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MOLECULAR CLONING, EXPRESSION, AND PURIFICATION OF RECOMBINANT HUMAN PPARγ IN *Escherichia coli*

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Abstract

In the presence of an increasing prevalence of obesity, diabetes, and cardiovascular diseases, the nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ) has emerged as a transcriptional regulator of metabolism whose activity can be modulated by direct binding of small molecules. PPARγ ligands are still clinically effective antidiabetic drugs, although side effects limit their use. Therefore, to investigate thoroughly the molecular interactions and the relationships between structure, function, and pharmacological properties, it would be desirable the use of full-length PPAR gamma protein. Nonetheless, considerable quantities of active protein are not able to be obtained from the original sources. In this study, a new strategy for human PPAR gamma (hPPARγ) expression in bacteria was developed using a fusion protein (CusF3H+) for purification with Immobilized Metal Affinity Chromatography (IMAC). hPPARγ was mainly expressed in soluble fraction at a low temperature (25ºC) and 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Protein identity was successfully confirmed by Western blot assay. Our results indicated the viability of using this approach to produce hPPARγ into *E. coli* Rosetta (DE3), which could be a useful methodology for the study of this protein in *in vitro* assays.

Keywords: Escherichia coli, molecular cloning, peroxisome proliferator - activated receptor- γ (PPAR-γ), protein expression, purification

Clonación molecular, expresión y purificación de la proteína recombinante PPARγ humana en *Escherichia coli*

Resumen

Ante el incremento en la prevalencia de diabetes, obesidad y enfermedades cardiovasculares, el receptor gamma activado por proliferador de peroxisomas (PPARγ) ha emergido como un regulador transcripcional clave en el metabolismo cuya actividad puede ser modulada por unión directa con ligandos. Los ligandos de PPARγ siguen siendo fármacos antidiabéticos clínicamente efectivos, pero los efectos secundarios limitan su uso. Por lo tanto, para continuar investigando las interacciones moleculares y la relación entre la estructura, función y propiedades

farmacológicas, sería deseable el uso de la proteína PPARγ completo. Sin embargo, no es posible obtener cantidades considerables de la proteína activa a partir de la fuente original. Por ello, se desarrolló una nueva estrategia para la expresión de PPARγ humano (hPPARγ) en bacterias, empleando una proteína de fusión (CusF3H+) para la purificación por medio de cromatografía de afinidad con iones metálicos inmovilizados (IMAC). hPPARγ se expresó principalmente en la fracción soluble, a temperatura baja (25ºC) y con una concentración del inductor isopropil-β-D-tiogalactopiranósido (IPTG) de 0.1 mM. La identidad de la proteína fue confirmada satisfactoriamente por medio de Western blot. Estos resultados indican la viabilidad de usar esta estrategia para la producción efectiva de hPPARγ en *Escherichia coli* Rosetta (DE3), pudiendo ser una estrategia útil para el estudio de la proteína en ensayos *in vitro.*

Palabras clave: Escherichia coli, clonación molecular, receptor gamma activado por proliferador de peroxisomas (PPAR-γ), expresión proteica, purificación

Introduction

Regulation of the glucose and lipid metabolism and even inflammatory stimuli and differentiation of adipose tissue are important properties of the peroxisome proliferator-activated receptor (PPAR) gamma. This protein belongs to the nuclear hormone receptor superfamily of ligand-activated transcription factors including PPAR α, β/ δ, and γ, which cooperatively are responsible for the regulation of diverse metabolism pathways (*Desvergne & Wahli*, 1999). PPARγ is one of the main and well-known members of the family due to their close relationship as a pharmacological target in diseases like metabolic syndrome, type 2 diabetes, obesity, inflammation, and cancer (*Lehrke & Lazar*, 2005; *Kersten et al.,* 2000; *Michalik et al.*, 2004).

