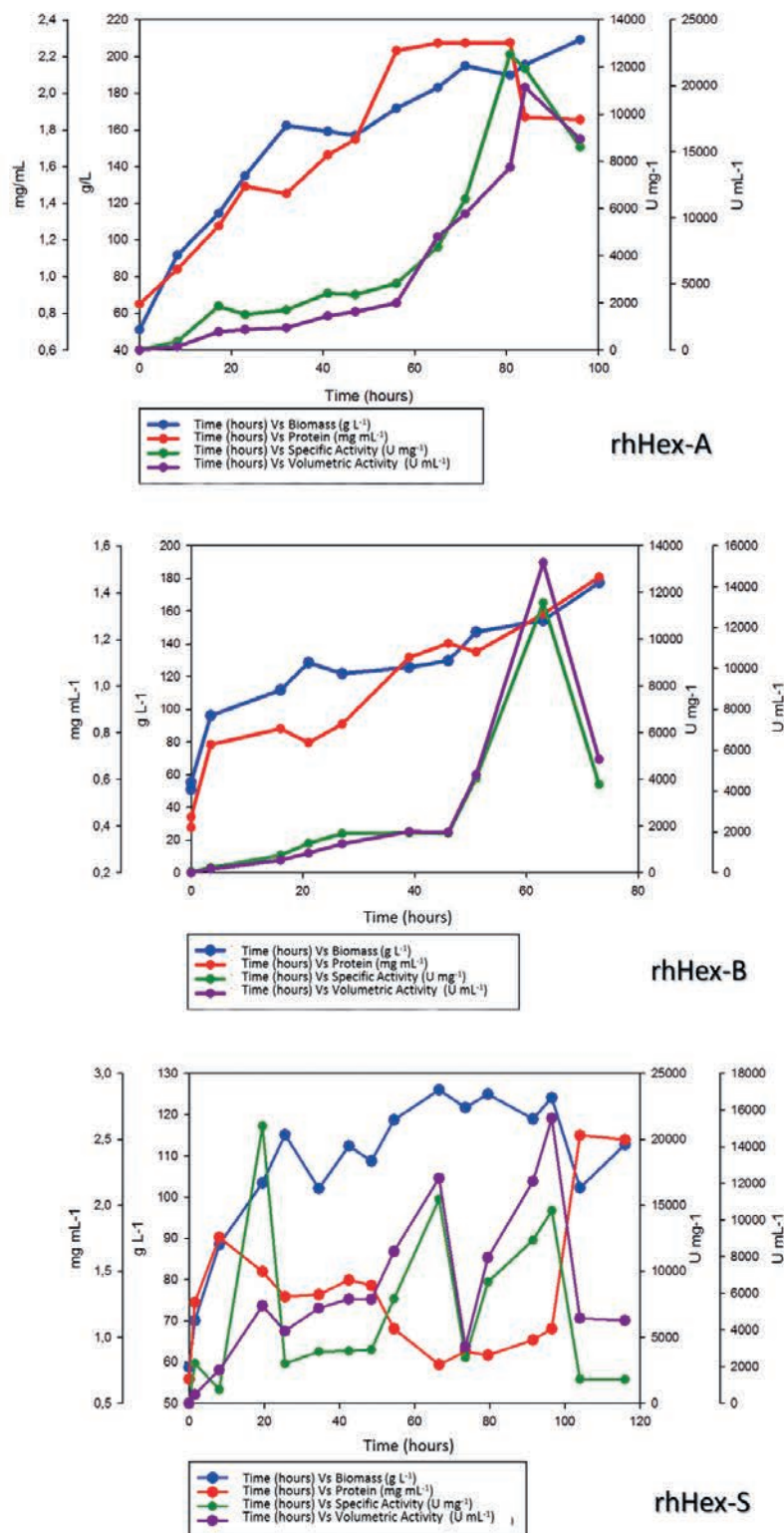


Figure 1. Production of rhHex-A (A), rhHex-B (B), and rhHex-S (C) in 1.65 L. Production of recombinant enzymes was induced with 0.5 % v/v methanol. **Outer Left axis:** protein concentration as determined by Lowry method (mg/mL). **Inner left axis:** biomass dry weight (g/L). **Inner right axis:** Specific enzyme activity in U .mg⁻¹. **Outer right axis:** Volumetric enzyme activity in U .mL⁻¹. Data is an average of duplicate cultures.n

