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Kinetic characterization of human thyroperoxidase. Normal and pathological enzyme expression in Baculovirus System: A molecular model of functional expression

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ABSTRACT

Background: Human thyroperoxidase (hTPO) is a membrane-bound glycoprotein located at the apical membrane of the thyroid follicular cells which catalyzes iodide oxidation and organification in the thyroglobulin (TG) tyrosine residues, leading to the thyroid hormone synthesis by coupling of iodotyrosine residues. Mutations in hTPO gene are the main cause of iodine organification defects (IOD) in infants.
Methods: We investigated the functional impact of hTPO gene missense mutations previously identified in our laboratory (p.C808R, p.G387R and p.P499L). In order to obtain the whole wild-type (WT) coding sequence of hTPO, sequential cloning strategy in pGEMT vector was carried out. Then, site-directed mutagenesis was performed. WT and mutant hTPOs were cloned into the pAcGP67B transfer vector and the recombinant proteins were expressed in Baculovirus System, purified and characterized by SDS–PAGE and Western blot. Moreover, we report for the first time the kinetic constants of hTPO, of both WT and mutant enzymes.
Results: The functional evaluation of the recombinant hTPOs showed decreased activity in the three mutants with respect to WT. Regarding to the affinity for the substrate, the mutants showed higher Km values with respect to the WT. Additionally, the three mutants showed lower reaction efficiencies (Vmax/Km) with respect to WT hTPO.
Conclusions: We optimize the expression and purification of recombinant hTPOs using the Baculovirus System and we report for the first time the kinetic characterization of hTPOs.

1. Introduction

One of the most frequent neonatal disorders associated with preventable mental retardation is congenital hypothyroidism. This prevalent infant disease affecting 1:2000–1:4000 newborns (Gruters, 1992; Rastogi and LaFranchi, 2010) and can be associated with genetic dysembryogenesis (80%) or defective thyroid hormone synthesis (15–20%) (Mangklabruks et al., 1991). Mutations in human thyroperoxidase (hTPO) gene are the main factor of dyshormonogenesis leading to iodine organification defects (IOD) in infants (Belforte et al., 2012; Rivolta et al., 2003).

hTPO is a membrane-bound glycoprotein located at the apical membrane of the thyroid follicular cells that catalyzes iodide

Abbreviations: hTPO, human thyroperoxidase gene (according to the recommendations of the HUGO Gene Nomenclature Committee) and protein; TG, thyroglobulin; IOD, iodine organification defects; WT, wild-type; EGF, epidermal growth factor; RT-PCR, reverse transcription polymerase chain reaction; GFP, green fluorescent protein; MOI, multiplicity of infection.

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oxidation and organification in the thyroglobulin (TG) tyrosine residues, leading to the thyroid hormone synthesis by coupling of iodotyrosine residues. hTPO gene is located on the chromosome 2p25 and presents 17 exons, spanning 131 Kb of genomic DNA (Park and Chatterjee, 2005). The mRNA is 3048 nucleotides long and the preprotein is composed of a putative 14 amino acids signal peptide followed by a 919 amino acids polypeptide encoding a large extracellular domain, a transmembrane domain, and a short intracellular tail (Park and Chatterjee, 2005). The extracellular domain is responsible for the enzymatic activity as it encodes the heme-binding region, the animal peroxidase domain, the catalytic center of the hTPO protein which is reported to be encoded by exons 8, 9 and 10. Exons 13 and 14 appear to codify for two regions: the complement control protein like residues (742–795) and calcium-binding epidermal growth factor (EGF)-like residues (796–839) (Park and Chatterjee, 2005). Transmembrane residues are encoded in exon 15 and the cytoplasmic tail, in exons 16 and 17. Proper folding, adequate membrane insertion and an intact catalytic site are crucial features for enzyme activity.

Since hTPO plays a key role in thyroid hormone biosynthesis and consequently in the molecular pathophysiology of IOD, many researchers expressed TPO in different systems for decades, being baculovirus one of the selected strategies (Fan et al., 1996; Gardas et al., 1999; Hendry et al., 1999; Ogino et al., 2007).

The Baculovirus System has been widely used in recombinant protein production of various origins (bacterial, fungal, plant, animal, viral, human) during the last 30 years as a result of the progressive development of a variety of transfer vectors, viral titration methods and development of culture media (Altmann et al., 1999; Cox, 2012; Demain and Vaishnav, 2009; Kärkkäinen et al., 2009; Kost et al., 2005; Roldão et al., 2011). The versatility of the system lies in the ability to incorporate large fragments of foreign DNA without altering the infectivity of the virus (due to its flexible nucleocapsid). It is used to express soluble intracellular and extracellular as well as plasma membrane proteins; enables high expression levels due to the use of strong viral promoters. Given the cellular characteristics of the model, the system provides a suitable environment for eukaryotic postrasduccional maturation and modification of proteins of interest and favorable scaling of the production processes, increasing yields of recombinant protein production (Kendler et al., 1993).

