Expression and Purification of Human Membrane Progestin Receptor α (mPRα)

THESIS

Expression and Purification of Human Membrane Progestin Receptor α (mPRα)

MD. BABUL HOSSAIN

Graduate School of

Science and Technology, Educational Division

Department of Bioscience

Shizuoka University

December 2015

THESIS

Expression and Purification of Human Membrane Progestin Receptor α (mPRα)

ヒトプロゲスチン膜受容体α（**mPR**α）の発現と精製

エムデイーバブル ホシャイン

静 岡 大 学
大学院自然科学系教育部

バイオサイエンス専攻

２０１５年　１２月

CONTENTS

Page No.

RESULTS

LIST OF ABBREVIATIONS

mPR : Membrane progestin receptor

hmPRα : Human membrane progestin receptor alpha

gmPRα : Goldfish membrane progestin receptor alpha

nPR : Nuclear progestin receptor

MPF : Maturation promoting factor

HSPs : Heat shock proteins

SDS-PAGE : Sodium dodecyl sulfate polyacrylamide gel electrophoresis

PBS : Phosphate-buffered saline

TBS : Tris-buffered saline

TBST : Tris-buffered saline and Tween 20

MALDI-TOF : Matrix-assisted laser desorption /ionization-time of flight (MALDI-TOF) mass spectroscopy

MIH : Maturation-inducing hormone

MIS : Maturation- inducing steroid

Giα : Inhibitory GTP- binding protein α

AC : Adenylate cyclase

cAMP : Cyclic AMP

ORF : Open reading frame

EDC : Endocrine disrupting chemical

CBB : Coomassie brilliant blue

BSA : Bovine serum albumin

ABSTRACT

Membrane progestin receptors (mPRs) are identified as a member of G protein-coupled receptor (GPCR) family in vertebrates, including humans. The GPCR family proteins are target for more than 50% of modern medicinal drug. Thus, mPRs are considered as attractive proteins to draw a new medicinal prospect. mPRs are potential intermediaries responsible for fast and nongenomic progestin actions initiated at the cell surface. Five subtypes of mPRs, α, β, γ (γ-1, γ-2), δ and ε, are conserved in vertebrates. Recent studies suggested that the alpha subunit (mPRα) has physiological roles in various reproductive tissues. The mPRα acts as mediator of induction of the oocyte maturation and stimulation of sperm hyper-motility in fish by progestin. In mammals, the mPRαs have been reported to be involved in progesterone regulation in uterine function particularly parturition prior to labor. mPRs suggested to be involved in breast cancer growth in human and GnRH secretion in rodents. In order to know the precise mechanism of nongenomic actions induced by progestin and structure determination of human mPRα (hmPRα), expression and purification of large amount of mPRα protein is necessary.

Previously, a method for the expression and purification of goldfish mPRα (gmPRα) protein using methylotropic yeast, *Pichia pastoris*, was established in my laboratory. The gmPRα protein was expressed after 24 hours induction in 20^0C with the cell densities in OD_{600} of 1.0-3.0. Subsequently the yeast cells were broken by MS 100 Micro Smash equipments and solubilized the gmPRα protein using DDM and purified the gmPRα protein on Ni-NTA, Cellufine Amino and anti-c-Myc-Tag beads column chromatography. However highly active gmPRα was purified by the established method, the yield was very low. It was hard to apply for further analysis.

In my study, for the expression and purification of recombinant hmPRα protein, several conditions were optimized on the methods of gmPRα. I optimized mainly 4 points, induction time with cell densities, cells breaking machine, buffer conditions for Ni-NTA column chromatography and binding assay method for purified mPR. By these optimizations, I could establish the way to collect 100 times higher amount of hmPRα than previous method.

Firstly, the timing and conditions for induction of expression was optimized. Several conditions in changing the temperature and cell density were tested. Highest amount of hmPRα protein was expressed in yeast at 6 hours of methanol induction at 20^0C with the optimum cell density of OD_{600} at 21 to 23. This optimization increased the level of production of hmPRα compared to gmPRα expression system. Crude membrane fractions containing

expressed hmPRα exhibited binding activity of Kd=3.8 nM and Bmax=288.8 fmol/mg for progesterone. This result indicated that hmPRα expressed in yeast was active.

Secondly, I applied stainless Ball Mill (PM 100, Verder Scientific Co., Ltd., Haan, Germany) instead of beads crusher (Micro-Smash MS100, TOMY Seiko), which could disrupt cells from 500 ml culture within 12 minutes under the freezing condition at once. By this step we could get even clear membrane fraction. Thus this step contributed for purification.

Thirdly, I optimized conditions for nickel-nitrilotriacetic acid (Ni-NTA). Buffer used for (Ni-NTA) column chromatography was selected among the 16 buffers tested, 50 mM NaH_2PO_4, 300 mM NaCl, 40 mM Imidazole pH 6.0 showed the best performance to purify hmPRα.

Fourth, I established a method of steroid binding assay for solubilized membrane receptor. There was difficulty for the binding assay of solubilized and purified mPR protein, because mPR protein can pass through the membrane. But, I succeeded to establish a strategy for the binding assay of solubilized and purified mPR protein, with supplemented the Ni-NTA resin (100 µl of 50% vol) in the reaction mixture of binding assay. The purified hmPRα protein was demonstrated its binding activity for progesterone (Kd = 5.2 nM and Bmax = 111.6 fmol/mg) which indicated that I succeeded in solubilizing and purification of hmPRα in active form.

By these optimizations, I established the procedures for hmPRα production. The DDM solubilized hmPRα was purified through three different column chromatography steps. Firstly, a Ni-NTA column was used. Then the hmPRα proteins were bound to cellulose resin with free amino groups (Cellufine Amino column) and finally passed through a SP-Sepharose column. By the optimized expression and purification conditions, higher amount of hmPRα (1.2-1.5 mg) obtained from 1 L culture (0.8 – 1% of total hmPRα). The identity of the purified protein was confirmed by MALDI-TOF/MS analysis.

By this method it was became possible to get relatively higher amount of hmPRα in low cast. In addition this study developed a new strategy to measure the binding activity of purified mPR proteins. As well the purified recombinant hmPRα could be a promising tool for screening of ligands of hmPRα and be a source of structure resolution approach and monoclonal antibody production.

INTRODUCTION

1.1 Progestin

Progestins are steroid compound that mimics the effects of progesterone, such act as a key regulating factor to control the reproductive tissues in female and male vertebrates. It is classified in two types; one is natural and another is synthetic type. Natural progestin, progesterone identified in humans and certain animals (Stanczyk 2003), the chemical structure and biosynthesis pathway is shown in Fig. 1A & 2. On the other hand, synthetic progestin is produced and frequently used in medical purpose. Progesterone is commonly known as steroid hormone which is produced by the ovary depending on the physiological conditions and the level of gonadotropin stimulation (Ashley et al. 2009). Progesterone deals with a number of physiological effects in different tissues. Thus research focused on the mechanism, how progesterone intervent its action into cells. Classically, physiological effects of progesterone have been known to be mediated by regulation of gene expression by the nuclear progesterone receptors (nPR) (Boonyaratanakornkit and Edwards 2004). However, new concept on actions of progestin was raised by identification of membrane progestin receptor (mPR) (Zhu et al. 2003). It is now widely appreciated that progestin can exert rapid actions (initiated within minutes) on cell surface through activation of membrane receptors and their association between intracellular signaling pathways (Revelli et al. 1998, Watson and Gametchu 1999, Norman et al. 2004, Thomas 2012). For example, non-classical progesterone actions on oocyte meiotic maturation, sperm motility, granulosa cell apoptosis, immunosuppression of T cells, breast and ovarian cancer cells, GnRH secretion, and reproductive behaviors have been demonstrated. And the progesterone-receptors mediating these effects have been biochemically characterized (Ke and Ramirez 1987, DeBold and Frye 1994, Zhu et al. 2003, Chien et al. 2006, Frye et al. 2006, Peluso et al. 2006, Carnevali et al. 2007, Tubbs and Thomas 2009, Charles et al. 2010, Zuo et al. 2010, Dressing et al. 2012).

Two distinctive naturally occurred maturation-inducing hormone (MIH), the progestins have been identified in several teleost fish species (Fig. 1B & C), the 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-DHP) was identified from amago salmon (*Oncorhynchus rhodurus*) and 17α,20β,21-trihydroxy-4-pregnen-3-one (20β-S) was identified in Atlantic croaker (*Micropogonias undulatus*) and spotted seatrout (*Cynoscion nebulous*) (Nagahama and Adachi 1985, Trant et al. 1986, Patino and Thomas 1990, Nagahama and Yamashita 2008). It was predicted and demonstrated in a series of studies that these progestins do not act via the classical intracellular mechanism of steroid action through

nuclear steroid receptors, instead initiate their actions by binding to specific membrane receptors on the external surface of oocytes (Nagahama et al. 1995, Thomas et al. 2002). The first evidence that showed receptors for MIH exist on the cell surface had been described in frog oocytes (Ishikawa et al. 1977, Godeau et al. 1978). Subsequently, this MIH receptors have been recognized on the oocytes membranes of several teleost species including spotted seatrout (Patino and Thomas 1990, Yoshikuni et al. 1993, King et al. 1997, Rahman et al. 2002). However the precise molecular structure and mechanisms of action of the membrane receptors remained unclear until 2003. Eventually after a series of experiment a strong candidate for a MIS membrane receptor was identified in spotted seatrout (Zhu et al. 2003).

1.2 Membrane progestin receptor (mPR)

Several decades after the initial suggestions of a membrane receptor for steroids, a novel cDNA was cloned and sequenced from a spotted seatrout ovarian cDNA library. A protein was cloned and referred as membrane progestin receptor (mPR). Characteristics of mPR was revealed that can mediate the initiation of the MIH-induction of oocyte maturation (Zhu et al. 2003). This novel gene meet seven criteria for its designation as a membrane progestin receptor, which were plausible structure, tissue specificity, cellular localization, steroid binding capability, signal transduction, hormonal regulation and biological significance. Computer modeling predicts that the protein has typical seven transmembrane domains (Fig. 3) of G protein-coupled receptors (GPCRs) (Zhu et al. 2003), the largest class of hormone receptors (Bockaert and Pin 1999).

Genome-wide phylogenetic analysis revealed that mPRs are members of a group of novel G protein-coupled receptors belonging to the new protein family, progestin and adipoQ receptor (PAQR) family (Tang et al. 2005). PAQRs consist of 11 close related genes of 7-transmembrane receptors. By their structure and binding characteristics, they are separated into three classes. Class II PAQRs consist of five members that present only in vertebrates, which including mPRα, mPRβ and mPRγ (correspond to PAQR7, 8 and 5, respectively) (Thomas et al. 2007). Firstly mPRα was identified in spotted sea trout (Zhu et al. 2003). Then mPRβ and mPRγ were found by homology search on Genebank and characterized in other vertebrates including human (Zhu et al. 2003). It is also identified two γ subtypes (PAQR5) in goldfish ovarian cDNA library named γ-1 and γ-2 (Tokumoto et al. 2012). Furthermore, PAQR6 and PAQR9 were classified as mPRδ and mPRε respectively depending on the analysis of expressed protein in human breast cancer cell (Smith et al. 2008) .