$388.18 \pm 10 \text{ U} \cdot \text{mg}^{-1}$ and volumetric activity $431.02 \pm 10 \text{ U mL}^{-1}$). Akeboshi *et. al.* [22], using the methylotrophic yeast *Ogataea minuta*, reported specific activities of 51,000 and 29,000 $\text{U} \cdot \text{mg}^{-1}$ with non-sulfated and sulfated substrates, respectively. In this sense, the enzyme activity rhHex-A produced in *P. pastoris* at 1.65 L was approximately 3.9-fold lower than that reported for *O. minuta* at 4 L scale. Since these enzyme activity levels are in the same order of magnitude ($10^3 \text{ U} \cdot \text{mg}^{-1}$) than that of *O. minuta* Hex-A, these results determine *P. pastoris* potential as an expression system for recombinant Hex-A. Expression of recombinant Hex-B in *Saccharomyces cerevisiae* showed that the enzyme was directed to the vacuole and was active despite a different glycosylation pattern [48]. Nevertheless, rhHex-B activity produced in this study was $\sim 8,000$ -fold higher compared with that reported for *S. cerevisiae* ($1.6 \text{ U} \cdot \text{mg}^{-1}$) [48]. Recently, production of recombinant Hex-B in *P. pastoris* KM71H was described, yet activity results are not comparable since enzyme activity assay substrate (p-nitrophenyl 2-acetamido-2-deoxy- β -d-glucopyranoside) was different to that used in this study (4-methylumbelliferyl- β -D-acetyl-glucosaminide) [23]. Last, for recombinant Hex-S Akeboshi *et. al.* [22] reported its production during recombinant Hex-A production, where *O. minuta* yeast was co-transformed both with *HEXA* and *HEXB* genes. Although this recombinant Hex-S represented 74 % of total isozymes produced, they did not describe Hex-S activity values nor recombinant enzyme characterization [22]. Therefore, the present study represents the first report for production, purification, and characterization of recombinant Hex-S in yeast.

Purification of recombinant Hexosaminidases

Although human β -hexosaminidases cDNAs were designed to encode an N-terminal 6x His-tag, after several attempts of purification through His-tag affinity chromatography, it was not possible to obtain the purified recombinant enzymes. These results were associated with a probable loss of 6xHis-tag through proteolytic processing during enzyme maturation. In fact, Edman protein sequencing confirmed lack of N-terminal 6x-His tag for all three recombinant β -hexosaminidases. Hence, recombinant β -hexosaminidases were purified by ion exchange chromatography, according to reported isoelectric points for hexosaminidases extracted from human tissues [7, 12, 26]. rhHex-A, rhHex-B, and rhHex-S clones were cultured in 1.65 L and harvested at the highest production time for enzyme purification. rhHex-A and rhHex-S were purified by anion exchange chromatography at pH 6.0 and 5.5, respectively; while rhHex-B was purified by cation exchange chromatography at pH 5.0.

Final specific enzyme activity for purified rhHex-A, rhHex-B, and rhHex-S was 1.35×10^6 ; 1.27×10^6 ; and $1.39 \times 10^6 \text{ U} \cdot \text{mg}^{-1}$, respectively. Results showed a 136-, 127-, and 139-fold of purification for rhHex-A, rhHex-B, and rhHex-S, respectively (**Table 1**). Reports in literature are limited regarding β -hexosaminidase purification, and most reports are derived from human data. For instance, Conzelmann *et. al.* [9], reported an enzyme activity of $5.5 \times 10^6 \text{ U} \cdot \text{mg}^{-1}$ after purification of Hex-A (from human liver of a patient with GM2 variant AB) by concanavalin affinity chromatography and DEAE cellulose. Nevertheless, they did not report enzyme activity values for Hex-B purified from human liver, due to low protein concentration. Tallman *et. al.* [49], after six purification steps for human placenta β -hexosaminidases, reported enzyme activity values of 30.6×10^6 and $28.1 \times 10^6 \text{ U} \cdot \text{mg}^{-1}$ for Hex-A and Hex-B, respectively. Regarding the recombinant production of human β -hexosaminidases, rhHex-B

Table 1. Purification of rhHexosaminidases produced in *Pichia pastoris*.

Sample	Total Protein (mg/mL)	Total activity U (nmol/h) x 10 ⁶	Specific activity (nmol/mg/h)	% yield	fold
rhHex-A					
Crude extract	2716.60	34.28	0.01	100	1
Ultrafiltration retentate	150.74	19.36	0.13	56	13
Purified protein	12.96	17.59	1.36	51	136
rhHex-B					
Crude extract	1890.00	19.56	0.01	100	1
Ultrafiltration retentate	325.15	6.53	0.02	33	2
Purified protein	4.20	5.33	1.27	27	127
rhHex-S					
Crude extract	1605.00	23.33	0.01	100	1
Ultrafiltration retentate	72.73	15.08	0.21	65	21
Purified protein	9.60	13.38	1.39	57	139

produced in insect cells and purified by two chromatographic steps showed a final enzyme activity of 6.5×10^6 U .mg⁻¹ [20]; while rhHex-A produced in *O. minuta* and purified by affinity chromatography showed a final activity of 1.4×10^6 U .mg⁻¹ [21]. Although in this study a one-step chromatography purification process was employed, purified rhHex-A and rhHex-B produced in *P. pastoris* GS115 showed similar enzyme activity levels to those of the recombinant enzymes produced in other expression systems, but lower than those levels reported for native enzymes. On the other hand, specific activity of purified rhHex-S was 50–fold higher than that reported by Potier *et al.* [10] for liver Hex-S (26820 U .mg⁻¹), and considerably higher than that purified from brain (1494 U .mg⁻¹). Last, specific activity of purified rhHex-S was 2.2–fold lower than that reported by Hepbildikler *et al.* (3.1×10^6 U .mg⁻¹) produced in insect cells [19].