The aim of our study was to clone the hTPO and use the Baculovirus System to define the undescribed kinetic constants and the functional characteristics of the proteins, comparing the WT versus mutagenized enzymes carrying mutations responsible for IOD previously identified in our laboratory (Kitts and Possee, 1993; Rivolta et al., 2007).

2. Materials and methods

2.1. Cloning strategy

2.1.1. WT hTPO cDNA construct

Total mRNA from human thyroid tissue of a healthy individual was isolated by Trizol method. Three primers sets were designed in order to get three cDNA fragments which sequences were overlapped between each other. Primers next to 5′ and 3′ contained restriction sites for EcoRI. The cDNA fragments were obtained by reverse transcription of total mRNA and subsequent polymerase chain reaction of the samples (RT-PCR). Primers sequence and RT-PCR conditions are shown in Table 1. Reverse transcription (RT) was performed in 20 μl using standard RT buffer (Invitrogen Life Technologies, Gaithersburg, MD, USA) containing 400 ng of total isolated RNA preheated at 90° for 5 min, 200 mM of each deoxy (d)-NTP (dATP, dCTP, dTTP, and dGTP), 20 U RNAsin (Promega, Madison, WI, USA), 50 pmol of reverse primer, 0.1 μM Dithiothreitol, 200 U M-MLV RT BRL (Invitrogen Life Technologies). Samples were incubated at room temperature for 10 min, 42 °C for 60 min and 95 °C for 5 min. Then, the whole volume of cDNA obtained was added to the PCR reaction which was performed in 100 μl, using a standard PCR buffer (Invitrogen Life Technologies), 200 mM of each deoxy (d)-NTP (dATP, dCTP, dTTP, and dGTP), 2.5 mM of $MgCl_2$, 50 pmol of each forward and reverse primers, 4% dimethylsulfoxide and 0.5 U Taq polymerase (Invitrogen Life Technologies). Samples were denatured at 95 °C for 1.5 min followed by 40 cycles of amplification. Each cycle consisted of denaturation at 95 °C for 30 s, primer annealing at 55 °C–58 °C for 30 s, and primer extension at 72 °C for 1 min. After the last cycle, the samples were incubated for additional 10 min at 72 °C to ensure that the final extension step was complete. RT-PCR products analyzed by agarose gel electrophoresis were recovered from gel using GFX PCR DNA Purification Kit (GE Healthcare, Little Chalfont, Buckingham Shire, UK), analyzed by directed sequencing and cloning into the pGEMT Easy vector (Promega, Madison, WI, USA). Then, E. coli TG1 competent cells were transformed with the recombinant vectors pGEMT TPO1, pGEMT TPO2 and pGEMT TPO3 according to the manufacturer's specifications. Bacteria colonies were selected on Luria–Bertani medium (LB medium) containing ampicillin, IPTG and X-Gal and grown on LB medium for 16 h at 37 °C, 250 rpm. Plasmid extraction was performed with Wizard Plus SV minipreps columns (Promega, Madison, WI, USA). The recombinant plasmids characterization was first done for insert size by digestion with EcoRI and then confirmed by sequencing. Sequential cloning of fragments was proceeded in order to obtain the complete cDNA of hTPO in the pGEMT vector (pGEMT- hTPOWT) (Supplementary Fig. S1). EcoRI, FseI and Sma I restriction endonucleases (Promega, Madison, WI, USA) were used for this purpose and the complete hTPO cDNA obtained was confirmed by sequencing (hTPO signal peptide and stop codon sequence were removed by the RT-PCR strategy, in order to use Baculovirus System signals).

2.1.2. Mutant hTPO cDNA constructs

The mutant clones were generated from pGEMT-hTPOWT using Quick Change II Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). Mutagenesis primers were designed using QuickChange Primer Design (http://www.genomics.agilent.com). The primers secuences used to obtain the M1 (p.C808R), M2 (p.G387R) and M3 (p.P499L) insertion mutations are summarized in Table 2. All final constructs (pGEMT-hTPOM1, pGEMT-hTPOM2 and pGEMT-hTPOM3) were verified by direct DNA sequencing.