The mPRs can transduce a range of rapid, cell surface-mediated actions of nongenomic intracellular signaling pathways (Thomas and Pang 2012). The alpha subtype

(mPRα) is the most well characterized among membrane progestin receptors. It is expressed in all the tissues in vertebrate and has suggested to be involved in important physiological functions (Zhu et al. 2003, Tang et al. 2005, You et al. 2010, Aparicio et al. 2011). For example, mRNA of mPRα is present in the different parts of the body in goldfish including brain, eye, gill, heart, intestine, kidney, muscle, spleen, ovary and testis (Tokumoto et al. 2006). Physiological role of mPRα is demonstrated primarily in teleost fish species that mPRα mediate the oocyte meiotic maturation induced by progestin (Zhu et al. 2003, Tokumoto et al. 2006, Tubbs et al. 2010, Hanna and Zhu 2011). Also stimulation of teleost sperm hypermotility by progestin is mediated through mPRα (Tubbs and Thomas 2009). On the other hand, progesterone has significant effects for the inhibition of apoptosis in human breast cancer cells which activate through mPRα, as well mPRα is involved in the growth of breast tumor (Dressing et al. 2012). Moreover it is also demonstrated that induction of oocyte maturation in goldfish and zebrafish by the treatment with an endocrine disrupting chemical (EDC), diethylstilbestrol (DES) is mediated by mPRα (Fig. 6). Thus mPRs have been recognized as a new target for EDCs also (Tokumoto et al. 2007).

1.3 Signal transmission into cells by mPR

It is demonstrated in a broad range of target tissues that steroid hormone can act as chemical messengers, mediating not only slow genomic actions but also rapid nongenomic actions. Researchers have been performed analysis on the mechanism of mPR function. It has been investigated and characterized that the nongenomic action of progestins is mediated by the membrane progestin receptors (mPRα, mPRβ and mPRγ) on plasma membrane in several fish species and mammals including frogs, rats, pigs and humans (Hanna and Zhu 2009). Moreover a lot of rapid nongenomic actions of progestins was recognized, induction of oocyte maturation, quick activation signaling of breast cancer cell, mammalian sperm hypermotility, modulational function during the reproductive signaling in the brain and initiation of the acrosomal response (Zhu et al. 2008). Oocyte maturation (OM) in fish induced by progestin is a well characterized and precious model for investigating the rapid and nongenomic actions through mPR (Thomas et al. 2004, Mourot et al. 2006, Tokumoto et al. 2006, Hanna and Zhu 2009). Oocyte maturation is activated by maturation inducing hormone (MIH), which is secreted from ovarian follicle cells stimulated by maturation promoting factor (MPF), Cyclin B (Nagahama et al. 1995, Yamashita et al. 1995). MIHs, the progestins act on the external surface, plasma membrane of oocytes by binding to the specific membrane progestin receptor (mPR) and initiate the oocyte maturation through non-genomic actions (Nagahama et al. 1995, Thomas et al. 2002, Tokumoto et al. 2006).

Furthermore, the induction of oocyte maturation by MIH requires protein synthesis but is not blocked by transcription inhibitors, which is indicating a nongenomic mechanism of action (Jalabert et al. 1976, Goetz and Theofan 1979, Patino and Thomas 1990). The main pathway for the induction of oocyte maturation, meiotic cell division can be induced by nongenomic action through a decrease of intracellular cyclic AMP (cAMP) levels within a few minutes (Fig. 5). It is reported, during the response of MIH in frog oocyte maturation, cAMP levels have been significantly decreased (Cicirelli and Smith 1985), as well as the same results has been demonstrated in teleost oocytes (Finet et al. 1988). It has been reported in spotted seatrout that MIH activates a pertusis toxin (PTX) sensitive inhibitory G (Gi) protein, and that activation of this pathway is necessary for the completion of oocyte maturation (Pace and Thomas 2005). As well it has been reported that progesterone, a mPRα ligands, decrease the intracellular cyclic adenosine monophosphate (cAMP) level by the nongenomic steroid actions (Nakashima et al. 2015). Thus, it is suggested that mPR is coupled to inhibitory G (Gi) protein and mediated rapid nongenomic actions in a wide range of target tissues (Fig. 5). On the other hand, the ovulation–inducing pathway is thought to be activated by genomic actions through the classical nuclear progesterone receptor (nPR) where involving new mRNA and protein synthesis mechanism and is relatively slow (Fig. 4). However in *Xenopus* oocyte maturation, both nPR and mPR are involved in mediating the nongenomic action of progestin (Zhu et al. 2008). Moreover, it was demonstrated in human myometrium that transactivation of nPRs leaded by activation of mPRs (Karteris et al. 2006). Although it is still remained unclear, the details of signaling pathway through the mPRs pathways and their potential interactions with the nPRs are interesting research question to address in the future.

1.4 Production of mPR protein in yeast (*Pichia pastoris*) expression system.

Multiple host systems are commonly used to produce recombinant human proteins, this include *E. coli* cells, yeast cells, insect cells and mammalian cells. *Pichia pastoris* (*P. pastoris*), the methylotrophic yeast is a highly successful determinate for the production of various heterologous protein. *P. pastoris* is an efficient host for the expression of membrane proteins (Shimamura et al. 2011, Shiroishi et al. 2011) and secretory proteins (Cregg 1985, Cregg et al. 1993, Mizutani et al. 2004, Mizutani et al. 2010) along with high cell densities fermentation capabilities and can carry out of many eukaryotic post translational modifications. Recently, three dimensional structure of human histamine H1 receptor, one of the GPCR, was determined using a recombinant protein expressed by Pichia (Shimamura et al. 2011). Previously it had been reported that different human cancer cell lines and *Escherichia coli* were used as a medium for the expression of recombinant mPR protein

(Tokumoto et al. 2007). However, large scale culture of *E. coli* was possible but not resulted in expression of active form of recombinant mPR protein (Oshima et al. 2014). In addition, expression level of mPRs in the mammalian cells is very low and it was not reach the sufficient amount for purification and structural analysis. *P. pastoris* is a successful determinate for recombinant protein expression (Asada et al. 2011, Mizutani et al. 2012). More than one hundred (100) of G protein coupled receptors (GPCRs) had been succeeded on the expression and its large scale purification with functional form using *P. pastoris*. For example, natural ligand binding activity has been assessed by the expression of mammalian GPCRs in *P. pastoris* (Lundstrom et al. 2006, Zeder-Lutz et al. 2006). Thus, now *P. pastoris* has been widely used for the expression of GPCRs. In 2011 and 2012, structures of two human GPCRs (the histamine H1 and the adenosine A_{2a} receptor) were determined by using recombinant protein expressed in *P. pastoris* (Shimamura et al. 2011, Hino et al. 2012). As well, yeast expression systems have been used for producing large amount of proteins for biopharmaceuticals application. Thus, *P. pastoris* was selected for the expression of large amount of mPR protein.

mPR can exhibit a range of rapid and nongenomic cell surface steroid actions which have been demonstrated in a wide variety of cell models, tissues and animals (Thomas and Pang 2012). However, complete knowledge of molecular structure and mechanism of steroid binding has still unknown, despite intensive research efforts in many laboratories to purify the receptor proteins. Since the receptor proteins are present in minute quantities in target tissues and its binding activity decrease during solubilization. As well, receptor protein purification is a great challenge. Therefore, we improved the method, which concerned the expression and purification of hmPRα protein in an active form.

MATERIALS AND METHODS

2.1 Materials

Pichia pastoris strain X33 was purchased from Invitrogen. [^{3}H]1,2,6,7 progesterone was bought from PerkinElmer Inc. Modified trypsin (sequencing grade) was collected from Promega (Tokyo, Japan). The CHCA was collected from Bruker Daltonics (Billerica, MA). Digitonin was purchased from Sigma Aldrich Chemicals (St. Louis, MO). DNA polymerase and Ligation Kit were from Takara Bio (Siga, Japan). DNA fragment extraction kit from agarose gel was purchased from QIAGEN (Tokyo, Japan). The Molecular weight marker for SDS-PAGE was from Bio-Rad (Hercules, CA). The anti-rabbit antibody conjugated with peroxidase and yeast nitrogen base without amino acids were obtained from Invitrogen (Carlsbad, CA). Anti-His-tag antibody was from Medical & Biological Laboratories (Nagoya, Japan). Other chemicals were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan).

2.2 Construction of recombinant plasmid

For the expression of recombinant human mPRα protein in *P. pastoris* (wild strain X33), the cDNA for human mPRα was prepared from human blood and amplified by polymerase chain reaction (PCR) using the primer set Hs mPRα normal F, GTCACCTGGCTTTGCCTTTG and Hs mPRα normal R, ATGCCATCCCCCTTCACTTG with KOD plus neo DNA polymerase (TOYOBO, Japan). The PCR program was set as heat soak at 95^{0}C, 10 min x 1 cycle; (denaturation at 95^{0}C, 30 sec, primer annealing at 70^{0}C, 30 sec, and extension at 72^{0}C, 1 min) x 45 cycles; and final extension at 72^{0}C, 10 min x 1 cycle. Amplified fragments were purified with phenol-chloroform. The purified DNAs were electrophoresed in 1.2% agarose gel and extracted from the gel using QIAEX-II Gel Extraction Kit. Subsequently, the DNAs were phosphorylated using Blunting Kination Ligation kit and again electrophoresed in 1.2% agarose gel and extracted from the gel using QIAEX-II Gel Extraction Kit. Then the pBluescript-II KS(+) vector was linearized by EcoRV to produce blunt ends and treated with Thermo-sensitive Alkaline Phosphatase (TSAP) for dephosphorylation. The phosphorylated DNAs were inserted into dephosphorylated pBluescript-II KS(+) Plasmid (Fig. 7). The recombinant vector was transformed into *E. coli* (XL1 Blue) cells and cultivated for the cloning of hmPRα gene at 37^{0}C for 16h. After the cloning, the hmPRα fragment in pBluescript-II KS(+) was again amplified by PCR using primer set Hs mPRα EcoRI, 5'-

CGGAATTCATGGCCATGGCCCAGAAACTCAGCCACCTCCTGCCGAG-3' and Hs mPRα-NotI 5'-ATAAGAATGCGGCCGCCTTGGTCTTCTGATCAAGTTTGCGCTGT ACCAGC-3'. The PCR program set as same as Hs mPRα normal F and normal R. The PCR amplified DNAs also electrophoresed in 1.2% agarose gel and extracted from the gel using QIAEX-II Gel Extraction Kit. Then the DNAs (QIEX-II extracted) and vectors (pPICZA and pPICZαA) were digested by Eco RI and Not I. The digested DNAs and vectors were purified by phenol-chloroform and the DNAs were electrophoresed in 1.2% agarose gel and extracted from the gel pieces using QIAEX-II. Then the DNAs were ligated into the *P. pastoris* expression vectors, pPICZA and pPICZαA (Invitrogen) (Fig. 9). The DNA ligation kit (Mighty Mix, Takara 6023) was used for the ligation at 16^0C for 16 h. Then the recombinant plasmids were washed by phenol/chloroform. Subsequently, the recombinant vectors were transformed into *E. coli* cells (TOP10F´) by incubation at 42^0C for 1 min. After transformation the *E. coli* cells were cultured in a 500 µl of Luria-Bertani (LB) medium (1% Tryptone, 0.5% Yeast Extract, 0.5% NaCl, pH 7.5), incubated for 3 hours at 37^0C with shaking at 200 rpm. The cultured cells were collected by the centrifugation at 3000 x g for 1 min at 20^0C. The cell pellets of 100 µl were placed on LB plates (LB with 1.5% agar) contained 25 µg/mL Zeocin and cultivated at 37^0C for 16 h. The Zeocin resistant transformants (*E. coli* colonies) were cultured on 2 ml of LB medium (contained of 25 µg/mL Zeocin) at 37^0C for 16h. The recombinant vectors were isolated from *E. coli* cells, which were confirmed by gel electrophoresis (Fig. 9E). The DNA sequences of the ORF region of the expression vectors were verified by DNA sequencing. The complete hmPRα DNA sequence along with α-factor, His-tag and C-Myc-tag is shown in Fig. 8.