Since *P. pastoris* transformed with α - and β -Hex cDNAs (rhHex-A) can produce the three isozymes (rhHex-A, rhHex-B, and rhHex-S), we estimated the presence of these enzymes within the rhHex-A crude extract and eluted fractions from anionic chromatography (DEAE sepharose) (Figure 2). Compared to other β -hexosaminidases, recombinant enzymes produced in this study showed a different pattern using DEAE sepharose chromatography. As observed in Figure 2A, rhHex-B and rhHex-A were detected in lower proportion in the non-bound protein (NBP) than in eluted protein (EP) fractions measured with non-sulfated substrate (MUG). The evaluation of the

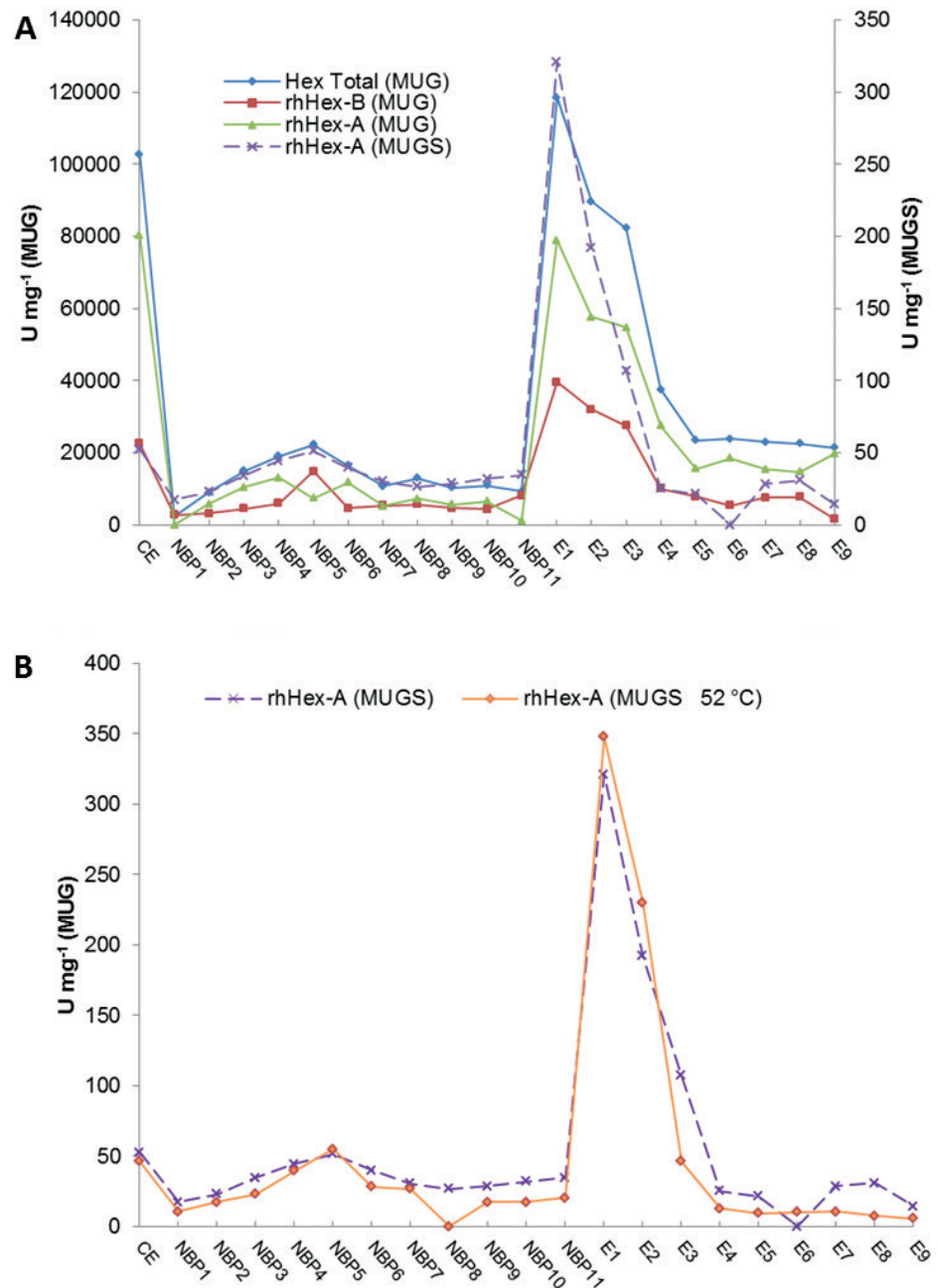


Figure 2. rhHex-A:rhHex-B ratio in rhHex-A crude extract. rhHex-A:rhHex-B ratio from rhHexA crude extract was determined by DEAE anion exchange chromatography. **A.** CE: rhHexA crude extract; NBP: non-bound protein fractions; E: eluted protein fractions. Total rhHex (red line): activity assayed with MUG substrate; rhHexB (green line): activity assayed with MUG substrate after 52°C inactivation; rhHex-A (MUG) (blue line): difference between total rhHex and rhHex-B activity assayed with MUG substrate; rhHex-A (purple dotted-line): activity assayed with MUGS substrate. **B.** Presence of rhHex-S within rhHexA crude extract was ruled out by measuring β -hexosaminidase activity with MUGS substrate with or without sample inactivation at 52°C, according to rhHex-S thermostability profile.