2.1.3. hTPO cDNAs cloning in the baculovirus transfer vector

The cDNA for WT hTPO was excised from pGEMT-hTPOWT and the cDNA of mutant hTPO, from pGEMT-hTPOM1, pGEMT-hTPOM2 and pGEMT-hTPOM3 using the EcoRI restriction site. So, the 2772 bp

Table 1
Summary of TPO primers used for RT-PCR amplification and sequencing and PCR conditions.

Fragment	Forward primers		Amplicon (bp)	Reverse primers		Annealing temperature
	Position of 5′ end	Nucleotide sequence (5′ → 3′)		Position of 5′ end	Nucleotide sequence (5′ → 3′)	
1	127	GGAATTCGTTGCACAGAAGCCTTCTTCCCC	1067	1184	ACGAAGGGCAGGTAGGCG	58 °C
2	789	ATACATCGACCACGACATCG	959	1747	GCTGATCCTGCACCTGCAGT	55 °C
3	1568	ATGTGTGTTAGTTGGTGGGG	1325	2883	AGGAAAGGGAACCTTTGG	56 °C

Primers are designated according to human TPO mRNA reference sequence (GenBank Accession Number: NM_000547.5).

Table 2
hTPO enzymatic activity and kinetic characterization.

hTPO	Specific activity 2 mM guaiacol ($U \cdot min^{-1} \cdot mg^{-1}$)	KM (mM)	Vmax ($U \cdot min^{-1} \cdot mg^{-1}$)	Vmax/Km ($U \cdot min^{-1} \cdot mg^{-1} \cdot mM^{-1}$)
WT	7.22 ± 1.46	5.552 ± 1.616	26.040 ± 2.160	4.69
M1: p.C808R	3.06 ± 1.73	9.670 ± 4.198	8.905 ± 1.203	0.92
M2: p.G387R	2.42 ± 1.14	7.026 ± 2.196	13.840 ± 1.309	1.97
M3: p.P499L	4.60 ± 0.01	7.396 ± 0.909	16.090 ± 0.629	2.18

cDNA that encode human TPO were subcloned into the EcoRI site of the baculovirus transfer vector pAcGP67-B (BD Biosciences, San Diego, CA, USA). For this purpose the transfer vector was digested with EcoRI, gel purified using GFX PCR DNA Purification Kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and treated with alkaline phosphatase (Fermentas, ThermoScientific, Waltham, MA, USA) to prevent self-ligation according to standard molecular cloning protocol. Both, vector and insert, were ligated with T4 DNA ligase enzyme and transformation of competent *E. coli* TG1 bacterial strain was subsequently performed. A variable number of colonies were obtained for each case and plasmidic DNA was purified with Wizard Plus SV minipreps columns. The plasmid characterization was first done for insert size by digestion with EcoRI and then confirmed by sequencing. As it shows Fig. 1, four different transfer vectors were constructed (pAcGP67-B-hTPOWT, pAcGP67-B-hTPOM1, pAcGP67-B-hTPOM2 and pAcGP67-B-hTPOM3).

2.2. Cell culture, infection and recombinant baculoviruses obtaining

A monolayer of one million Spodoptera frugiperda (Sf9) insect cells (Invitrogen) grown at 27 °C in Sf900 II medium (Invitrogen) supplemented with 1% (v/v) fetal bovine serum (FBS) (Internegocios S.A., Mercedes, Buenos Aires, Argentina), and 1% (v/V) antibiotic–antimycotic 100× solution (Invitrogen) were co-transfected with 1 μg of each construction (pAcGP67-B-hTPOWT, pAcGP67-B-hTPOM1, pAcGP67-B-hTPOM2, pAcGP67-B-hTPOM3) and 250 ng linearized Baculogold Bright™ AcMNPV DNA containing the green fluorescent protein (GFP) coding sequence (BD Biosciences) in the presence of Cellfectin (Invitrogen). After 4 hours of incubation period, the co-transfection supernatant was removed and replaced by 2 ml of culture medium supplemented with 1% FBS and 1% antibiotic–antimycotic solution (Invitrogen). The cultures were incubated for 96 hours at 27 °C and grown in darkness. After incubation period, culture supernatants were collected and clarified by centrifugation at 8000 rpm.

Co-transfection efficiency was determined by measuring GFP expression by fluorescence under UV light. The co-transfection supernatant was used to infect Sf9 monolayer cultures at a multiplicity of infection (MOI) of 0.02 (first amplification). After 4 days of incubation at 27 °C, the cell culture supernatant was collected and three rounds of virus amplification were assessed. The virus titer was determined by a plaque assay (Kendler et al., 1993).