2.3 Transformation of recombinant plasmids into yeast cells.

The recombinant plasmids (pPICZA and pPICZαA) were transformed into yeast cells by electroporation. The *P. pastoris* strains X-33 (Invitrogen) were used for transforming the hmPRα-expression construct. The constructs were linearized using two different restriction enzymes (BstXI and PmeI). The NE Buffer CutSmart (10X) Biolab B7204S was used for restriction reaction. After digestion, the linearized plasmids were purified by phenol/chloroform and checked by electrophoresis. Subsequently, 10 µg of BstXI and 168 µg of PmeI linearized plasmid DNA were used to transform into the competent *P. pastoris* cells through electroporation (Fig. 10). The electroporation was performed at 1500 V, 25µF, resistor high 800 Ω and low 200 Ω, time 4.82 mSec by Gene pulser (Bio-Rad), following protocols established previously (Oshima et al. 2014).

After transformation, the recombinant *P. pastoris* were cultivated on Yeast extract-peptone-dextrose medium (YPD) plates (1% yeast extract, 2% peptone, 2% dextrose, 2% agar), which were contained 100 μg/mL, 500 μg/mL and 1000 μg/mL Zeocin. After 3 days later, the colonies were found on YPD plates. Genomic amalgamation of hmPRα-expression construct was verified by PCR using Ex Tag polymerase (Takara Bio, siga, Japan) and primer sets of (5′ AOX1, 5'-GACTGGTTCCAATTGACAAGC-3' and 3′ AOX1, 5'-GCAAATGGCATTCTGACATCC-3') that was amplified between AOX1 promoter and terminator regions (Fig. 14). Production of recombinant hmPRα protein was confirmed by checking several Zeocin-registant clones. Best expressed clones were determined by western blot analysis using anti His-tag antibodies (Fig. 13), which was kept and stored on MD plate contained 1.34% yeast nitrogen base, 4 x 10^{-5}% biotin, 2% dextrose, 1.5% agar at 4^0C (Oshima et al. 2014).

2.4 Expression of hmPRα in *P. pastoris*

For the production of hmPRα, I picked the expressed single colony from the MD plate and inoculated in 100 ml BMGY medium (1% yeast extract, 2% bactopeptone, 100 mM potassium phosphate, pH6.0, 1.34% yeast nitrogen base without amino acids, 4 x 10^{-5}% biotin, 1% glycerol) in a 500 mL baffled flask and incubated for 21 hours at 30^0C with shaking at 180 rpm. The volume was increased to 500 mL BMGY medium in a 2 L baffled flask and incubated for 16.5 hours at 30^0C with shaking at 180 rpm where the Yeast cells OD_{600} nm was reached up to 17-19. For the determination of culture cell densities, 1 mL aliquot was used from the culture medium. The remaining culture was harvested for the induction. Cells were collected by the centrifugation at 3000 x g for 5 min and washed once by 300 ml BMMY (1% yeast extract, 2% bactopeptone, 100 mM potassium phosphate, pH6.0, 1.34% yeast nitrogen base without amino acids, 4 x 10^{-5}% biotin, 0.5% methanol) medium. For induction of hmPRα protein expression, the cells were re-suspended in 400 ml BMMY medium to the densities in OD_{600} nm of 21-23. The medium was kept in a 2 L baffled flask and incubated at 20°C for 6 hours with shaking at 180 rpm. After 6 hours, the cells were harvested with the centrifugation at 3,000 × *g* for 5 min. The cell precipitates were collected and frozen with liquid nitrogen and stored at -80°C. To establish the best condition for the expression of hmPRα, 1 ml aliquot of the induction culture cells were collected after 0h, 1h, 2h, 4h, 6h and 24h. Production of hmPRα was examined by western blotting using ant His tag antibody.

2.5 Membrane preparation and solubilization of membrane proteins

For the purification, yeast cells were broken and membrane fractions were collected and then the membrane proteins were solubilized. For the breaking of yeast cells, firstly frozen cell pellets (≅20 g harvested from 800 ml culture) were melted and re-suspend in 80 ml ice-cold breaking buffer (50 mM sodium phosphate, 1 mM PMSF, 1 mM EDTA, 5% glycerol, pH7.4). Then re-frozen the cells using liquid nitrogen as shape of tubules in the stain-less chamber of Ball Mill, for the breaking of cells with 5 stain-less balls. Consecutively, cells were disrupted by Retsch Ball Mill PM 100 (Verder Scientific Co., Ltd., Haan, Germany) instrument with six rounds shaking with an interval of chilling with liquid nitrogen (Fig.17). Each round breaking was fixed at 400 rpm for 3 minutes. Broken cells like as white powder were collected into centrifuge tubes. Unbroken cells and debris were separated from the fractions containing the membranes by low speed centrifugation (1000 x g, 4^0C, 7 min). After collecting the supernatant once, the pellet was resuspended in 30 ml ice cold breaking buffer for a further round of supernatant collection. The supernatants from two subsequent rounds were combined and the membrane fractions were recovered by centrifugation at 20,000 × *g*, 4°C, for 20 min. The precipitates were re-suspended with buffers for the steroid binding assay or purification according to their expected use. For the purification, the membrane fractions were solubilized using 1mM PMSF, 10% Glycerol and 0.1% DDM by incubation for 30 min. Subsequently the insoluble materials were separated by high speed centrifugation at 20,000 × *g*, 4°C, for 20 min, following the previous developed method (Oshima et al. 2014).

2.6 Purification of hmPRα.

Solubilized proteins were kept on ice and separated from the dissolved debris by filtration by Mixed Cellulose Ester filter paper. The clear sample was applied for column chromatography. In the first step an 80ml Ni-NTA Agarose (QIAGEN, Gaithersburg, MD, USA) column (ϕ 4.5 × 5.0 cm) was used. The column had been equilibrated with 800 ml lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 40 mM Imidazole pH 6.0) containing 1 mM PMSF and 0.01% DDM. After applying, the column was washed with 800 ml of same buffer, and then the bound proteins were eluted with a 500 mL gradient of 40–400 mM imidazole in same buffer, and washed with 100ml of the same buffer containing 400mM imidazole. The fractions were collected in 30 tubes. The fractions that contained recombinant hmPRα were identified by Coomassie Brilliant Blue (CBB) staining and western blot analysis (using anti-His-tag antibodies), that were collected and diluted for 4.5 times with DDW (up to conductivity 18, 50 mmho), which was used for next step of purification. In the second

purification step, the samples were loaded onto a 5 mL Cellufine Amino (JNC Corporation, Tokyo, Japan) column (ϕ 1.6 × 10 cm) that was equilibrated with CA buffer (50 mM Tris-HCl buffer, pH 8.0 containing 0.01% DDM and 1 mM PMSF). After applying, the column was washed with 15 mL of same buffer and eluted with a 120 mL gradient of 0–0.5 M NaCl in CA buffer. The fractions containing hmPRα was detected by CBB staining and western blotting using anti-His-tag antibodies. The collected fractions were diluted upto 4.5 times with DDW (conductivity up to 22, 50 mmho), that were passed through a 1 ml of SP-Sepharose column and applied to a 1.5 ml of a Cellufine Amino column. The proteins were eluted with CA-buffer containing 0.5 M NaCl. The fractions that contained the purified hmPRα protein were detected by CBBR staining and immuno-blotting assay, which were collected and concentrated with Centriprep YM-3 filter units (Millipore, Billerica, MA). All the purification steps were done at 4^0C.

2.7 SDS-PAGE and Western blot analysis.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 12% polyacrylamide gel under denaturing conditions according the method of Laemmli, by which the proteins were separated. For western blot, the separated proteins were transferred to Immobilon membranes (Millipore, Billerica, MA). Then the membranes were blocked in 5% non fat powdered milk in 20mM Tris buffer saline, pH 7.6 (TBS) containing 0.1% Tween 20 (TBST) for 1-2 hours at room temperature. After blocking, membranes were washed with TBST buffer, three times for 5 min each, subsequently the membranes were incubated 1 hour with primary antibodies (polyclonal anti-His-tag antibody, anti mPRα antibody from mouse serum) that was 1000-fold dilution in TBS buffer. After that the blots were washed with TBST buffer, three times for 5 min each and then incubated for 1 hour with secondary antibodies (HRP rabbit antibody conjugated with peroxidise, Invitrogen and anti-mouse antibody, MBL, Japan) that was 2000-fold dilution in TBS buffer. The visualization of target protein was performed by enhanced chemiluminescence using an ECL detection kit (PerkinElmer, Waltham, MA), a method using based upon a chemiluminescent reaction mediated by peroxidase conjugated to a secondary antibody. The signals were digitized using CCD camera system (Luminescent image analyzer LAS-4000 mini, Fujifilm, Tokyo, Japan). The image was analyzed by densitometry, by which it was evaluated the relative amount of protein and quantified the results.

2.8 Peptide mass fingerprint analysis by MALDI-TOF/MS

MALDI-TOF/MS is used for the identification of hmPRα protein. Firstly the SDS-PAGE of purified hmPRα protein was stained by CBBR. The SDS-PAGE gel-slices containing purified recombinant hmPRα were digested by trypsin at 37^0C for 16 h. Then, the peptide fragments were collected using a ZipTip (Millipore) and it is eluted through a solution (2 µl) which was containing 60% acetonitrile, 0.1% TFA and 5 mg/ml of CHCA (Bruker Daltonics), as described previously for goldfish mPRα (Oshima et al. 2014). A 384-well plate was used for loading the samples, which layered with CHCA. A MALDI-TOF/MS Autoflex (Bruker Daltonics, Billerica, USA) was used to detect the peptide mass spectrum, in a positive ion mode. The spectra that were obtained from MALDI-TOF/MS were calibrated by a mixture of molecular weight standard (Bruker Daltonics). At least 300 laser shots were used to take the average spectrum result. The peptide fingerprint was analyzed using the MASCOT software (Matrix Science, London, UK). It was searched against peptides from human taxonomy using NCBInr database, parameters were used as, cysteine's modification by carbamidomethylation (C), trypsin digest zero missed cleavage and peptide mass tolerance ± 0.4 Da. Probability-based MOWSE (Molecular Weight Search) score was used for identifying the human mPRα protein.

2.9 Radiolabeled ligand binding assays

For the ligand binding assay analysis, the plasma membrane pellet was collected by disrupting the yeast cells. The membrane preparation procedures are described in membrane preparation and solubilization section. Prior to assay analysis the frozen membrane pellet was resuspended in HAED buffer (25 mM HEPES, 10 mM NaCl, 1 mM Dithiothreitol, 1 mM EDTA, pH 7.6) containing 0.1% digitonin. GF/B filters were used for the assay analysis. The progestin receptor binding in the membrane fractions were measured by the following procedures established previously (Tokumoto et al. 2007).

Progestin receptor binding assay analysis for the purified hmPRα including solubilized proteins, Ni-NTA fractions, Cellufine Amino fractions; the Ni-NTA resin (100µl of 50% vol) was supplemented in the reaction mixture. GF/B filters were used and pre-soaked in wash buffer without Tween 80. 400µl volume of reaction mixture was used for the assay analysis.

2.10 Competition studies

Steroid binding assays was performed to investigate the hormone binding activity of progestin membrane receptor. One set (3 replicates) of assay tubes was used for measuring

the total bound and another set was used for the non-specific binding. The assay tubes for the total binding contained 1.5 nM of [^{3}H] 1,2,6,7 progesterone alone. On the other hand the nonspecific binding (NSB) assay tubes contained [^{3}H] 1,2,6,7 progesterone with 100 fold greater concentration of cold progestin (nonradioactive) as competitor. After a 30 min incubation of binding reaction at 4^0C with the membrane fractions, the reaction was stopped by filtration over Whatman GF/B filters that was presoaked in wash buffer (25mM HEPES; 10mM NaCL, 1mM EDTA, pH 7.4) containing 2.5% Tween 80. The filters were washed 3 times with 5 ml of wash buffer at 4^0C. The bound radioactivity was counted in a liquid scintillation counter. The specific binding result was calculated and visualized by Graph Pad PRISM software version 4.0c.