activity assay using the MUGS substrate, with and without 52 °C inactivation (see Figure 5 rhHex-S thermostability), suggested rhHex-S is not present within the rhHex-crude extract (Figure 2B). These results contrast with previous reports, using DEAE

spharose, which determined Hex-B and Hex-A/Hex-S were obtained in NBP and EP fractions, respectively [12, 26, 49-51]. Taking in to account these results, the Hex-A:Hex-B ratio was established using EP fraction specific activity levels measured with MUG substrate, obtaining a Hex-A:Hex-B ratio of 2.17. Although the ratio was calculated in a different manner compared with previous reports, this value was similar to that reported by Okochi *et. al.* [52] for β -hexosaminidases purified from kidney (2.5 to 4.52), and higher than that reported for β -hexosaminidases purified from placenta (0.89 to 1.24). Furthermore, in this study rhHex-A represents a 67 % of total isozymes present in the rhHex-A crude extract, which was 3-fold higher than that reported for the recombinant Hex-A produced in *O. minuta* (about 23 % of the total β -hexosaminidases after DEAE purification process [22]).

Molecular weights of most tissue extracted proteins have been established using gel filtration chromatography. Hex-A purified from human placenta displayed a molecular weight of 112.5 kDa [15]; while molecular weights between 100 and 110 kDa have been reported for human liver purified enzyme [7, 8]. Similar results were reported for Hex-B purified from liver and placenta, with a molecular weight between 108 and 112 kDa, respectively. In addition, a 103 kDa Hex-S was described in human liver [7]. In contrast, Potier *et. al.* reported for Hex-S, purified from liver and brain, a molecular weight of 154 and 152 kDa, respectively [10]. In this study, using a SDS-PAGE analysis under reducing conditions, for rhHex-B bands of ~200 kDa were observed, and for rhHexA and rhHex-S of ~100 kDa (**Figure 3A**). This high molecular weight could be mainly associated with differences in N-glycosylations among purified proteins [22, 53]. For instance, for recombinant proteins produced in *P. pastoris*, high mannose N-glycans (Man8-12) have been described [54, 55], while low mannose N-glycans (Man5) have been described for proteins produced in CHO cells [54]. Although Akeboshi *et. al.* determined two bands at 46 and 43 kDa after EndoH treatment, we consider that the marked difference in molecular weight observed between β -hexosaminidases expressed in *P. pastoris* GS115 and those purified from other sources (native or recombinant) could be also associated with interactions between subunits through non-covalent bonds that may prevent dissociation under reducing conditions and favor protein aggregate formation [56].

Immunodetection of recombinant β -hexosaminidases was attempted by Western-blot using commercial antibodies against α - and β -subunits. After several attempts and Western-blot modifications (e.g. protein and antibodies concentration and incubation times), it was possible to observe faint bands that correlated with those observed in the SDS-PAGE. However, these results did not allow for reliable evidence regarding recombinant protein identity, due to low band intensity (data no shown). Therefore, to confirm recombinant human β -hexosaminidase identity, SDS-PAGE gel bands obtained under reducing conditions from purified fractions, were analyzed through MALDI-TOF (**Figure 3B**). Tryptic fragments from the 100 kDa band of rhHex-A sample corresponded with both α - and β -subunit sequences of human β -hexosaminidases. Furthermore, tryptic fragments from rhHex-B (200 kDa) and rhHex-S (100 kDa) bands coincided with β - and α -subunits of human β -hexosaminidases, respectively.

Taken together, these results confirm recombinant β -hexosaminidases produced in *P. pastoris* identity and suggest these recombinant β -hexosaminidases have