2.3. Protein expression

Sf9 cell suspension cultures were grown in sterile Erlenmeyer flasks under continuous shaking at 100 rpm in Sf900 II medium supplemented with 1% (v/v) FBS and 1% antibiotic–antimycotic solution (Invitrogen) at 27 °C. Sf9 independent suspension cultures in log-phase at a cell density of 1.5×10^6 cells were infected at MOI 2 with recombinant tittered viruses: AcMNPV-hTPOWT, AcMNPV-hTPOM1, AcMNPV-hTPOM2 and AcMNPV-hTPOM3, in order to express hTPOWT, hTPOM1, hTPOM2 and hTPOM3 respectively. The cultures had a 100 ml final volume and were supplemented with 7.5 μM of hemin (Targovnik et al., 2010).

In addition two negative controls of hTPO expression were assessed: one uninfected Sf9 cell culture (C-naive) and other infected with recombinant baculovirus without hTPO cDNA insertion (C-infection). Infected cultures were incubated in an orbital shaker at 100 rpm at 27 °C for 6 days. All assays were performed in triplicate.

2.4. Membrane isolation

Infected cultures were harvested at 6 days post-infection and the cells were centrifuged at 2500 *g* for 5 min in bench top centrifuge (Rolco SRL) in falcon tubes. Culture supernatants were discarded and the cell pellets were resuspended in 6 ml of phosphate saline buffer (PBS: pH = 7.4). Cells were centrifuged at 2500 × *g* for 5 min. The supernatants were discarded and pellets were resuspended in 2 ml of buffer RIPPA (150 mM NaCl, 1% Triton 100-X, 0.5% sodium

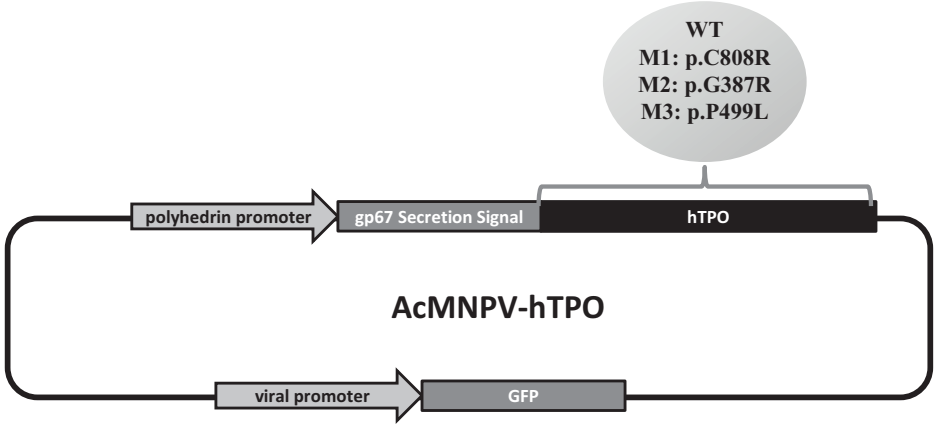

Fig. 1. Recombinant baculovirus polyhedrin-minus (AcMNPVTPOWT, AcMNPVTPOM1, AcMNPVTPOM2, AcMNPVTPOM3). The hTPO cDNAs were fused in-frame to the viral signal peptide GP67. hTPO expression is under control of the polyhedrin promoter.

deoxicolate, 0.1% SDS, 50 mM Tris–HCl pH = 8.0) and 20 μl of protease inhibitor cocktail was added (Sigma Aldrich, Saint Louis, MO, USA). Samples were incubated for 15 min on ice and then sonicated for 30 s at 25 W (Cole-Parmer Instrument Co). The sonicated extracts were incubated on ice for additional 15 min and then transferred to 2 ml Eppendorf tubes. Low speed centrifugation was performed for 5 min at 1000 *g* (4 °C). An aliquot of 50 μl of supernatant (SN1) was reserved while the remaining was collected and transferred to a new tube. The pellet (P1) was re-suspended in 300 μl of PBS to be then assessed by SDS–PAGE. The SN1 was centrifuged at 21,000 × *g* for 15 min (4 °C) and the resulting pellet (P2: enriched membrane fraction) was re-suspended in 300 μl of store solution (50 mM Tris–HCl pH = 7.5, 0.5% SDS) to which 3 μl of protease inhibitor (Sigma Aldrich) were added. The supernatant (SN2) was kept to be then assessed by SDS–PAGE.

2.5. Total protein measurement

Protein concentration was measured using the Bradford assay (Bradford, 1976) of protein estimation with bovine serum albumin as standard.