2.11 Saturation analyses and Scatchard plots

Saturation analysis of progesterone binding was measured over a range of [^{3}H] 1,2,6,7 progesterone (specific activity, 96.6 Ci/mmol) concentrations (0.5-12.5nM), which was added in the assay tubes of total and nonspecific binding. A 100–fold molar excess cold progesterone (P4) also contained in assay tubes for measuring the nonspecific binding. Linear and nonlinear regression analyses for all receptor binding assays, and calculations of dissociation constant (K_d) and binding capacity (Bmax) were conducted using GraphPad Prism for Macintosh (version 4.0c; Graph Pad Software, San Diego, CA). The results are shown on Scatchard plots.

2.12 Anti hmPRα (α-hmPRα) antibody production

The purified hmPRα proteins in CA buffer with 0.5 M NaCl were emulsified in an equal volume of Freund's adjuvant (complete for the initial injection, incomplete for subsequent injections). For each injection 12.5 μg of protein in a total volume of 0.5 ml was used for a mouse. BALB/c mice were injected at 10 day intervals. Three weeks after the third injection, titer of serum was checked as primary antibody. Mouse with higher titers of serum was used as primary antibody (α-hmPRα) for the detection of recombinant hmPRα (Fig. 23B).

2.13 Statistical Analysis.

All the experiments were repeated three times. One-way analysis of variance (ANOVA) was calculated using GraphPad Prism (San Diego, CA).

RESULTS

3.1 Recombinant human mPRα protein expression

pPICZA and pPICZαA expression vector and the wild yeast *P. pastoris* X33 variety is used for the expression and production of recombinant hmPRα protein. For the expression, the cDNA of hmPRα was fused to the secretion signal sequence of the α-factor from *S. cerevisiae* in the expression cassette (Fig. 11A). The hmPRα expression construct was transformed by electroporation that was inserted into the host genome of yeast by homologous recombination. The successful insertion of the expression cassette was confirmed by PCR using *AOX1* primer set, along with its promoter and terminator regions in genomes that control the transcription of the heterologous hmPRα gene-fusion into the yeast cells (Fig. 14).

The expressed fusion hmPRα protein was also carried a c-Myc epitope and a histidine tag (His-tag) with its C-terminal end. Expression was induced by 0.5% methanol in the BMMY induction medium at 20^0C. The expression of the hmPRα protein was confirmed by western blot analysis using anti-His-tag antibodies. The cell membranes were prepared from *P. pastoris* cells that were carrying the expression cassette of hmPRα from pPICZA and pPICZαA construct. The construct was linearized using BstXI and PmeI enzyme before electroporation. When using the construct linearized by BstXI, gene insertion was confirmed but hmPRα protein production was not induced (Fig. 12). But, using PmeI linearized construct, gene insertion was confirmed but production of hmPRα protein was induced only in pPICZαA construct (Fig. 13). Amount of 168 μg of linearized pPICZαA construct was used for transformation, resulted a band for hmPRα protein, around 50 kDa was detected (Fig. 13D). The theoretical molecular mass of hmPRα along with α-factor signal peptide is approximately 52 kDa, which was consistent with the molecular mass of the detected band.

To decide the optimal conditions for achieving the high level of expression of hmPRα protein, 1 ml aliquots of the culture were collected after 0, 1, 2, 4, 6 and 24 hours and the amount of expressed hmPRα protein was analyzed by western blotting using anti-His-tag antibodies. The highest level of hmPRα was expressed at 6-hour (Fig. 15). The optimal cell density before the initiation of induction was also examined. We found that, when the cell density was increased, the amount of hmPRα was increased. By several trials, finally, it is determined that the cell density of OD_{600} at 21 to 23 was optimum before methanol induction (Fig. 16), and it is different to previously established conditions for the goldfish mPRα

protein production at an OD_{600} of 1.0-3.0 (Oshima et al. 2014) . As a result, using this higher induction density of cells, we successfully produced the recombinant hmPRα protein.

3.2 Specific binding of [^{3}H] 1,2,6,7 progesterone to the plasma membrane, prepared from the hmPRα expressed *P. pastoris.*

hmPRα protein was obtained in the *P. pastoris* cell membrane fraction. Digitonin was used for the preparation of cell-membranes for measuring the specific binding activity of [^{3}H] 1,2,6,7 progesterone on expressed recombinant hmPRα protein, because this glycoside facilitate receptor-access of the steroids (Alamae 1995) (Rae et al. 1998) (Ambhaikar and Puri 1998). Previously it was reported that a final concentration of 0.1% digitonin was optimal for the facilitating steroid binding to goldfish mPRα (Oshima et al. 2014) in a filter-binding assay (Ambhaikar and Puri 1998, Rae et al. 1998). After the treatment of the crude cell membrane fractions with 0.1% digitonin, specific [^{3}H]1,2,6,7 progesterone binding activity of membrane fractions from hmPRα expressed cells was significantly increased. On the other hand, membrane fractions from untransformed host cells showed lower binding activity in the same conditions (Fig. 18A). Saturation analysis demonstrated the progesterone binding to the cell membranes of hmPRα expressing cells is saturable and of limited capacity (Bmax = 288.8fmol/mg). Scatchard analysis represented the presence of a single site of high-affinity binding (Kd = 3.8 nM) in the cell membrane fraction of hmPRα (Fig. 18B). Therefore, these results demonstrated that heterologously produced recombinant hmPRα in yeast was active.

3.3 Solubilization and purification of hmPRα protein

In order to obtain pure hmPRα, the proteins were solubilized and separated from the other proteins in membrane fractions. For the lysis of a large amount of yeast cells, a new machine was applied. We applied stainless ball mill, PM 100, instead beads crusher, Micro-Smash MS 100. PM 100 can disrupt the samples by rotating stainless steel balls (5 balls) in the chamber under the freezing conditions (Domanski et al. 2012, LaCava et al. 2015) (Fig. 17). By using this PM 100 along with 5 balls, we could disrupt all cells from 500 ml culture at once, as well the proteins could be kept in stable conditions compare to MS-100 (Fig. 17C). After the disruption of the yeast cells by Ball Mill PM100, the membrane proteins were solubilized using 0.1% n-dodecyl-β-D-maltoside (DDM) as described previously (Oshima et al. 2014). For the optimization of the conditions for Ni-NTA affinity chromatography, 16 kinds of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 1mM PMSF,

10% glycerol, 0.1% DDM) with 4 different concentrations of imidazole (10, 20, 40 or 80mM) and of 4 different pH 5.0, 6.0, 7.0 or 8.0 were tested (Fig. 19). Membrane preparations were incubated with one of the 16 different lysis buffers for 30 min on ice, and then the solubilized supernatant was separated from insoluble materials by high speed centrifugation at 20,000 × *g*, 4^0C, 20 min. The solubilized hmPRα fraction (about 0.5 mg/ml) was applied on Ni-NTA resin. Unbound materials in the Ni-NTA resin were separated by low speed centrifugation and subsequently bound proteins were eluted by elution buffer. Remaining materials were solubilized by denaturing buffer for SDS-PAGE. The contents of hmPRα in each fraction were analyzed by western blotting using anti-His-tag antibodies. Out of the 16 buffers tested, lysis buffer of 40 mM imidazole, pH 6.0 showed the best separating performance of hmPRα from other proteins (Fig. 19C). As a result, this buffer was chosen as the lysis buffer and as well Ni-NTA chromatography running buffer.

Recombinant hmPRα was purified by three steps of column chromatography. Before applying on column, the insoluble particles were removed from the samples, using filtration on Mixed Cellulose Ester filter. This filter was selected out of the 3 filters (Glass filter, Cellulose Acetate and Mixed Cellulose Ester) (Fig. 20). The first steps of purification, the solubilized sample were separated on Ni-NTA column. The protein content of the eluted fractions was assessed by CBBR and immunoblotting using anti-His-tag antibodies. hmPRα protein was detected in the fractions 11 to 16 (Fig. 21) which corresponded to 160 mM imidazole in the buffer. These fractions were collected and applied on a Cellufine Amino column, which previously determined as an effective resin for the purification of mPRα protein (Oshima et al. 2014). The proteins were eluted from the column by linear gradient of sodium chloride which was visualized by CBBR staining and immunoblotting analysis (Fig. 22). In the third purification step, the hmPRα fractions passed through a SP-Sepharose column. The purified hmPRα proteins were concentrated and collected using Cellufine Amino resin. The SDS-PAGE stained with CBBR and immunoblotting assay clearly demonstrated that the recombinant hmPRα was successfully purified with higher purity (Fig. 23). Additionally, four different gel columns chromatography were tested for the hmPRα protein purification. A Sephacryl S-300 (SIGMA-ALDRICH) column (ϕ 1.6 × 35 cm) was tested for fractionation of the concentrated Ni-NTA fractions (Fig. 24). Also Sephadex G-75, G-150 and G-200 (Pharmacia Biotech, Sweden) columns (ϕ 1.6 × 35 cm) were tested for fractionation, where I applied the concentrated Ni-NTA fractions (Fig. 25A). The results of gel chromatography were not effective for the separation of hmPRα proteins from other impurities (Fig. 25). Then I applied vortex for 30 seconds or sonication for 1 minute on the concentrated Ni-NTA fractions in the presence of 0.1% DDM to dissociate protein complex

that might be formed after solubilization. Samples were subsequently applied on a Sephadex G-150 column (ϕ 1.6 × 35cm). This fractionation was also not effective for separating the hmPRα protein from other proteins (Fig. 26).

3.4 Characterization of purified recombinant hmPRα

To identify the purified protein, MALDI-TOF/MS analysis was conducted. Peptide mass fingerprint analysis of the purified 50-kDa protein was confirmed as a hmPRα (Fig. 27 and 28). In the peptide mass fingerprint analysis, mascot protein score was 85 (67 ≤ is significant). From the results, it is concluded that the recombinant hmPRα protein was successfully expressed and purified.

In order to examine the binding activity of solubilized hmPRα, I examined several components of reaction mixture for the steroid binding assay. Firstly I applied PBS buffer, PBS with BSA, PBS with albumin, Ni-NTA chromatography buffer, Ni-NTA buffer with BSA or Ni-NTA buffer with albumin. Among those components, the specific binding was measured in Ni-NTA-BSA reaction mixture (Fig. 29), but the activity measured was very low. Then, I checked the amount of mPR protein remained on binding assay membrane after application of reaction mixture and after washing the membrane with washing buffer. By the western blot, it is demonstrated that no significant amount of mPR proteins remained on membrane (Fig. 30). Then I tested two conditions, with or without wash membrane. After wash the membrane, the membrane bound protein was examined by western blot. The specific binding activity was detected in no wash samples (Fig. 31A). Western blot result showed that hmPRα protein was washed out from the membrane with washing buffer (Fig. 31B). Therefore, I modified the method of steroid binding assay for solubilized mPR proteins. When Ni-NTA resin was supplemented into the reaction mixture, the steroid binding activity of hmPRα could be detected (Fig. 32). Using this method, specific [^{3}H]1,2,6,7-progesterone binding activity was detected in the purified hmPRα fraction. Saturation analysis demonstrated that progestin binding to the purified hmPRα was saturable and has limited capacity (Bmax=111.6fmol/mg), moreover scatchard analysis showed the presence of a single class of high affinity binding sites (Kd=5.2 nM) (Fig. 33).