To modulate the transcriptional activity of the target genes, PPARγ requires the assembly with retinoid receptors (RXRs) as heterodimer in response to different synthetic or natural ligands (*Forman et al., 1995; Lehmann et al*., 1995; *Itoh et al.*, 2008). Among the natural ligands of PPARγ are fatty acids and derivatives of eicosanoids, while synthetic ligands include thiazolidinediones, which are used as antidiabetic drugs that improve insulin sensitization. Nevertheless, relevant adverse effects have been reported, so it is important to keep studying the precise molecular interaction of the receptor with their ligands. PPARγ still remains an attractive target for the development of drugs against diabetes, obesity, and even cancer and inflammatory diseases due to its great plasticity of functions, since it is a nuclear regulator of gene transcription and because it is associated with the metabolism of lipids, carbohydrates, and other molecules. It is relevant to produce the molecule in large quantities for its study in both *in vitro* and *in vivo* models.

For this purpose, it would be necessary to obtain a large amount of protein through a simple and accessible method. To investigate the molecular

interactions and the relationship between structure and function, it would be adequate to use full-length human PPARγ. However, considerable quantities of active protein are not able to be obtained from the original sources. Therefore, it is essential to develop new methods for full-length PPARγ production. In this study, human PPARγ (*hPPARγ*) was cloned in a pET-30a(+)/CusF3H+ plasmid and expressed in *Escherichia coli* Rosetta (DE3) at low temperature. hPPARγ protein was obtained from soluble fractions and purified through Immobilized Metal Affinity Chromatography (IMAC). Protein identity was confirmed by western blot analysis.

Materials and Methods

Materials

The Human PPAR gamma (*hPPARγ*) gene was synthesized by General Biosystem (USA). The *hPPARγ* gene was cloned into pET-30a(+)/CusF3H+ vector (generated by X. Zarate-Kalfópulos). The *Escherichia coli* Rosetta (DE3), protein molecular weight marker, tryptone, yeast extract, DNAse and restriction enzymes were purchased from Promega (USA). The agarose, acrylamide and other chemicals were obtained from Sigma Aldrich (USA). Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from AG Scientific Inc (USA). The water used in the experiments was ultrapure (18.2 MΩ) from Simplicity UV System, Merck Millipore (USA). Polyclonal rabbit anti-PPARγ antibody and goat anti-rabbit IgG secondary antibody were purchased from Sigma Aldrich (USA).

Methods

DNA constructs

The *hPPARγ* gene (NM_138712.3, 1434 bp) was obtained from General Biosystem (USA). hPPARγ gene was amplified by PCR with Platinum SuperFi DNA Polymerase, (ThermoFisher Scientific, USA) and the forward and reverse primers were 5´-CGTCCATGGCTATGACAATGGTTG-3´ and 5´-GTCCTCGAGTCTAGTACAAGTCCTTG-3´, respectively. The hPPARγ sequence was inserted into the pET-30a(+)/CusF3H+ vector [8] using the sites *NcoI* and *XhoI* of the multiple cloning site (MCS).

hPPARγ expression

Escherichia coli Rosetta (DE3) pLysS competent cells were transformed with the resulting pET-30a(+)/CusF3H+/hPPARγ and were allowed to grown in Luria Broth (LB) at 37°C to OD_{600nm} of 0.6. Afterward, 0.1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) was used overnight at 25°C and 220 rpm to induce protein expression. Two mL cells culture were harvested by centrifugation (24,400 x*g* for 15 min at 4°C). The cell pellet was dissolved with a binding buffer (50 mM Tris-HCl, 500 mM NaCl, pH 8.0) and lysed with 0.1 mm glass beads (Cat. No. 11079101, Biospec Products, USA). Then, it was centrifuged (48,400 x*g* for 20 min at 4°C) and protease inhibitors cocktail was added (cOMplete Tablets Protease Inhibitor Cocktail, Mini EASYpack, Roche, Germany). The supernatant and insoluble fraction (dissolved with 100 µL of 8M urea) were analyzed by SDS-PAGE.