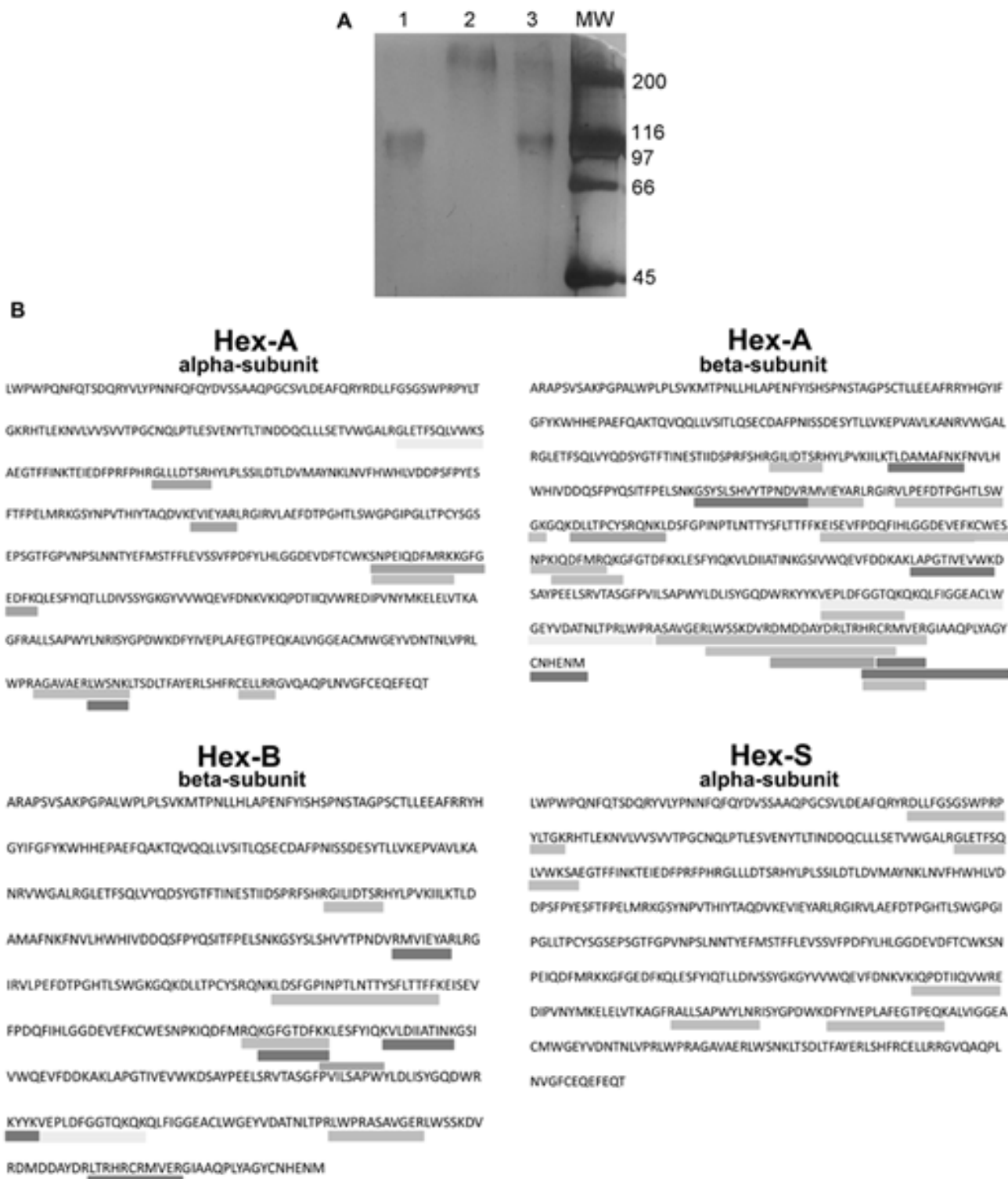


Figure 3. A. SDS-PAGE of purified recombinant hexosaminidases. Polyacrylamide gel was run under reducing conditions. 10 μ g of protein was loaded in each lane. Gels were stained with silver nitrate. Lanes 1 to 3: rhHex-S, rhHex-B, and rhHex-A purified fractions, respectively. **B. Analysis of Tryptic fragments of purified proteins by MALDI-TOF.** Recombinant proteins were analyzed by SDS-PAGE and gel bands were cut, de-stained and treated to obtain tryptic fragments, followed by MALDI-TOF evaluation. Tryptic fragments from rhHex-A 100 kDa band showed a correspondence with α - and β -subunits of human β -hexosaminidases. Bars under amino acid sequences represent peptide fragments identified after MALDI-TOF analysis. The tryptic fragments from the 200 and 100 kDa bands for rhHex-B and rhHex-S, respectively, showed a correspondence with the β - and α -subunits of human β -hexosaminidases, respectively.

different molecular weights than those reported for both native and recombinant β -hexosaminidases. These results might agree with dimer association description during native hexosaminidase A crystallization [15]. However, further assays are necessary to confirm these results.

Characterization of recombinant Hexosaminidases

Recombinant β -hexosaminidases were characterized for their stability at different pH (3.5 to 7.5) and temperature (4 °C, 37 °C, and 45 °C) conditions, as well as in human serum. Regarding pH stability, rhHex-A, rhHex-B, and rhHex-S reduced their activity by 35 %, 50 % and 15 % of activity respectively at pH 3.5 (Figure 4). rhHex-A depicted its highest activity between pH 4.5 and 5.0, with a reduction between 10 and 55 % at pH above 5.5, while at pH 4.0 enzyme activity was reduced by 15 % compared with levels observed at pH 5.0 (Figure 4). On the other hand, rhHex-B showed highest activity at pH 4.5, with a marked reduction at pH values above (60 %) or below (50 %) this pH (Figure 4). Last, rhHex-S showed the highest activity at pH 4.0, with an up to 15 % reduction at pH values below 4.0 and up to 6.0. Nevertheless, a marked activity reduction between 80 and 100 % was observed at pH greater than 6.0 (Figure 4). There are few reports showing the effect of pH on enzyme activity for β -hexosaminidases. However, these results are in agreement with those reported by Geiger *et al.* [7], who described a maximum pH activity in a range from 4.0 to 5.0, for β -hexosaminidases extracted from liver.

Temperature stability was evaluated up to 72 h at 4, 37 and 45 °C (Figure 5). Under these conditions, rhHex-A did not show a significant reduction in enzyme activity at 4 and 37 °C throughout the evaluated time (72 h). In contrast, at 45 °C enzyme remained stable during the first 10 h, after which a marked reduction was observed reaching