DISCUSSION

G-Protein coupled receptors (GPCRs) play the essential roles to regulate the physiological processes and these receptors are the major target （approximately 50%） of modern medicinal drugs and there is a wide consent that drug development activities against GPCRs will continue for the near future. The 3D structure and screening of GPCRs can provide essential information to understand its functions and the design of new drugs. However, the success of structure determination and development of the screening system relies mostly on the production of recombinant GPCRs, because the expression levels of GPCRs are very low and difficult to purify from the native tissue (Shiroishi et al. 2011). On the other hand, recombinant GPCR protein production in a functional form is known to be difficult partly for their structural flexibility (Lundstrom et al. 2006). However it is demonstrated that *P. pastoris* yeast is proficient to express heterologous genes at high levels, due to its strong and tightly regulated AOX1 promoter and it is very effective for the production of various recombinant heterologous proteins (Sears et al. 1998, Cregg et al. 2000, Macauley-Patrick et al. 2005, Asada et al. 2011). It is also reported that 94% of a total of 103 recombinant GPCRs were expressed in yeast cell membranes (Lundstrom et al. 2006).

Previously, a method was established in my laboratory for the expression and purification of gmPRα protein using *Pichia* (Oshima et al. 2014). The gmPRα protein was expressed by the induction for 24 hours at 20^0C with the cell densities in OD_{600} of 1.0-3.0. MS 100 Micro Smash equipment was used for the disruption of yeast cells and DDM was used for the solubilization of gmPRα protein. The gmPRα protein was purified on Ni-NTA, Cellufine Amino and anti-c-Myc-Tag beads column chromatography accordingly. However, highly purified gmPRα was obtained by the established method but the yield was very low. The amount of purified protein was hard to apply for further analysis.

Based on the procedure of gmPRα, in my study, several conditions were optimized for the expression and purification of higher amount of recombinant hmPRα protein. Here I optimized mainly 4 conditions

Firstly, the time and conditions for induction of expression was optimized. Several conditions, temperature and cell densities were tested. The production of hmPRα in *P. pastoris* was detected from 4 hours after start of induction and the amount was increased to 6 hours. After 24 hours, the expressed hmPRα was degraded in *P. pastoris* (Fig. 15). Thus, the induction of hmPRα protein was stopped at 6 hours. Various cell densities of *P. pastoris* (estimated by OD_{600} 5, 10 and 20 to 23) before the start of induction were tested. When the

cell density was increased the amount of hmPRα was increased (Fig 16B) and OD_{600} at 20 to 23 was the best. That was indicated two possibilities. First, short induction time reduced the protein degradation during induction period. Second, in higher cell densities the yeast will not proliferate than lower conditions. Thus it is speculated that cells consumed much energy for production of recombinant proteins than proliferations. Thus, OD_{600} at 21 to 23 was selected for the production of hmPRα. This optimization increased the level of production of hmPRα compared to gmPRα expression system.

On the progesterone binding assay of the crude membrane fractions of yeast containing hmPRα, the fraction showed binding activity of Kd=3.8 nM and Bmax=288.8 fmol/mg for progesterone. The Kd value of hmPRα expressing yeast membrane fractions was almost same as reported Kd =4.17 nM of MDA-MB-231 breast cancer cell membrane fractions which express hmPRα (Thomas et al. 2007). The result indicated that hmPRα expressed in yeast was active. The steroid binding activity for crude membrane fractions prepared from hmPRα-expressing *P. pastoris* cells was detected in the presence of digitonin. The digitonin was effective to perform the binding assay of mPR on bovine ovarian membrane fractions (Rae et al. 1998). Binding activity of membrane fraction of human sperm with progestin has also been detected in the presence of digitonin, (Ambhaikar and Puri 1998). A similar effect was demonstrated in gmPRα expressing *P. pastoris* membrane fractions (Oshima et al. 2014).

Secondly, I applied a new method for disrupting the yeast cells using a stainless Ball Mill (PM 100, Verder Scientific Co., Ltd., Haan, Germany) (Domanski et al. 2012, LaCava et al. 2015) instead of beads crusher (Micro-Smash MS100, TOMY Seiko). The Ball Mill could disrupt the cells from 500 ml culture within 12 minutes under the freezing condition at once. Higher amount of mPR protein could be obtained in the membrane fractions by using Ball Mill PM 100 than beads crusher (Fig. 17). By this step, we could get even clear membrane fractions compare to beads crusher. It is speculated two reasons for this proper result. One possible reason was that disruption of cells could be conducted in freezing condition. Thus protein degradation of expressed protein was reduced. Furthermore, we can pool the crushed samples in freezer until used for purification. This way realized the large amount purification at once. Another reason was the possibility that only cell surface of yeast was breakdown. In freezing condition, only the yeast cell surface might be broken by the attack with balls. Thus, I could obtained more clear solubilized samples and this step even contributed for purification.

Thirdly, I optimized the buffer conditions for nickel-nitrilotriacetic acid (Ni-NTA) column. The Ni-NTA is commonly used for the first step during purification of His-tagged

protein. In the gmPRα purification, general conditions for Ni-NTA fractionation was used (Oshima et al. 2014), but gmPRα eluted as a broad peak and separation from other proteins was not good. Thus I tested 16 kinds of buffers with low to high pH (pH5.0-8.0) and lower to higher imidazole concentration (10-80mM). Among them, low pH (pH 6.0) and high imidazole (40 mM) containing buffer was the most effective for the purification of hmPRα (Fig. 19). Thus I applied the buffer (50 mM NaH_2PO_4, 300 mM NaCl, 40 mM Imidazole pH 6.0) on Ni-NTA column chromatography. By which, I could fractionate the hmPRα within a short duration and resulted in the proper performance to purify hmPRα.

By combined above mentioned optimizations, I could establish the way to collect 100 times higher amount of hmPRα than previous method (Table 2).

Fourth, I established a method to measure the steroid binding activity of solubilized and purified hmPRα. There was difficulty of steroid binding assay on filter for solubilized and purified mPR proteins, because mPR proteins can pass through the filter membrane. Previously, in the binding assay analysis of solubilized gmPRα, BSA was added in the reaction mixture, where it helped to trap the gmPRα on the filter (Oshima et al. 2014). Thus I added BSA with mPRα in the binding reaction mixture and measured the specific binding of steroids. I also measured without BSA, with or without wash. Among them no wash (without BSA) showed the higher specific binding activity, compared to BSA supplemented (Fig 29, 31). On the other hand, when washed the samples did not get any binding activity (Fig. 31). That mean, mPR protein passed through the filter with washing buffer. So, I tried to add Ni-NTA resin (100 μl of 50% vol) in the reaction mixture, which helped to trap the hmPRα on filter membrane. By this way, I succeeded to establish a strategy for the binding assay of solubilized and purified mPR protein. The purified hmPRα protein was demonstrated its binding activity for progesterone (Kd = 5.2 nM and Bmax = 111.6 fmol/mg). The Kd value of purified hmPRα did not change significantly, compared to the value of membrane fractions. This result indicated that I succeeded in solubilizing and purification of hmPRα in an active form.

By these optimizations, I established the procedures for hmPRα production and purification. The DDM solubilized hmPRα was purified through three different column chromatography steps. Firstly, a Ni-NTA column was used. Then, the hmPRα proteins were bound to cellulose resin with free amino groups (Cellufine Amino column) and finally passed through a SP-Sepharose column. By the optimized expression and purification conditions, higher amount of hmPRα (1.2-1.5 mg) obtained from 1 L culture (0.8 – 1% of total hmPRα) (Table 2). The yield of activity was increased by more than 100 times compared to that of

goldfish mPRα expression and purification (Table 1). The identity of the purified protein was confirmed by MALDI-TOF/MS analysis.

By this method it was became possible to get relatively higher amount of hmPRα in low cast. In addition this study developed a new strategy to measure the binding activity of purified mPR proteins. The purified recombinant hmPRα could be a promising tool for screening of ligands of hmPRα and be a source of structure resolution approach and monoclonal antibody production.

The mPRs are expressed in a broad range of organisms from fish to humans (Thomas 2008, Tokumoto et al. 2012), and particularly progestin binding activity in these group animals was revealed; for example, fish (goldfish, seatrout and zebrafish), frog and mammals (cattle, rat, mouse, human) (Tokumoto et al. 2006, Josefsberg Ben-Yehoshua et al. 2007, Smith et al. 2008, Tubbs and Thomas 2009). The mPRs were found to be expressed in the reproductive tissues (ovary, uterus and testes), kidneys, brain and spinal cord among vertebrates, including fish, mice and humans (Zhu et al. 2003, Zhu et al. 2003, Hanna et al. 2006, Labombarda et al. 2010). The broad distribution of mPRs suggests that these receptor proteins play a role in a wide variety of steroid related functions in tissues.

Researchers reported that mRNA of mPRα and β were expressed in the central nervous system of mice (Intlekofer and Petersen 2011). These findings suggested that mPRα and β are involved to mediate neural actions of progesterone. The expression of 5 subtypes of mPR were analyzed by q-PCR in human brain (Pang et al. 2013). Among the mPR subtypes, mPRε was the most abundant subtype in the brain and is a potential intermediary of the antiapoptotic effects of neurosteroids in the central nervous system. The roles of brain mPRs in the regulation of mammalian sex behavior have also been investigated (Frye et al. 2013, Frye et al. 2014).

Researchers investigated the progesterone signaling through mPRs in human breast cancer cell and it is demonstrated that mPRs are participated for the development of breast tumor growth through inhibit apoptosis in cancer cell (Dressing et al. 2012). It is proposed that the gene expression level of mPRα could be a biomarker for breast cancer survival (Xie et al. 2012). Recently, it is demonstrated that progesterone activated the pathway to generate cancer stem cells through mPRs in mammary cells (Vares et al. 2015).

Progesterone works as immuno-modulator which may interact with mPRα, mPRβ and mPRγ and make a rapid nongenomic responses to inhibit the human T-cells proliferation that may attack fetus during pregnancy (Chien et al. 2009). It is investigated that progesterone signaling by mPRα is associated with the inflammatory responses in the murine macrophage

cells function, parturition and this affiliation may contribute to the functional withdrawal of progesterone, shows the way to labor (Lu et al. 2015).

In view of the fact that mPRs are the potential mediator for the various cellular responses to progesterone, attentions to discover new drugs or facilitate to the remedial way of diseases like as reproductive problems, cancers and encephalitis.

Very recently, the three–dimensional structure of PAQR1 and its functional characteristics was reported (Tanabe et al. 2015). HmPRα belongs to a GPCR family and classified into the protein family, progestin and adipoQ receptor (PAQR) family. The strategy of structure resolution could be applied for mPRs. The active recombinant hmPRα protein produced in this study will be useful for such an approach on the fields of GPCR study and drug development.