Protein purification

One liter of bacterial culture medium was collected by centrifugation at 24,400 *xg* for 15 min at 4°C. Cell pellet was dissolved in 50 mL of binding buffer (50 mM Tris-HCl, 500 mM NaCl, pH 8.0) and lysed using 0.1 mm glass beads in a bead-beater (Biospec Products, USA). The lysate was centrifuged at 48,400 x*g* for 20 min and at 4°C to obtain clarification, to which a cocktail of protease inhibitors (Roche, Germany) was added. Purification was carried out by Immobilized Metal Affinity Chromatography (IMAC) using the Äkta Primer Plus FPLC system (GE Healthcare, USA). The clarification was passed through a one mL HisTrap Fast Flow column (GE Healthcare, USA) previously equilibrated with five column volumes (CV) of binding buffer. Subsequently, the column was washed with five CV of the wash buffer (50 mM Tris-HCl, 500 mM NaCl, 10 mM Imidazole, pH 8.0) until obtaining an absorbance value close to zero. Elution was carried out through a 20% to 100% imidazole gradient with an elution buffer (50 mM Tris-HCl, 500 mM NaCl, 250 mM Imidazole, pH 8.0). Fractions with increased absorbance were analyzed by SDS-PAGE 10% to determine their purity. Moreover, the fractions containing the CusF3H + / hPPARγ protein were dialyzed against a buffer (50 mM Tris-HCl, pH 7.4) to remove excess of imidazole. Finally, samples were concentrated using an Amicon Ultra centrifugal filter (MWCO 30 kDa, Merck-Millipore, USA).

CusF3H+ fusion protein removal

CusF3H+ is a metal-binding protein formed by the addition of three histidine residues at the N-terminus. These residues can bind Ni (II) ions allowing improved purity after affinity chromatography (*Vargas-Cortez et al.,* 2017). The CusF3H+ fusion protein present in the N-terminal region was removed from the hPPARγ protein by using Enterokinase Max ™ (Invitrogen, USA). 0.1 units of enzyme were used in an EkMax buffer (500 mM Tris-HCl pH 8.0, 10 mM CaCl2, 1% Tween 20) for 16 h at 25°C. The digestion product was removed by incubating the reaction with 200 µL of High Affinity Ni-Charged Resin (GenScript, USA) and analyzed by 10% SDS-PAGE to determine hPPARγ purity. The obtained samples were dialyzed and concentrated through a buffer exchange (50 mM Tris pH 7.4, 100 mM KCl, 10% Glycerol) using an Amicon Ultra centrifugal filter (MWCO 30 kDa, Merck-Millipore, USA). The hPPARγ concentration was determined according to the method described by Bradford (*Bradford*, 1976), with bovine serum albumin as standard.

Western blot assay

The hPPARγ protein and total lysate of *Escherichia coli* Rosetta (DE3) pLysS (negative control) were transferred from a 10% SDS-PAGE gel to a 0.22 µm nitrocellulose membrane (Sigma Aldrich, USA) by means of a western blot transfer. Transfer sandwich was immersed in a cold buffer (39 mM Glycine, 48 mM Tris-base and 20% Methanol) and transference was

carried out for 90 min and 100 volts. Membrane was stained with a solution of Ponceau Red S (Sigma Aldrich, USA) to corroborate the transfer and the staining was reversed by washing with distilled water. Subsequently, the membrane was incubated with a blocking solution (1X Tris-Buffered Saline (TBS), 0.1% Tween 20 and 5% Fat-free milk) for 1 h and 30 rpm.

The polyclonal rabbit anti-PPARγ antibody (1:1,000 in TBS-T buffer, 1% fat-free milk and 0.02% sodium azide) was added and incubated for 1 h at 25°C and 30 rpm. Three washes were made with TBS-T buffer and after that, goat anti-rabbit IgG secondary antibody conjugated with alkaline phosphatase (1:10,000) was added and incubated for 1 h at 25ºC and 30 rpm. Finally, the membrane was washed with TBS-T buffer for 5 times and the substrate BCIP / NBT (Boehringer Mannheim, Germany) was added until a brown precipitate was developed. The reaction was stopped by washing with distilled water.

