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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.
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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).

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