2.6. SDS–PAGE and western blot

Ten micrograms of protein purification fraction (SN1, P1, SN2, P2) from each harvested culture (hTPOWT, hTPOM1, hTPOM2, hTPOM3)/line were separated by SDS–PAGE under reducing conditions on 10% polyacrylamide gels. A prestained SDS–PAGE MW marker was used (PageRuler, ThermoScientific). One of the gels was subjected to staining with Coomassie Blue for protein detection. The other was used for Western blot. For this purpose the gel was equilibrated for 20 min with the transfer buffer (25 mM Tris–HCl pH 8.8, 190 mM glycine, 20% methanol) and then transferred to a nitrocellulose membrane. After transfer, the membrane was incubated overnight at 4 °C in blocking solution (PBS, 3% milk). The membrane was washed with wash buffer (PBS–0.05% Tween 20) and incubated with a 1/300 dilution of anti-hTPO polyclonal serum (obtained from a sera pool of patients with thyroiditis), in buffer dilution (PBS, 0.05% Tween 20, 1% milk). Then, it was incubated for 2 h at room temperature with constant stirring. Finalized this time, the antibody solution was recovered and the membrane washed 3 times with wash solution for 10 min each time. The membrane was then incubated with a 1/2000 dilution of the second monoclonal anti-human IgG antibody (Sigma Aldrich) in buffer dilution for 1 h at room temperature with constant stirring. Finalized this time the antibody solution was recovered and the membrane was washed 3 times with wash solution for 10 min each time. The developing solution was prepared by dissolving 15 mg of diaminobenzidine (DAB) (Sigma Aldrich) in 7 ml distilled H_2O and 10 μl of 30% H_2O_2. The preparation was placed on the membrane to banding. The reaction was stopped by rinsing with distilled H_2O.

2.7. Enzyme activity determination

For determination of the enzymatic activity of hTPO, guaiacol as a chromogenic substrate for oxidation reaction was used (Hosoya and Nobuo, 1961). The protocol was adapted according to the system needs, to which 15 μl of enzyme preparation were diluted in 800 μl of 100 mM phosphate buffer, pH 7.4 containing different concentrations of guaiacol (between 0 and 50 mM). The oxidation reaction was initiated with the addition of 100 μl of 892 mM H_2O_2 and the absorbance was monitored spectrophotometrically at 470 nm for 60 s. In all experimental conditions the reaction velocity was estimated from the slope of the initial-linear part (dA/dt) of the curves obtained and the volumetric activity of the preparations was calculated using the extinction coefficient of the reaction product, the tetra-guaiacol (25.6 $cm^{-1}\cdot\mu mol^{-1}$) and reaction and sample volumes used (Tjissen, 1985). The volumetric activity was expressed in arbitrary units/ml (U/ml), which represents the activity present in 1 ml of sample capable of converting a substrate mol in 1 min. As to compare the enzyme activity of the different preparations tested, the hTPO concentrations were estimated from scanning stained SDS–PAGE gel and image analysis. We used the ImageJ software (http://imagej.nih.gov/ij/features.html) which allows the quantification of image color intensities, generating a graph of the lane peaks and calculating the area corresponding to each peak. From these data the percentage of area corresponding to the band of 106 kDa (size compatible with recombinant hTPO) out of the total area of proteins was calculated. Knowing the value of total protein concentration measured by the Bradford technique and hTPO percentage in the fraction, it was possible to estimate hTPO concentration.

2.8. Kinetic hTPO characterization

hTPO specific activity was estimated for each hTPOWT, hTPOM1, hTPOM2, hTPOM3 and negative controls purified from membrane fraction at different guaiacol concentrations (50, 35, 25, 15, 10, 6, 3.5, 2, 1 and 0 mM). The values of apparent Km and Vmax were determined using the Gauss–Newton algorithm, adjusting Specific Activity *f* [guaiacol] to a hyperbola applying a nonlinear regression method.