Results

Protein expression analysis

The *hPPARγ* sequence (1434 bp) was obtained from the NCBI database and cloned in pET-30a(+)/CusF3H+. A schematic representation of the obtained vector is shown in Figure 1. CusF3H+ acts as a fusion protein and CusF3H + / hPPARγ was obtained mainly as a soluble protein with an estimated molecular weight of 64 kDa (Figure 2). Induction conditions were maintained at 0.1 mM IPTG, 25 ° C and 220 rpm overnight (~ 16 hours). In addition, insoluble fractions were also analyzed by SDS-PAGE, and only a small proportion of total protein was present in that fraction (Figure 2, lanes 7-9).

hPPARγ purification and removal of the CusF3H+ fusion protein

The CusF3H + fusion protein was allowed to purify hPPARγ through nickel metal ion affinity chromatography. Twenty-five elution fractions were obtained in an imidazole gradient and the highest protein elution was obtained at a concentration of 150 mM imidazole. These fractions were visualized through 10% SDS-PAGE (Figure 3). CusF3H + fusion protein binds with high affinity to the chromatographic column and is not showed to come off the column in the washing process (Figure 4).

Western Blot Analysis

The hPPARɣ protein identification was corroborated by western blot, showing a positive signal on the nitrocellulose membrane at a weight close to 50 kDa compared to the pre-stained molecular weight marker. Antibodies did not show interaction against the native proteins of the expression system (Figure 5).

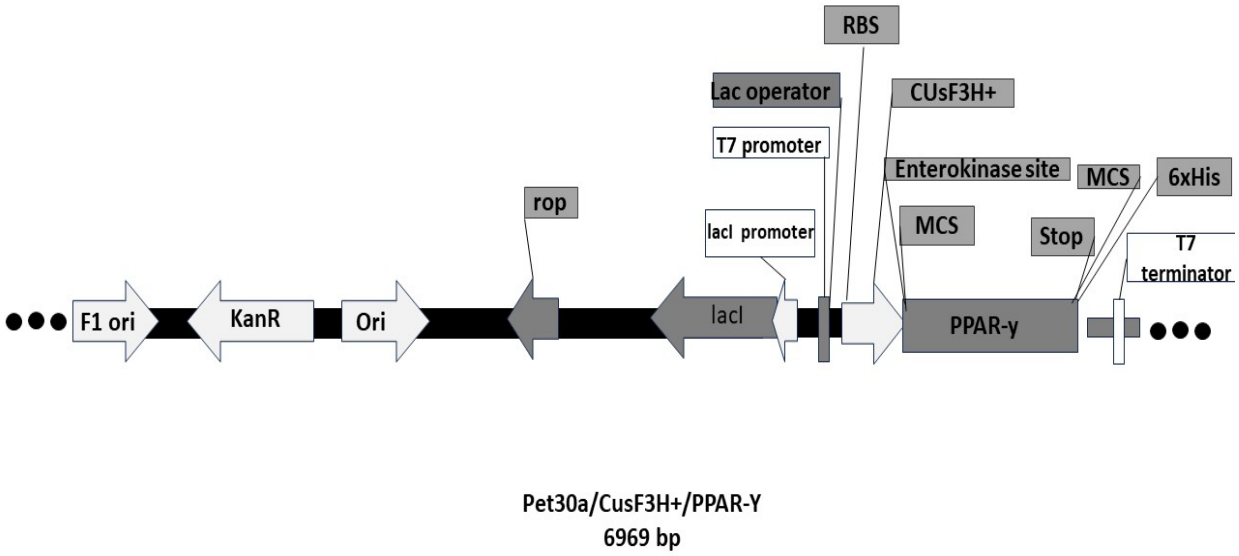

Fig.1 Schematic structure of the pET30(a+)/CusF3H/PPARγ vector (6963 bp). PPARγ was inserted into the pET30(a+)/CusF3H+ vector through *NcoI* at the 5' end and *XhoI* at the 3' end.