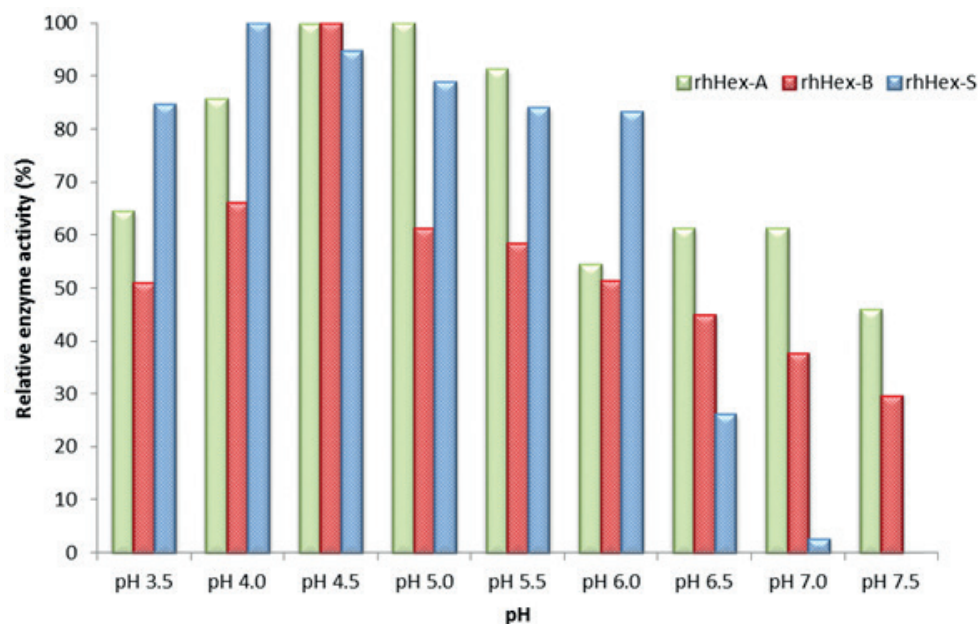


Figure 4. Effect of pH on recombinant hexosaminidase enzyme activity. Enzyme activity was evaluated for rhHex-A, rhHex-B, and rhHex-S after 1 h incubation at 37°C and different pH values. Results are shown as relative enzyme activity (%) based on the highest enzyme activity value obtained for each enzyme.