3. Results

By sequential cloning strategy into the pGEMT vector, it was possible to obtain the complete hTPO cDNA which was used for subsequent mutagenesis using the Quick Change System, obtaining the pGEMT-hTPOM1, pGEMT-hTPOM2 and pGEMT-hTPOM3 constructs. The WT and mutant cDNAs obtained were sub-cloned into the transfer vector pAcGP67-B. In order to assess the presence of the inserts we proceeded to the digestion of clones with EcoRI enzyme and sequencing (Fig. 2A). Recombinant plasmids were subjected to co-transfection with linearized commercially available baculovirus in independent cell cultures of the Sf9 cell line, where recombinant baculoviruses were generated. Suspension cultures of 100 ml final volume with a cell concentration of 1.5×10^6cells/ml were infected with the recombinant titrated viruses AcGP67-B-hTPOWT, AcGP67-B-hTPOM1, AcGP67-B-hTPOM2 or AcGP67-B-TPOM3. On day 6, the cultures were harvested and membrane fractions (enriched in recombinant hTPO) were purified. As shown in Fig. 2B–D, Western blot and SDS–PAGE were assessed to evaluate hTPO presence (105 kDa) in each membrane fraction obtained. So, P2, was the most enriched fraction in hTPO as expected (Fig. 2). All infected cultures expressed recombinant hTPO (Fig. 2C). It was also possible to prove that the uninfected cell cultures did not show protein expression in the 105 kDa molecular weight range (naive control), and that virus infection did not up regulate the expression of proteins with hTPO size nor peroxidase activity (infection control) (Fig. 2D). The former results were demonstrated both by Western blot and SDS–PAGE assays and by enzyme activity determination. These data confirmed that the 105 kDa bands correspond to recombinant hTPO, allowing the calculation of its percentage in each fraction using scanning densitometry of the SDS–PAGE gels with the ImageJ program. Considering the value obtained from $mg\cdot ml^{-1}$ of total proteins for each fraction using the Bradford technique and the corresponding hTPO calculated percentage, it was possible to estimate hTPO concentration of each membrane fraction.

Spectrophotometrical monitoring of guaiacol oxidation reaction was conducted from enriched hTPO fractions (WT and mutants), obtaining the tetraguaiacol formation rate (dAbs/dtpo). Such monitoring was performed at different guaiacol concentrations, which allowed to obtain volumetric activity data for each substrate

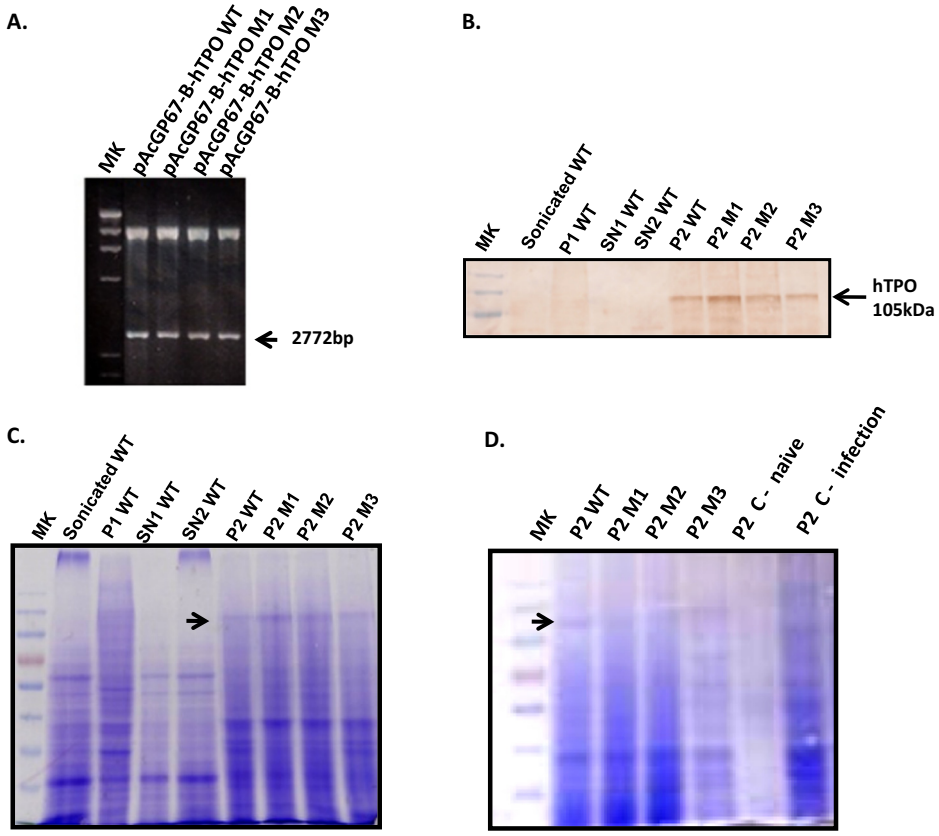

Fig. 2. (A) EcoRI restriction assay. Evaluation of the presence of the insert in pAcGP67-B transference vector (1% agarose gel). (B) Western blot assay of wild-type (WT) and M1 (p.C808R), M2 (p.G387R) and M3 (p.P499L) mutants. Evaluation of the presence of hTPO in different sub-cellular fractions resulting from cell lysates purification process. (C and D) SDS–PAGE assay. hTPO presence in different sub-cellular fractions resulting from cell lysates purification process. (10% denaturing polyacrylamide gels). SN: supernatant, P: pellet, MK: Marker (PageRuler, ThermoScientific).