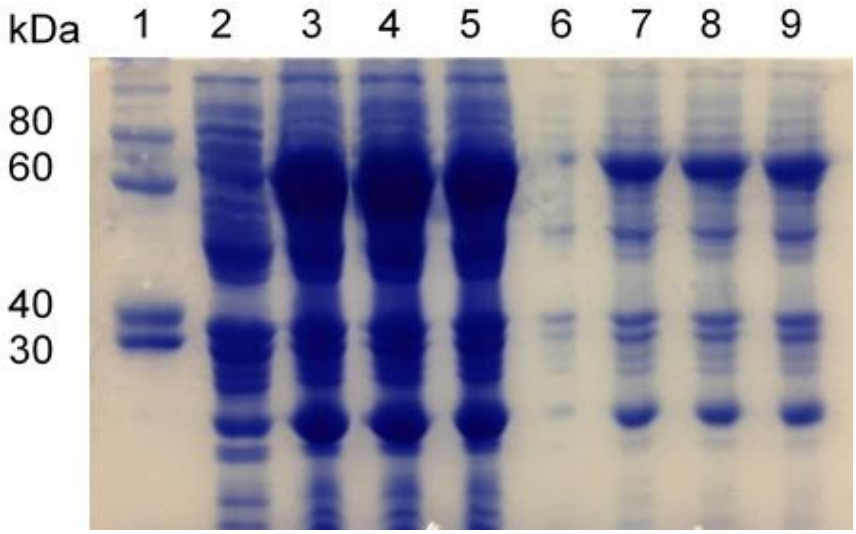

Fig.2 CusF3H+/PPARγ (64 kDa) analysis expression in 10% SDS-PAGE. Lane 1: Protein MW marker, lane 2: non-induced bacterial culture (soluble fraction), lane 3-5: CusF3H+/PPARγ protein expression (soluble fraction), lane 6: non-induced bacterial culture (insoluble fraction), lane 7-9: CusF3H+/ PPARγ protein expression (insoluble fraction)

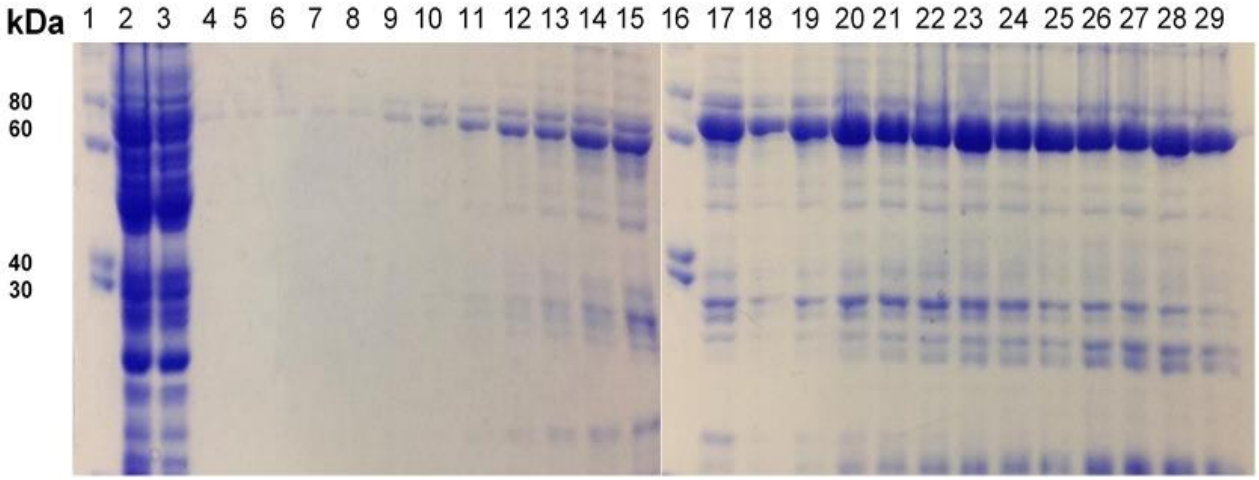

Fig. 3 Elution fractions analysis in SDS-PAGE. Lane 1 and 16: molecular weight marker, lane 2: non-binding fraction, lane 3: wash fraction and lanes 4-29: elution fractions in imidazole gradient

Subsequently, digestion with 0.1 U of enterokinase allowed hPPARγ protein showing a weight of 54 kDa (Figure 4). Purified protein concentration was 1.8 mg / mL according to the method described by Bradford, with an r^2 of 0.99 for the bovine serum albumin standard.