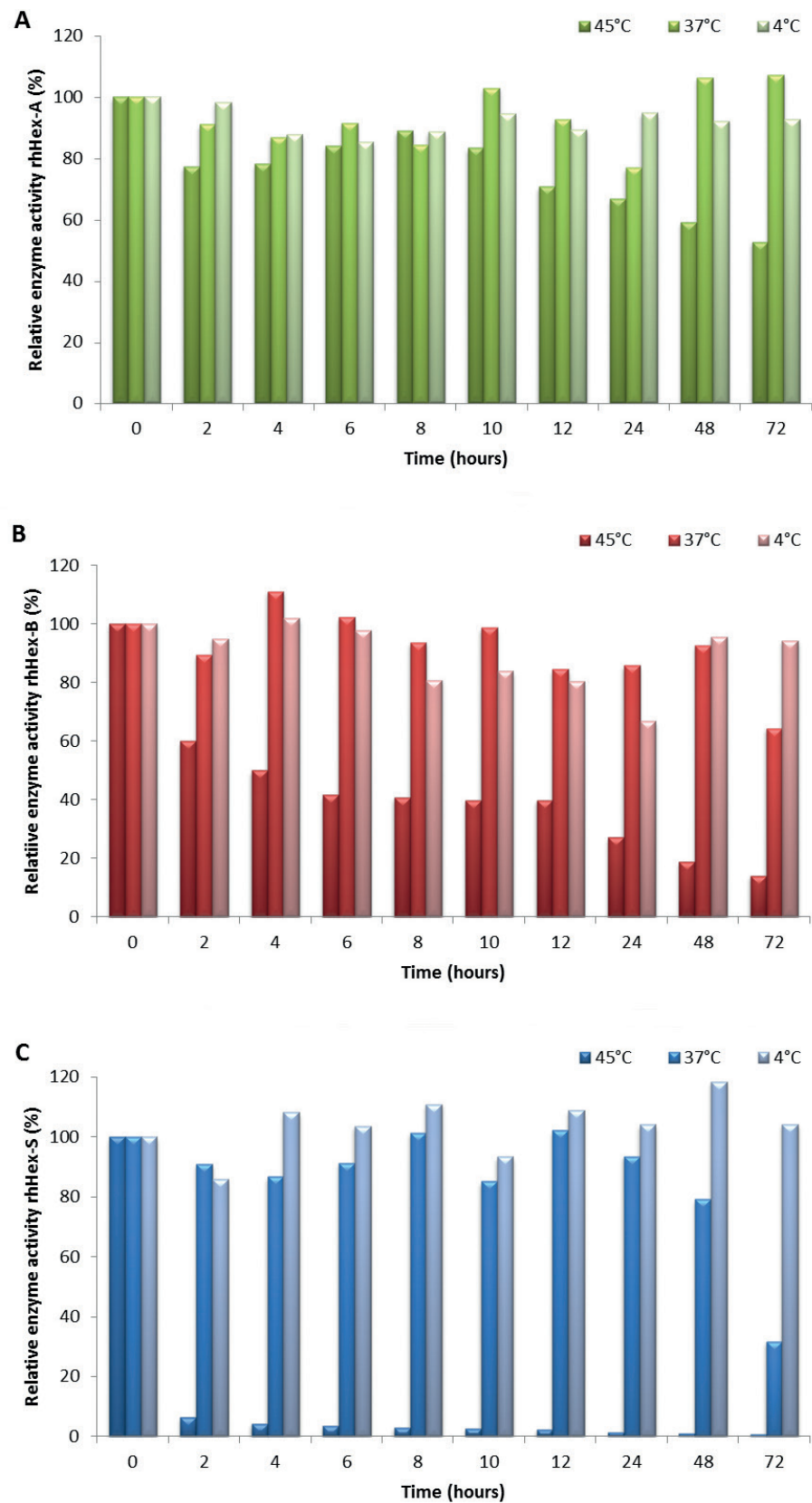


Figure 5. Effect of temperature on recombinant hexosaminidase enzyme activity. Enzyme activity was evaluated for rhHex-A (A), rhHex-B (B), and rhHex-S (C) after 72 h incubation at 4, 37, and 45 °C. Results are presented as relative enzyme activity (%) based on enzyme activity obtained at 0 h.

a final activity of 35 % its initial activity levels after 72 h of incubation (Figure 5A). rhHex-B showed a remarkable stability at 4 and 37 °C, although after 72 h at 37 °C enzyme activity was reduced by 40 % compared with initial levels. At 45 °C rhHex-B there was a stark reduction in enzyme activity after 2 h of incubation (40 % of initial levels), reaching a 90 % decrease after 72 h (Figure 5B). Last, rhHex-S also showed high stability at 4 and 37 °C, with an 80 % reduction after 72 h of incubation at 37 °C. At 45 °C rhHex-S showed the highest thermolabile behavior of the studied enzymes, with enzyme activity reduction above 90 % after the first 2 h of incubation (Figure 5C).

β -subunit has been reported as the most stable intracellular subunit [57]. Therefore, a 52 °C inactivation is commonly used during Hex-A enzyme activity assay in plasma samples [40]. Taken together, the results obtained in this study suggest β -subunit rhHex-B produced in *P. pastoris GS115* has a profile slightly different to previous thermostability reports. Geiger *et. al.* showed that at 40 and 50 °C, Hex-B was the most stable among hexosaminidases, losing its activity at 60 °C, as well as Hex-A and Hex-S [7]. In contrast, Krejsová *et. al.* described a recombinant Hex-B produced in yeast with stability at 65 °C [23].

Regarding rhHex-S, temperature stability profile is in agreement with previous reports showing low α -subunit stability [57]. Last, rhHex-A obtained in this study showed an important stability at 45 °C during 8 hours of incubation, which contrasts with liver extracted isozyme that lost a 10 % activity just after 10 min incubation at 40 °C [7].

Lastly, stability of recombinant β -hexosaminidases was evaluated in human serum spiked with recombinant enzymes. Activity of Hex-A and Hex-B in non-spiked human serum was between 700 and 3,000 U · mg⁻¹ and 1,000 and 1,800 U · mg⁻¹, respectively. Hex-S was not determined by this method since it is considered a less important isozyme in human blood samples. Addition of recombinant β -hexosaminidases to human serum increased the activity between 5- and 83-fold in comparison with levels

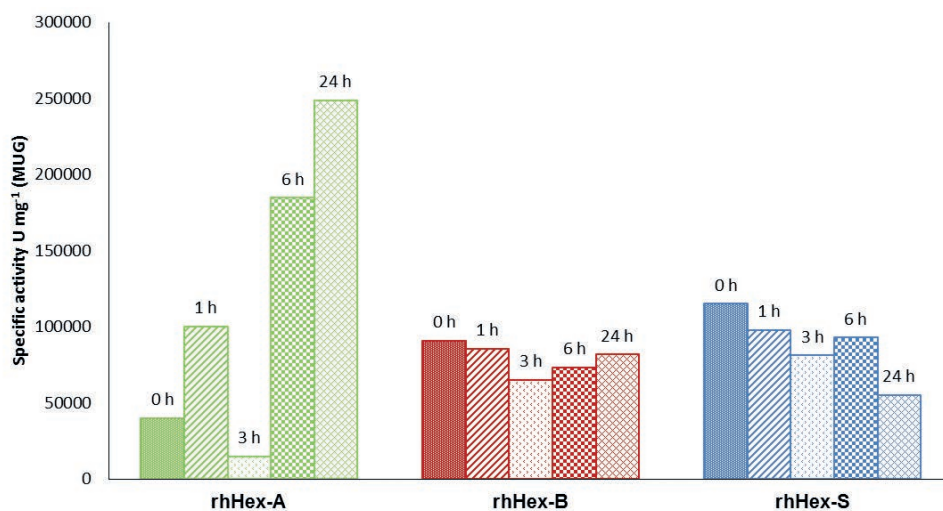


Figure 6. Effect of pH on recombinant hexosaminidase enzyme activity. Enzyme activity was evaluated for rhHex-A, rhHex-B, and rhHex-S after 1 h incubation at 37 °C and different pH values. Results are shown as relative enzyme activity (%) based on the highest enzyme activity value obtained for each enzyme.

observed in human control serum. It was observed that rhHex-B and rhHex-S did not show an important reduction in enzyme activity within 24 h of incubation, while rhHex-A presented an unexpected activity profile, since an increase in enzyme activity was observed throughout time (**Figure 6**). Explanation regarding this unexpected increase is still under evaluation.