CONCLUTION AND FUTURE PROSPECTS

Although in this dissertation, the key focus deployed on the production of recombinant human membrane progestin receptor alpha (hmPRα) in an active form using yeast (*P. pastoris*) expression system and a method improved to purify the hmPRα protein with keeping its active status. A novel method is developed for expression and production of the goldfish mPRα (gmPRα) by yeast (*P. pastoris*) expression system that was first reported by Oshima et al. in 2014. This expression system compared to gmPRα expression is less time consuming and might be used for the production of mPRs in a large scale. This study also emphasized on the disruption of yeast cells, Ball Mill PM100 can be used for the powerful and quick grinding of yeast cells. Cells were disrupted in a freeze environment, by which the hmPRα proteins were kept in stable conditions. The developed purification procedures by which large amount of pure and functional mPR proteins could be produced that will facilitate the mPR research.

mPRs are likely to be involved in various functions induced by progestin's because they are expressed in a wide variety of tissues, as well progestin's have been shown to exert rapid, nongenomic actions. As a probable and new promising field, progestins signaling for many aspects through membrane progestin receptors (mPRs) remain unresolved and delimited by controversy. However, remarkable progress for understanding the characteristics and physiological functions of mPRα have been made. Recently in six vertebrate species; seatrout, goldfish, zebrafish, *Xenopus*, sheep and human, progestin binding characteristics with recombinant mPRαs has been established. Researchers demonstrated the signal transduction pathways activated by progestin in mPRα-transfected cell lines. mPRα is expressed on the plasma membrane and functions as a mediator in progestin's activation of intracellular signaling pathways. Moreover high expression levels of mRNA for mPRα has been reported in breast cancer cells and ovarian cancer cells that mediated for the inhibition of cancer cell apoptosis. mPRα is also involved to progestin stimulation for the teleost sperm hypermotility.On the other hand putative progesterone receptors have been identified on mammalian sperm, but it is still complicated for receptor roles in progesterone actions for the induction of acrosome reaction and hyper-activation. Another important role for the MIS induction of oocyte maturation in fish via mPRα, associated with inhibitory G protein (Gi) pathway has been characterized. An unusual ligand for goldfish mPRα diethylstilbestrol (an EDC) showed its effectiveness for the inducing oocyte maturation. Thus, mPRα has

prospects for the potential role to cellular response associated with progesterone interaction which has been drawn some attention to discover some new drugs or cause and fact finding approach like as reproductive problem, breast cancer, male contraception and some therapies of encephalitis. My study would be applicable for the screening of agonist and antagonist ligands for hmPRα. Moreover the sufficient amount of purified hmPRα can be useful for the structure resolution approach, as well it can be useful for the production of monoclonal antibody, by which open the new door to elucidate the central role of hmPRα.

Dedicated to my Beloved Father

ACKNOLEDGEMENTS

First and foremost I express my sincerest gratitude to Almighty Allah for blessings me. I heartily express my deepest gratitude and indebtedness to my beloved and honorable Prof. Dr. Toshinobu Tokumoto, Chairman, Department of Biology, Shizuoka University, Japan for his unwavering support, supervision and mentorship as well as provided me a wonderful laboratory for doing research in Shizuoka University. His guidance and help which is un-measurable and without it I would not be where I am today. He has enlightened my life. His immense knowledge, commitment, hard-work and inventiveness are the landmarks to follow in my life. I would like to extend my thanks for his valuable advice and guidance to start my graduate career on the right way and built up the foundation for becoming a researcher.

I am very grateful to the Environmental Leaders Program (ELSU) of Shizuoka University, provided the fellowship for my PhD study and lead my daily life smoothly in Shizuoka, Japan.

I am also very grateful to the Ministry of Education, Government of the People's Republic of Bangladesh, for providing me the deputation to accomplish this research.

Special thanks to Dr. Takayuki Oshima and Shizuoka Hirose for their support during my research.

I would like to thank all of my lab members (past and present) for their helps and suggestions.

I am also thankful to my parents, who raised me with a love of science and supported me in all my pursuits. Recently my father has died. No words are sufficient to describe my father’s contribution in my life. This dissertation is dedicated for his memory.

My grateful thanks and dedication to my wife Shamima Pervin, daughter Fahmida Faiza and son Shoaib Hossain, for their continuous solacing support and patience.

I am also grateful to the stuff of Radiochemistry Research laboratory and Research Support Office in the Research Institute of Green Science and Technology of Shizuoka University for the use of equipments.

I also express my thanks to ELSU friends and Bangladeshi friends and well wishers in Shizuoka University for their inspiration and encouragement.

The author

REFERENCES

Alamae (1995). "Permeabilization of the methylotrophic yeast Pichia pinus for intracellular enzyme analysis: a quantitative study." J Microbiol Methods **22**: 193–205.

Ambhaikar, M. and C. Puri (1998). "Cell surface binding sites for progesterone on human spermatozoa." Mol Hum Reprod **4**(5): 413-421.

Aparicio, I. M., M. Garcia-Herreros, L. C. O'Shea, C. Hensey, P. Lonergan and T. Fair (2011). "Expression, regulation, and function of progesterone receptors in bovine cumulus oocyte complexes during in vitro maturation." Biol Reprod **84**(5): 910-921.

Asada, H., T. Uemura, T. Yurugi-Kobayashi, M. Shiroishi, T. Shimamura, H. Tsujimoto, K. Ito, T. Sugawara, T. Nakane, N. Nomura, T. Murata, T. Haga, S. Iwata and T. Kobayashi (2011). "Evaluation of the Pichia pastoris expression system for the production of GPCRs for structural analysis." Microb Cell Fact **10**: 24.

Ashley, R. L., J. A. Arreguin-Arevalo and T. M. Nett (2009). "Binding characteristics of the ovine membrane progesterone receptor alpha and expression of the receptor during the estrous cycle." Reprod Biol Endocrinol **7**: 42.

Bockaert, J. and J. P. Pin (1999). "Molecular tinkering of G protein-coupled receptors: an evolutionary success." Embo J **18**(7): 1723-1729.

Boonyaratanakornkit, V. and D. P. Edwards (2004). "Receptor mechanisms of rapid extranuclear signalling initiated by steroid hormones." Essays Biochem **40**: 105-120.

Carnevali, O., L. Tosti, C. Speciale, C. Peng, Y. Zhu and F. Maradonna (2007). "DEHP impairs zebrafish reproduction by affecting critical factors in oogenesis." PLoS One **5**(4): e10201.

Charles, N. J., P. Thomas and C. A. Lange (2010). "Expression of membrane progesterone receptors (mPR/PAQR) in ovarian cancer cells: implications for progesterone-induced signaling events." Horm Cancer **1**(4): 167-176.

Chien, C. H., J. N. Lai, C. F. Liao, O. Y. Wang, L. M. Lu, M. I. Huang, W. F. Lee, M. C. Shie and E. J. Chien (2009). "Mifepristone acts as progesterone antagonist of non-genomic responses but inhibits phytohemagglutinin-induced proliferation in human T cells." Hum Reprod **24**(8): 1968-1975.

Chien, E. J., C. P. Chang, W. F. Lee, T. H. Su and C. H. Wu (2006). "Non-genomic immunosuppressive actions of progesterone inhibits PHA-induced alkalinization and activation in T cells." J Cell Biochem **99**(1): 292-304.

Cicirelli, M. F. and L. D. Smith (1985). "Cyclic AMP levels during the maturation of Xenopus oocytes." Dev Biol **108**(1): 254-258.

Cregg, J. M. (1985). "Pichia pastoris as a host system for transformations." Mol. Cell. Biol **5**: 3376–3385.

Cregg, J. M., J. L. Cereghino, J. Shi and D. R. Higgins (2000). "Recombinant protein expression in Pichia pastoris." Mol Biotechnol **16**(1): 23-52.

Cregg, J. M., T. S. Vedvick and W. C. Raschke (1993). "Recent advances in the expression of foreign genes in Pichia pastoris." Biotechnology (N Y) **11**(8): 905-910.

DeBold, J. F. and C. A. Frye (1994). "Genomic and non-genomic actions of progesterone in the control of female hamster sexual behavior." Horm Behav **28**(4): 445-453.

Domanski, M., K. Molloy, H. Jiang, B. T. Chait, M. P. Rout, T. H. Jensen and J. LaCava (2012). "Improved methodology for the affinity isolation of human protein complexes expressed at near endogenous levels." Biotechniques **0**(0): 1-6.

Dressing, G. E., R. Alyea, Y. Pang and P. Thomas (2012). "Membrane progesterone receptors (mPRs) mediate progestin induced antimorbidity in breast cancer cells and are expressed in human breast tumors." Horm Cancer **3**(3): 101-112.

Finet, B., B. Jalabert and S. K. Garg (1988). "Effect of defolliculation and 17 alpha-hydroxy, 20 beta-dihydroprogesterone on cyclic AMP level in full-grown oocytes of the rainbow trout, Salmo gairdneri." Gamete Res **19**(3): 241-252.

Frye, C. A., C. J. Koonce and A. A. Walf (2013). "Pregnane xenobiotic receptors and membrane progestin receptors: role in neurosteroid-mediated motivated behaviours." J Neuroendocrinol **25**(11): 1002-1011.

Frye, C. A., K. Sumida, J. P. Lydon, B. W. O'Malley and D. W. Pfaff (2006). "Mid-aged and aged wild-type and progestin receptor knockout (PRKO) mice demonstrate rapid progesterone and 3alpha,5alpha-THP-facilitated lordosis." Psychopharmacology (Berl) **185**(4): 423-432.

Frye, C. A., A. A. Walf, A. S. Kohtz and Y. Zhu (2014). "Progesterone-facilitated lordosis of estradiol-primed mice is attenuated by knocking down expression of membrane progestin receptors in the midbrain." Steroids **81**: 17-25.

Godeau, J. F., S. Schorderet-Slatkine, P. Hubert and E. E. Baulieu (1978). "Induction of maturation in Xenopus laevis oocytes by a steroid linked to a polymer." Proc Natl Acad Sci U S A **75**(5): 2353-2357.

Goetz, F. W. and G. Theofan (1979). "In vitro stimulation of germinal vesicle breakdown and ovulation of yellow perch (Perca flavescens) oocytes. Effects of 17 alpha-hydroxy-20 beta-dihydroprogesterone and prostaglandins." Gen Comp Endocrinol **37**(3): 273-285.

Hanna, R., Y. Pang, P. Thomas and Y. Zhu (2006). "Cell-surface expression, progestin binding, and rapid nongenomic signaling of zebrafish membrane progestin receptors alpha and beta in transfected cells." J Endocrinol **190**(2): 247-260.

Hanna, R. N. and Y. Zhu (2009). "Expression of membrane progestin receptors in zebrafish (Danio rerio) oocytes, testis and pituitary." Gen Comp Endocrinol **161**(1): 153-157.

Hanna, R. N. and Y. Zhu (2011). "Controls of meiotic signaling by membrane or nuclear progestin receptor in zebrafish follicle-enclosed oocytes." Mol Cell Endocrinol **337**(1-2): 80-88.

Hino, T., T. Arakawa, H. Iwanari, T. Yurugi-Kobayashi, C. Ikeda-Suno, Y. Nakada-Nakura, O. Kusano-Arai, S. Weyand, T. Shimamura, N. Nomura, A. D. Cameron, T. Kobayashi, T. Hamakubo, S. Iwata and T. Murata (2012). "G-protein-coupled receptor inactivation by an allosteric inverse-agonist antibody." Nature **482**(7384): 237-240.

Intlekofer, K. A. and S. L. Petersen (2011). "Distribution of mRNAs encoding classical progestin receptor, progesterone membrane components 1 and 2, serpine mRNA binding protein 1, and progestin and ADIPOQ receptor family members 7 and 8 in rat forebrain." Neuroscience **172**: 55-65.

Ishikawa, K., Y. Hanaoka, Y. Kondo and K. Imai (1977). "Primary action of steroid hormone at the surface of amphibian oocyte in the induction of germinal vesicle breakdown." Mol Cell Endocrinol **9**(1): 91-100.

Jalabert, B., C. Bry, B. Breton and C. Campbell (1976). "[Effect of 17 alpha hydroxy-20 beta dihydroprogesterone and progesterone on maturation and ovulation in vivo and on the plasma level of gonadotropic hormone t-GtH in rainbow trout Salmo gairdneri]." C R Acad Sci Hebd Seances Acad Sci D **283**(10): 1205-1208.

Josefsberg Ben-Yehoshua, L., A. L. Lewellyn, P. Thomas and J. L. Maller (2007). "The role of Xenopus membrane progesterone receptor beta in mediating the effect of progesterone on oocyte maturation." Mol Endocrinol **21**(3): 664-673.

Karteris, E., S. Zervou, Y. Pang, J. Dong, E. W. Hillhouse, H. S. Randeva and P. Thomas (2006). "Progesterone signaling in human myometrium through two novel membrane G protein-coupled receptors: potential role in functional progesterone withdrawal at term." Mol Endocrinol **20**(7): 1519-1534.