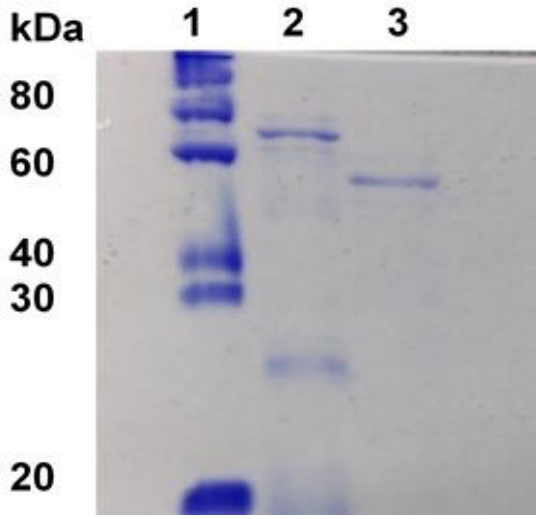

Fig. 4 Analysis of enterokinase digestion in SDS-PAGE. Lane 1: Protein MW marker, lane 2: pooled elution fractions, lane 3: hPPARγ (54 kDa) after removal of CusF3H+ fusion protein (~10 kDa)

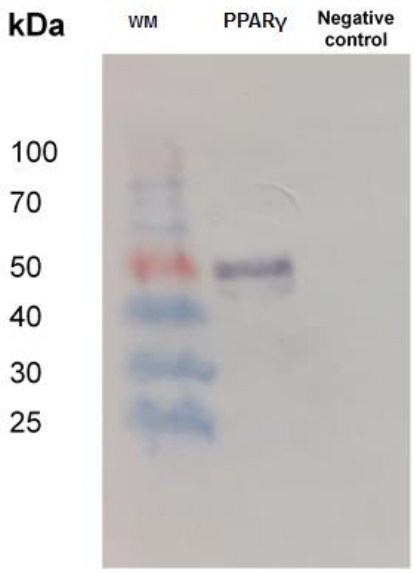

Fig. 5 Western blot analysis. Lane 1: prestained molecular weight marker. Lane 2: hPPARγ protein (54 kDa). Lane 3: complete protein fraction of *Escherichia coli* Rosetta (DE3) pLysS, negative control.

Discussion

In the present work, hPPARγ protein was successfully expressed in *Escherichia coli* Rosetta (DE3) at low temperature and purified from soluble fraction through IMAC. Related to cloning, several usage codons were found that were different to those used in bacteria, and for this reason, an improvement in the sequence of the gene was performed. Despite the fact that it is well known that *Escherichia coli* Rosetta (DE3) is capable of using uncommon codons for bacteria, the assurance of a successful expression in making the change of codons in the gene sequence was preferred. We performed several variations in the classical parameters affecting the expression of recombinant proteins, like temperature and time of incubation. We also tried other parameters; however, none of them substantially modified the expression of heterologous protein. According to other reports, inclusion bodies can be formed when higher temperatures are used for expression conditions (*Sanchez de Groot & Ventura*, 2006; *Hunke & Betton*, 2003). The use of low temperatures has been reported to ease proper folding of the expressed proteins and to diminish the formation of inclusion bodies. For this reason, we decided to evaluate the expression at 25 ºC, finding a better expression at this temperature rather than at 32-37ºC (data not shown).

Literature reports have shown that expression of PPARγ in prokaryotic systems like *Escherichia coli* strains could demand a change of culture media, using Terrific broth (TB) instead of LB (*Li et al*., 2010). In spite of the fact that they reported a better expression with TB instead of LB, we do not find significant differences between these two media (data not shown). The main

difference between LB and TB is the high concentration of phosphate salts that the latter has, and according to the authors, TB is a suitable media to avoid the formation of inclusion bodies (*Moore et al.*, 1993).

There are few reports about full-length hPPARγ expression in bacteria, and protein expression was often described to take place mainly in inclusion bodies. Previously, GST- hPPARγ fusion protein generation was reported in *Escherichia coli* BL21. Induced overexpression was achieved through the addition of 0.2 mM IPTG at room temperature for 2-5 hours, and fusion proteins were purified from the cell pellet using glutathione-Sepharose beads (*Berger et al*., 1999). Furthermore, *Nagai* et al. 2011 reported hPPARγ expression as a fusion protein with GST in *Escherichia coli* and was purified using a GSTrap HP column. Temperature and IPTG concentration were not described (*Nagai et al.*, 2011).