concentration according to Tijssen equation (Tjissen, 1985). Considering the estimated mass of hTPO for each P2 fraction, specific activity was calculated. Fig. 3A shows the specific activity of each TPO (WT and mutants) at 2 mM of guaiacol, demonstrating significant enzyme activity reduction in all three mutants assayed (Student T test, $P < 0.05$). Fig. 3B shows the dependency of the specific activity of each TPO (WT and mutants) with the concentration of guaiacol. By non-linear regression, a rectangular hyperbola (as Michaelis–Menten equation) was fitted to each curve. The best fitting values of Km and Vmax or each recombinant enzyme are shown in Table 2. In all three cases, mutants showed higher KM values with respect to the WT therefore lower apparent affinity for the substrate, as well

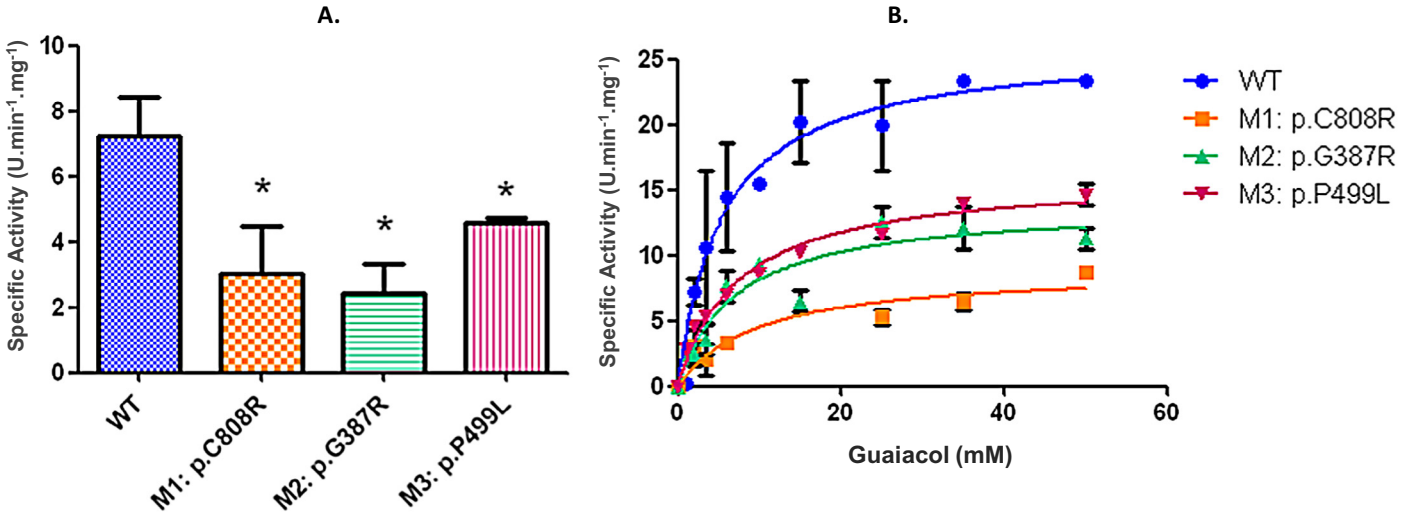

Fig. 3. Enzymatic activity evaluation. (A) Specific activity of each TPO (WT and mutants) at 2 mM of guaiacol. (B) hTPO kinetic curves. Dependence of specific activity of WT, M1 (p.C808R), M2 (p.G387R) and M3 (p.P499L) with the concentration of guaiacol in the reaction media. The continuous lines are plots of Michaelis–Menten equation using the best fitting values of Km and Vmax showed in Table 2. *$P < 0.05$ compared with control using the Student T test.

as lower catalytic efficiency (Vmax/KM) with respect to WT-hTPO. Likewise hyperbolas obtained were comparatively evaluated, showing that the fitted curves for each hTPO (WT and mutants) were significantly different from each other (Fisher's test, $P < 0.0001$).