Ke, F. C. and V. D. Ramirez (1987). "Membrane mechanism mediates progesterone stimulatory effect on LHRH release from superfused rat hypothalami in vitro." Neuroendocrinology **45**(6): 514-517.

King, W. t., S. Ghosh, P. Thomas and C. V. Sullivan (1997). "A receptor for the oocyte maturation-inducing hormone 17alpha,20beta,21-trihydroxy-4-pregnen-3-one on ovarian membranes of striped bass." Biol Reprod **56**(1): 266-271.

Labombarda, F., D. Meffre, B. Delespierre, S. Krivokapic-Blondiaux, A. Chastre, P. Thomas, Y. Pang, J. P. Lydon, S. L. Gonzalez, A. F. De Nicola, M. Schumacher and R. Guennoun (2010). "Membrane progesterone receptors localization in the mouse spinal cord." Neuroscience **166**(1): 94-106.

LaCava, J., K. R. Molloy, M. S. Taylor, M. Domanski, B. T. Chait and M. P. Rout (2015). "Affinity proteomics to study endogenous protein complexes: pointers, pitfalls, preferences and perspectives." Biotechniques **58**(3): 103-119.

Lu, J., J. Reese, Y. Zhou and E. Hirsch (2015). "Progesterone-induced activation of membrane-bound progesterone receptors in murine macrophage cells." J Endocrinol **224**(2): 183-194.

Lundstrom, K., R. Wagner, C. Reinhart, A. Desmyter, N. Cherouati, T. Magnin, G. Zeder-Lutz, M. Courtot, C. Prual, N. Andre, G. Hassaine, H. Michel, C. Cambillau and F. Pattus (2006). "Structural genomics on membrane proteins: comparison of more than 100 GPCRs in 3 expression systems." J Struct Funct Genomics **7**(2): 77-91.

Macauley-Patrick, S., M. L. Fazenda, B. McNeil and L. M. Harvey (2005). "Heterologous protein production using the Pichia pastoris expression system." Yeast **22**(4): 249-270.

Mizutani, K., K. Hashimoto, N. Takahashi, M. Hirose, S. Aibara and B. Mikami (2010). "Structural and functional characterization of recombinant human serum transferrin secreted from Pichia pastoris." Biosci Biotechnol Biochem **74**(2): 309-315.

Mizutani, K., I. Okamoto, K. Fujita, K. Yamamoto and M. Hirose (2004). "Structural and functional characterization of ovotransferrin produced by Pichia pastoris." Biosci Biotechnol Biochem **68**(2): 376-383.

Mizutani, K., M. Toyoda, Y. Otake, S. Yoshioka, N. Takahashi and B. Mikami (2012). "Structural and functional characterization of recombinant medaka fish alpha-amylase expressed in yeast Pichia pastoris." Biochim Biophys Acta **1824**(8): 954-962.

Mourot, B., T. Nguyen, A. Fostier and J. Bobe (2006). "Two unrelated putative membrane-bound progestin receptors, progesterone membrane receptor component 1 (PGMRC1) and membrane progestin receptor (mPR) beta, are expressed in the rainbow trout oocyte and exhibit similar ovarian expression patterns." Reprod Biol Endocrinol **4**: 6.

Nagahama, Y. and S. Adachi (1985). "Identification of maturation-inducing steroid in a teleost, the amago salmon (Oncorhynchus rhodurus)." Dev Biol **109**(2): 428-435.

Nagahama, Y. and M. Yamashita (2008). "Regulation of oocyte maturation in fish." Dev Growth Differ **50 Suppl 1**: S195-219.

Nagahama, Y., M. Yoshikuni, M. Yamashita, T. Tokumoto and Y. Katsu (1995). "Regulation of oocyte growth and maturation in fish." Curr Top Dev Biol **30**: 103-145.

Nakashima, M., M. Suzuki, M. Saida, Y. Kamei, M. B. Hossain and T. Tokumoto (2015). "Cell-based assay of nongenomic actions of progestins revealed inhibitory G protein coupling to membrane progestin receptor alpha (mPRalpha)." Steroids **100**: 21-26.

Norman, A. W., M. T. Mizwicki and D. P. Norman (2004). "Steroid-hormone rapid actions, membrane receptors and a conformational ensemble model." Nat Rev Drug Discov **3**(1): 27-41.

Oshima, T., R. Nakayama, S. R. Roy and T. Tokumoto (2014). "Purification of the goldfish membrane progestin receptor alpha (mPRalpha) expressed in yeast Pichia pastoris." Biomed Res **35**(1): 47-59.

Pace, M. C. and P. Thomas (2005). "Activation of a pertussis toxin-sensitive, inhibitory G-protein is necessary for steroid-mediated oocyte maturation in spotted seatrout." Dev Biol **285**(1): 70-79.

Pang, Y., J. Dong and P. Thomas (2013). "Characterization, Neurosteroid Binding and Brain Distribution of Human Membrane Progesterone Receptors delta and {epsilon} (mPRdelta and mPR{epsilon}) and mPRdelta Involvement in Neurosteroid Inhibition of Apoptosis." Endocrinology **154**(1): 283-295.

Patino, R. and P. Thomas (1990). "Characterization of membrane receptor activity for 17 alpha, 20 beta, 21-trihydroxy-4-pregnen-3-one in ovaries of spotted seatrout (Cynoscion nebulosus)." Gen Comp Endocrinol **78**(2): 204-217.

Patino, R. and P. Thomas (1990). "Gonadotropin stimulates 17 alpha,20 beta,21-trihydroxy-4-pregnen-3-one production from endogenous substrates in Atlantic croaker ovarian follicles undergoing final maturation in vitro." Gen Comp Endocrinol **78**(3): 474-478.

Peluso, J. J., A. Pappalardo, R. Losel and M. Wehling (2006). "Progesterone membrane receptor component 1 expression in the immature rat ovary and its role in mediating progesterone's antiapoptotic action." Endocrinology **147**(6): 3133-3140.

Rae, M. T., G. S. Menzies and T. A. Bramley (1998). "Bovine ovarian non-genomic progesterone binding sites: presence in follicular and luteal cell membranes." J Endocrinol **159**(3): 413-427.

Rahman, M., K. Ohta, M. Yoshikuni, Y. Nagahama, H. Chuda and M. Matsuyama (2002). "Characterization of ovarian membrane receptor for 17,20beta-dihydroxy-4-pregnen-3-one, a maturation-inducing hormone in yellowtail, Seriola quinqueradiata." Gen Comp Endocrinol **127**(1): 71-79.

Revelli, A., M. Massobrio and J. Tesarik (1998). "Nongenomic actions of steroid hormones in reproductive tissues." Endocr Rev **19**(1): 3-17.

Sears, I. B., J. O'Connor, O. W. Rossanese and B. S. Glick (1998). "A versatile set of vectors for constitutive and regulated gene expression in Pichia pastoris." Yeast **14**(8): 783-790.

Shimamura, T., M. Shiroishi, S. Weyand, H. Tsujimoto, G. Winter, V. Katritch, R. Abagyan, V. Cherezov, W. Liu, G. W. Han, T. Kobayashi, R. C. Stevens and S. Iwata (2011). "Structure of the human histamine H1 receptor complex with doxepin." Nature **475**(7354): 65-70.

Shiroishi, M., T. Kobayashi, S. Ogasawara, H. Tsujimoto, C. Ikeda-Suno, S. Iwata and T. Shimamura (2011). "Production of the stable human histamine H(1) receptor in Pichia pastoris for structural determination." Methods **55**(4): 281-286.

Smith, J. L., B. R. Kupchak, I. Garitaonandia, L. K. Hoang, A. S. Maina, L. M. Regalla and T. J. Lyons (2008). "Heterologous expression of human mPRalpha, mPRbeta and mPRgamma in yeast confirms their ability to function as membrane progesterone receptors." Steroids **73**(11): 1160-1173.

Stanczyk, F. Z. (2003). "All progestins are not created equal." Steroids **68**(10-13): 879-890.

Tanabe, H., Y. Fujii, M. Okada-Iwabu, M. Iwabu, Y. Nakamura, T. Hosaka, K. Motoyama, M. Ikeda, M. Wakiyama, T. Terada, N. Ohsawa, M. Hato, S. Ogasawara, T. Hino, T. Murata, S. Iwata, K. Hirata, Y. Kawano, M. Yamamoto, T. Kimura-Someya, M. Shirouzu, T. Yamauchi, T. Kadowaki and S. Yokoyama (2015). "Crystal structures of the human adiponectin receptors." Nature **520**(7547): 312-316.

Tang, Y. T., T. Hu, M. Arterburn, B. Boyle, J. M. Bright, P. C. Emtage and W. D. Funk (2005). "PAQR proteins: a novel membrane receptor family defined by an ancient 7-transmembrane pass motif." J Mol Evol **61**(3): 372-380.

Thomas, P. (2008). "Characteristics of membrane progestin receptor alpha (mPRalpha) and progesterone membrane receptor component 1 (PGMRC1) and their roles in mediating rapid progestin actions." Front Neuroendocrinol **29**(2): 292-312.

Thomas, P. (2012). "Rapid steroid hormone actions initiated at the cell surface and the receptors that mediate them with an emphasis on recent progress in fish models." Gen Comp Endocrinol **175**(3): 367-383.

Thomas, P. and Y. Pang (2012). "Membrane progesterone receptors: evidence for neuroprotective, neurosteroid signaling and neuroendocrine functions in neuronal cells." Neuroendocrinology **96**(2): 162-171.

Thomas, P., Y. Pang, J. Dong, P. Groenen, J. Kelder, J. de Vlieg, Y. Zhu and C. Tubbs (2007). "Steroid and G protein binding characteristics of the seatrout and human