Li *et al.* 2010 reported that hPPARγ was expressed as inclusion bodies in *Escherichia coli* BL21. Cells were allowed to grow at 37ºC to OD_{600nm} and induced with 0.8 mM IPTG. They obtained 176 mg of PPAR-gamma of at over 99% purity per liter of TB medium [*Li et al.,* 2010]. Moreover, Kong *et al.* 2006 studied PPAR gamma LBD as a fusion to the N-terminus of the g3p protein of filamentous phage of the phage lambda. Fusion protein was expressed in *Escherichia coli* strain BB4 as inclusion bodies at 30ºC and after induction with 1 mM IPTG (*Kong & Ma*, 2006).

In addition, bacterial expression of other variants of human PPAR such as PPAR delta have been reported. Hua *et al.* 2009 showed PPAR delta LBD expression in *Escherichia* coli TB1 in the soluble fraction (at 30ºC and 0.4 mM IPTG for 6 hours) (*Hua et al.*, 2009). Velkov, 2013 reported expression plasmid for human PPAR*δ* in *Escherichia coli BL21 (DE3)* after IPTG induction at a cell density of 0.6 for 6 hours (*Velkov*, 2013). Cho *et al.* 2006 reported PPAR delta expression in *Escherichia coli* BL21 (DE3) in LB medium with 1 mM of IPTG at 20ºC overnight (*Cho et al*. 2006).

In this study, a new strategy for bacterial human PPAR expression was developed using a fusion protein (CusF3H+) for purification with IMAC. hPPARγ was mainly expressed in soluble fraction at low temperature (25ºC), and protein identity was confirmed by Western blot assay. Our results shown the viability of using this strategy to produce human PPAR gamma in *Escherichia coli* Rosetta (DE3). To conclude, hPPARγ protein was successfully expressed in *Escherichia coli* Rosetta (DE3) at low temperature and purified from soluble fraction through IMAC. This methodology could be useful for PPARγ in *in vitro* assays.

Acknowledgements

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Conflict of interest

The authors declare that they have no conflict of interest.

Authors contribution

The experiments were performed by Dalila Balderas-Renteria, Sonia Montero-Molina and Jorge Solis-Estrada. Verónica Almaguer-Cantú supervised the molecular cloning. Katiushka Arevalo-Niño and Xristo Zarate-Kalfopulos supervised protein expression and purification process. Isaías Balderas-Rentería conceived the idea and guided the aim of study. All authors read and approved the final manuscript.

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Supplementary information

Furthermore, the chromatogram of the FPLC system followed a typical IMAC purification pattern, where a constant flow of 0.5 mL / min was maintained in all purification steps (Fig S1).

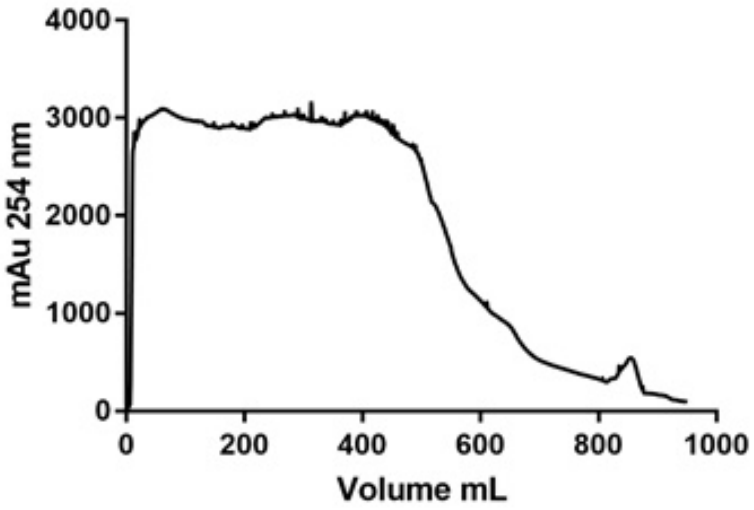

Fig S1. Chromatogram of purification in Äkta Primer Plus FPLC system.