4. Discussion

Since TPO plays a leading role in thyroid hormone biosynthesis and consequently in the molecular pathophysiology of IOD, the potential deleterious impact of three point substitutions previously described in our laboratory responsible for IOD was evaluated (M1: p.C808R, M2: p.G387R and M3: p.P499L) (Rivolta et al., 2003, 2007). For this purpose we decided to optimize and use the Baculovirus System as a model of enzyme expression of hTPO to evaluate its performance characteristics in terms of activity and kinetic constants associated. Given its vital importance, the hTPO has had a key role in thyroid function studies, therefore different researchers have proposed to express it in different systems for decades, being Baculovirus System one of the alternative choices. In 1993, Kendler and colleagues were able to obtain recombinant hTPO in the Baculovirus System in order to determine its antigenic potential for further use in diagnostic tests for autoantibodies in human thyroiditis (Kendler et al., 1993). A second study, published in 1996 by Ji-Lao-Fan and colleagues, evaluated not only hTPO antigenic potential but also introduced the partial structural evaluation of the protein and its correlation with biological activity from purified recombinant TPO expressed in the Baculovirus System (Fan et al., 1996). In 1999 Hendry and colleagues were able to obtain the first crystals of recombinant hTPO from baculovirus expression system, with the goal of solving the three-dimensional structure. While the results were promising, Hendry concluded that achieving larger crystals to promote proper X-ray diffraction was required (Hendry et al., 1999). At the same time, Andrej Gardas and collaborators described a high yield purification of full-length recombinant hTPO from baculovirus insect cells and compared it to native hTPO purified from human thyroid glands. In contrast to native specimen, recombinant hTPO was enzymatically inactive and resistant to solubilization with detergents, but both reacted with sera from patients with autoimmune thyroid disease. Accordingly, the conformational change of recombinant hTPO correlates with the difficulties associated with its purification strategy (Gardas et al., 1999).

In the present work it was possible to optimize the expression and purification of active recombinant hTPO both wt and mutants, achieving an average yield of protein of about 100 mg/ml. This satisfactory performance enabled not only the Western blotting and SDS–PAGE characterization of each of the protein fractions obtained, but also the determination of the dependence of the specific activity with guaiacol concentration, which facilitated its kinetic characterization not described so far in literature. The high performance is associated with the ability of insect cells to grow in suspension, optimizing the scale process of recombinant protein production. Furthermore, functional evaluation of the recombinant hTPOs showed significant differences in their specific activity, demonstrating decreased activity in the three mutants with respect to WT. Significantly lower activity was recorded for M1 (p.C808R), whose replacement is located in the EGF homology domain of hTPO, unlike M2 (p.G387R) and M3 (p.P499L) located in the catalytic site in the vicinity of two heme-binding sites (E399 and H494, respectively). This not only indicates that the chosen system is able to solve intermediate activity situations, but also that a point mutation outside the catalytic site of the enzyme may have a more deleterious effect than expected. This could be explained by the protein structure potential impact of cysteine to arginine substitution at codon 808. In EGF–Ca^{2+} binding domain of hTPO are located 6 cystein residues responsible for the formation of 3 disulfide bridges essential for protein structure: C800–C814, C808–C823 and C825–C838. Disruption of the C808–C823 disulfide bond, as a result of p.C808R mutation, would generate significant conformational changes which could on the one hand partially retain the hTPO on the endoplasmic reticulum, or it might be located within the membrane with conformational defects responsible for a lower net activity of the enzyme (Henderson et al., 1996; Rivolta et al., 2003). Likewise, the enzyme kinetic analyses allowed to provide more information about the affinity of the enzyme for its substrate (KM), as well as reaction efficiency (Vmax/KM) which gave valuable information about the intrinsic characteristics of the studied proteins (hTPO wt and mutants). In all three cases, the mutants showed higher KM values with respect to the WT therefore lower apparent affinity for the substrate, as well as lower Vmax/KM rate of the mutants with respect to WT indicating lower reaction efficiencies. In particular M1 presented the highest KM value and lowest Vmax/Km value, coinciding with the previously hypothesized conformational changes associated to p.C808R mutation, which could explain its lower affinity for substrate and a lower rate of product production per time unit.

However, the strategy used as a model only observes one aspect of the functionality of the hTPO. This poses as a perspective of the purification technique optimization of membrane fractions obtained, so to have pure samples of recombinant hTPO for possible biophysical structural tests such as circular dichroism and X-ray crystallography. This information would complement the data obtained from enzymatic activity, as it could provide structural impact of different sequence variants on the three-dimensional conformation of the protein. Likewise, there are adjustments of the Baculovirus System to allow the co-expression of proteins, which promise to determinate the hTPO activity in the context of DUOX2, closer to the real situation of thyroid follicle.

Obtaining hTPO cDNA achieved by the sequential cloning strategy in the pGEMT vector allowed the functional study of hTPO mutant alleles not previously characterized. Additionally, the expression, purification and kinetic characterization of recombinant hTPO were optimized using the Baculovirus System. We define here non-described kinetic constants of the protein, as well as also comparatively assess the impact of deleterious mutations not only in terms of the activity but also its intrinsic biochemical properties (Km and Vmax).

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.mce.2014.12.026.

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