Although recombinant human β -hexosaminidases have been produced separately in different yeasts [21-23, 48], this study reports the production and characterization of the three recombinant human β -hexosaminidases using the same yeast strain (i.e. *Pichia pastoris* GS115), which allows for better enzyme characteristic comparison. In summary, these results demonstrated recombinant hexosaminidases obtained from *P. pastoris* GS115 have similar stability characteristics compared to human tissue extracted enzymes (native enzymes). This is an important finding requiring further evaluation for future therapeutic use.

Conclusions

In this study, we described human recombinant Hex-A, Hex-B, and Hex-S production and characterization. These enzymes were produced within the same host by using the methylotrophic yeast *P. pastoris* GS115. Purified enzymes showed enzyme activities of 1.4×10^6 (rhHex-A); 1.3×10^6 (rhHex-B); and 1.4×10^6 (rhHex-S) U .mg⁻¹. These recombinant proteins were stable under different pH and temperature conditions, as well as in human serum. Nevertheless, rhHex-B showed a slightly lower thermostability, while rhHex-A was more stable than that reported for human native hexosaminidases. This is not surprising since it is well known that proteins could be slightly different depending on organism and/or tissue in which they are produced. Thus, these results offer valuable findings regarding *P. pastoris* importance as an expression host for recombinant enzymes production with potential application for Enzyme Replacement Therapy. However, further studies should focus on *in-vitro* and *in-vivo* recombinant enzyme GM2 ganglioside tissue metabolism evaluation, as well as the effect on disease phenotype.

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Conflicts of Interest

The authors declare no competing financial interests.

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Caracterización de beta-hexosaminidasas lisosomales humanas recombinantes producidas en la levadura metilotrónica *Pichia pastoris*

Resumen. Las β -hexosaminidasas (Hex) son enzimas diméricas involucradas en la degradación lisosomal de glicolípidos y glicanos. Estas enzimas están formadas por las subunidades α - y/o β -codificadas por los genes *HEXA* and *HEXB* respectivamente. Las mutaciones de estos genes conducen a las enfermedades de Tay Sachs o Sandhoff, que son desórdenes neurodegenerativos causados por la acumulación de glicolípidos no degradados. Aunque las Hex derivadas de tejido han sido ampliamente caracterizadas, la información disponible sobre las β -hexosaminidasas recombinantes es limitada. En este estudio se produjeron Hex recombinantes lisosomales (rhHex-A, rhHex-B y rhHex-S) en la levadura metilotrónica *Pichia pastoris* GS115. Las actividades específicas más altas de las enzimas fueron 13.124, 12.779, 14.606 U .mg⁻¹ para rhHex-A, rhHex-B y rhHex-S, respectivamente. Estos resultados fueron 25 a 50 veces más altos que los obtenidos de leucocitos humanos normales. Las proteínas se purificaron y se caracterizaron a diferentes condiciones de pH y temperatura. Todas las proteínas fueron estables a pH ácido y a 4°C y 37°C. A 45°C la rhHex-S se inactivó completamente, mientras que rhHex-A y rhHex-B mostraron alta estabilidad. Este estudio demuestra el potencial de *P. pastoris* GS115 para la producción de enzimas lisosomales poliméricas y presenta la caracterización de distintas β -hexosaminidasas recombinantes producidas en un único hospedero.

Palabras clave: β -N-acetylhexosaminidasas; caracterización; *Pichia pastoris*; hexosaminidasas recombinantes; enfermedad de Sandhoff; enfermedad de Tay Sachs.

Caracterização de beta-hexosaminidasas lisossomais humanas recombinantes produzidas na levedura metilotrónica *Pichia pastoris*

Resumen. As β -hexosaminidasas (Hex) são enzimas diméricas envolvidas na degradação lisossomal de glicolípídeos e glicanos. Essas enzimas são formadas por subunidades α - e/ou β -codificadas pelos genes *HEXA* e *HEXB*, respectivamente. As mutações nesses genes causam a doença de Sandhoff ou Tay Sachs, que são desordens neurodegenerativas causadas pela acumulação de glicolípídeos não degradados. Embora Hex derivadas de tecido hajam sido caracterizadas extensivamente, as informações disponíveis sobre as β -hexosaminidasas recombinantes são limitadas. Esse estudo produziu Hex recombinantes lisossomais (rhHex-A, rhHex-B e rhHex-S) na levedura metilotrónica *Pichia pastoris* GS115. As atividades específicas mais altas das enzimas foram 13.124, 12.779, 14.606 U .mg⁻¹ para rhHex-A, rhHex-B y rhHex-S, respectivamente. Esses resultados foram 25 a 50 vezes mais altos do que os obtidos a partir de leucócitos humanos normais. As proteínas foram purificadas e caracterizadas em diferentes condições de pH e temperatura. Todas as proteínas foram estáveis a pH ácido e a 4°C e 37°C. A 45°C a rhHex-S foi completamente inativada, enquanto rhHex rhHex-A e B se mostraram altamente estáveis. Esse estudo demonstra o potencial de *P. pastoris* GS115 para a produção de enzimas lisossomais poliméricas e apresenta a caracterização de diferentes β -hexosaminidasas recombinantes produzidas em único hospedeiro.

Palabras clave: β -N-acetylhexosaminidasas, caracterização, *Pichia pastoris*, hexosaminidasas recombinantes, Doença de Sandhoff, Doença de Tay Sachs.

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