progestin membrane receptor alpha subtypes and their evolutionary origins." Endocrinology **148**(2): 705-718.
Thomas, P., Y. Pang, Y. Zhu, C. Detweiler and K. Doughty (2004). "Multiple rapid progestin actions and progestin membrane receptor subtypes in fish." Steroids **69**(8-9): 567-573.
Thomas, P., Y. Zhu and M. Pace (2002). "Progestin membrane receptors involved in the meiotic maturation of teleost oocytes: a review with some new findings." Steroids **67**(6): 511-517.
Tokumoto, M., Y. Nagahama, P. Thomas and T. Tokumoto (2006). "Cloning and identification of a membrane progestin receptor in goldfish ovaries and evidence it is an intermediary in oocyte meiotic maturation." Gen Comp Endocrinol **145**(1): 101-108.
Tokumoto, T., M. Tokumoto, T. Oshima, K. Shimizuguchi, T. Fukuda, E. Sugita, M. Suzuki, Y. T. Sakae, Y. Akiyama, R. Nakayama, S. R. Roy, M. Saydur Rahman, Y. Pang, J. Dong and P. Thomas (2012). "Characterization of multiple membrane progestin receptor (mPR) subtypes from the goldfish ovary and their roles in the induction of oocyte maturation." Gen Comp Endocrinol **177**(1): 168-176.
Tokumoto, T., M. Tokumoto and P. Thomas (2007). "Interactions of diethylstilbestrol (DES) and DES analogs with membrane progestin receptor-alpha and the correlation with their nongenomic progestin activities." Endocrinology **148**(7): 3459-3467.
Trant, J. M., P. Thomas and C. H. Shackleton (1986). "Identification of 17 alpha,20 beta,21-trihydroxy-4-pregnen-3-one as the major ovarian steroid produced by the teleost Micropogonias undulatus during final oocyte maturation." Steroids **47**(2-3): 89-99.
Tubbs, C., M. Pace and P. Thomas (2010). "Expression and gonadotropin regulation of membrane progestin receptor alpha in Atlantic croaker (Micropogonias undulatus) gonads: role in gamete maturation." Gen Comp Endocrinol **165**(1): 144-154.
Tubbs, C. and P. Thomas (2009). "Progestin signaling through an olfactory G protein and membrane progestin receptor-alpha in Atlantic croaker sperm: potential role in induction of sperm hypermotility." Endocrinology **150**(1): 473-484.
Vares, G., S. Sai, B. Wang, A. Fujimori, M. Nenoi and T. Nakajima (2015). "Progesterone generates cancer stem cells through membrane progesterone receptor-triggered signaling in basal-like human mammary cells." Cancer Lett **362**(2): 167-173.
Watson, C. S. and B. Gametchu (1999). "Membrane-initiated steroid actions and the proteins that mediate them." Proc Soc Exp Biol Med **220**(1): 9-19.
Xie, M., X. Zhu, Z. Liu, M. Shrubsole, V. Varma, I. A. Mayer, Q. Dai, Q. Chen and S. You (2012). "Membrane progesterone receptor alpha as a potential prognostic biomarker for breast cancer survival: a retrospective study." PLoS One **7**(4): e35198.
Yamashita, M., H. Kajiura, T. Tanaka, S. Onoe and Y. Nagahama (1995). "Molecular mechanisms of the activation of maturation-promoting factor during goldfish oocyte maturation." Dev Biol **168**(1): 62-75.
Yoshikuni, M., N. Shibata and Y. Nagahama (1993). "Specific binding of [(3)H]17alpha,20beta-dihydroxy-4-pregnen-3-one to oocyte cortices of rainbow trout (Oncorhynchus mykiss)." Fish Physiol Biochem **11**(1-6): 15-24.
You, S., L. Zuo and V. Varma (2010). "Broad tissue expression of membrane progesterone receptor Alpha in normal mice." J Mol Histol **41**(2-3): 101-110.
Zeder-Lutz, G., N. Cherouati, C. Reinhart, F. Pattus and R. Wagner (2006). "Dot-blot immunodetection as a versatile and high-throughput assay to evaluate recombinant GPCRs produced in the yeast Pichia pastoris." Protein Expr Purif **50**(1): 118-127.
Zhu, Y., J. Bond and P. Thomas (2003). "Identification, classification, and partial characterization of genes in humans and other vertebrates homologous to a fish membrane progestin receptor." Proc Natl Acad Sci U S A **100**(5): 2237-2242.
Zhu, Y., R. N. Hanna, M. J. Schaaf, H. P. Spaink and P. Thomas (2008). "Candidates for membrane progestin receptors--past approaches and future challenges." Comp Biochem Physiol C Toxicol Pharmacol **148**(4): 381-389.

Zhu, Y., C. D. Rice, Y. Pang, M. Pace and P. Thomas (2003). "Cloning, expression, and characterization of a membrane progestin receptor and evidence it is an intermediary in meiotic maturation of fish oocytes." Proc Natl Acad Sci U S A **100**(5): 2231-2236.

Zuo, L., W. Li and S. You (2010). "Progesterone reverses the mesenchymal phenotypes of basal phenotype breast cancer cells via a membrane progesterone receptor mediated pathway." Breast Cancer Res **12**(3): R34.

TABLES AND FIGURES

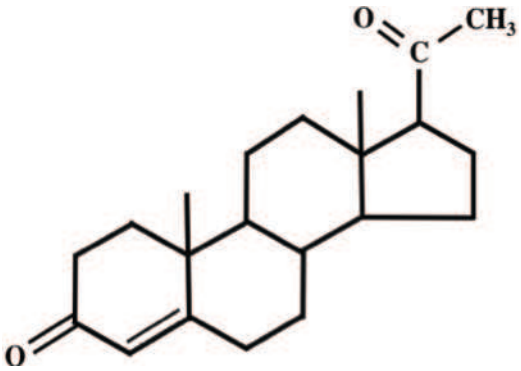
Table 1. Summary of the purification of recombinant human mPRα from *Pichia pastoris* expression system. The activity of every pooled fraction was measured with 4 nM of [^{3}H] 1,2,6,7 progesterone and the yield of total progesterone binding activities of each fractions are presented as percentages of the crude extract, which was assumed to have a binding activity of as 100%.

	Protein (mg)	Total activity [^{3}H] 1,2,6,7 progesterone (nmol)	Specific activity (pmol/mg)	-fold	Yield (%)
Crude extract	1264.5	0.534	0.42	1.0	100
DDM solubilization	197.2	0.232	1.18	2.8	43.4
Ni-NTA	68.4	0.104	1.52	3.6	19.5
Cellufine Amino	4.5	0.015	3.33	8.0	2.8
SP-Sepharose	1.2	0.012	10.00	23.8	2.3

Table 2. Summary of the amount of total protein production and purified human mPRα from *Pichia pastoris* expression system. The amount of total protein was measured by *DC* protein assay. Expressed and purified hmPRα protein was determined by western blot analysis using anti-His-tag antibody.

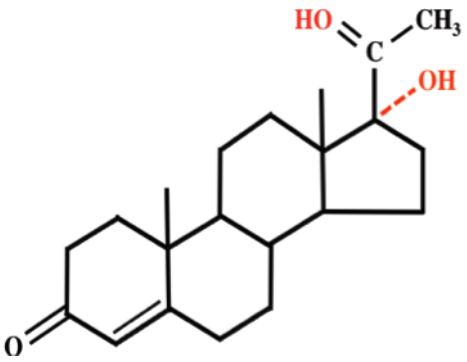
	Total Protein (mg)	hmPRα protein (mg)	Purified hmPRα yield
Crude extract	1264.5	145.5	0.8-1%
DDM solubilization	197.2	26.1	
Ni-NTA	68.4	6.1	
Cellufine Amino	4.5	2.7	
SP-Sepharose	1.2-1.5	1.2-1.5	

A



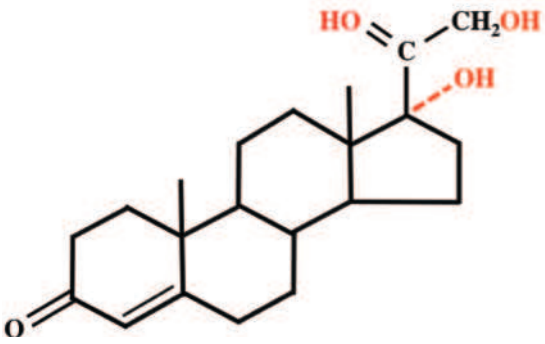
Progesterone

B



17α, 20β-dihydroxy-4 pregnen-3-one (17, 20β-DHP)

C



17α, 20β-trihydroxy-4 pregnen-3-one (17, 20β-S)

Fig. 1. Structure of the natural progestins. (A) Progesterone is an natural progestin of human body. (B) The natural progestins, 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-DHP) was identified from amago salmon (*Oncorhynchus rhodurus*) and (C) 17α,20β,21-trihydroxy-4-pregnen-3-one (17, 20β-S) was identified in Atlantic croaker (*Micropogonias undulatus*) and spotted seatrout (*Cynoscion nebulous*).

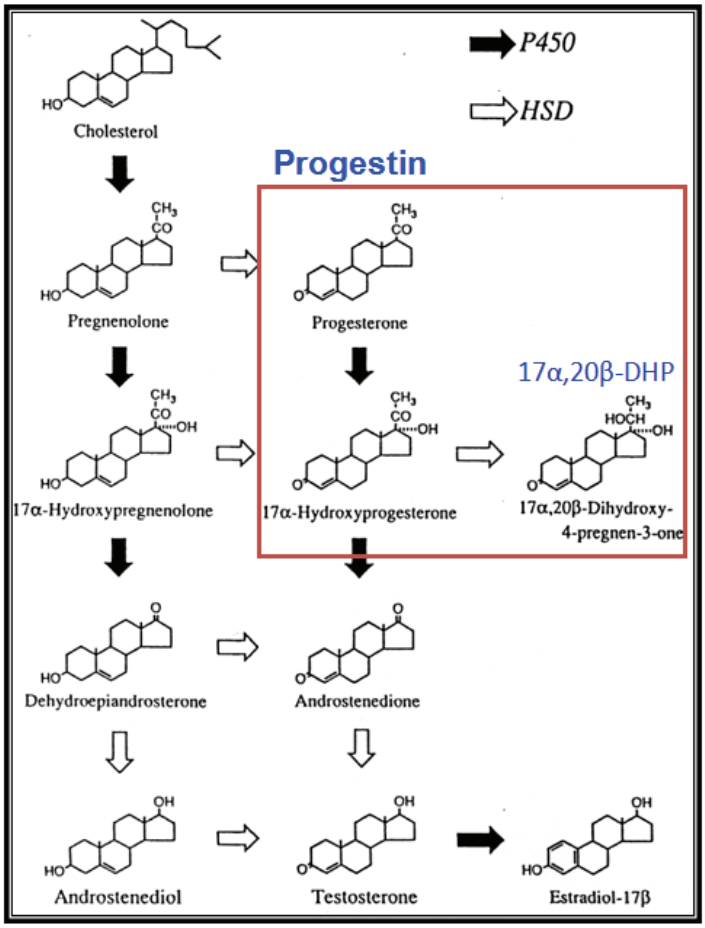


Fig. 2. A schematic representation for the biosynthesis of progestins (steroid hormone). Steroids are lipids derived from cholesterol; include the hormones testosterone, estradiol, progesterone. A series of enzymatic steps in the mitochondria and endoplasmic reticulum of steroidogenic tissues convert cholesterol into steroid hormones and intermediates.

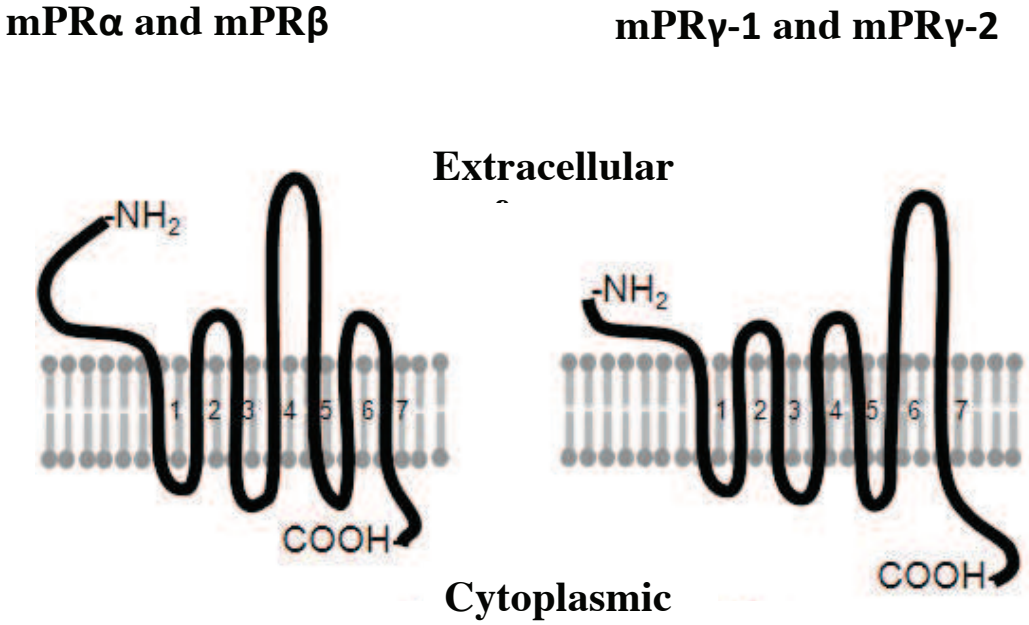


Fig. 3. Predicted topology of membrane progestin receptor (mPR) protein. For plausible structure, computer modeling predicts that the protein has seven transmembrane domains, typical structure of G protein-coupled receptors (several computer programs using as SOSUI, Tmpred, TMHMM)

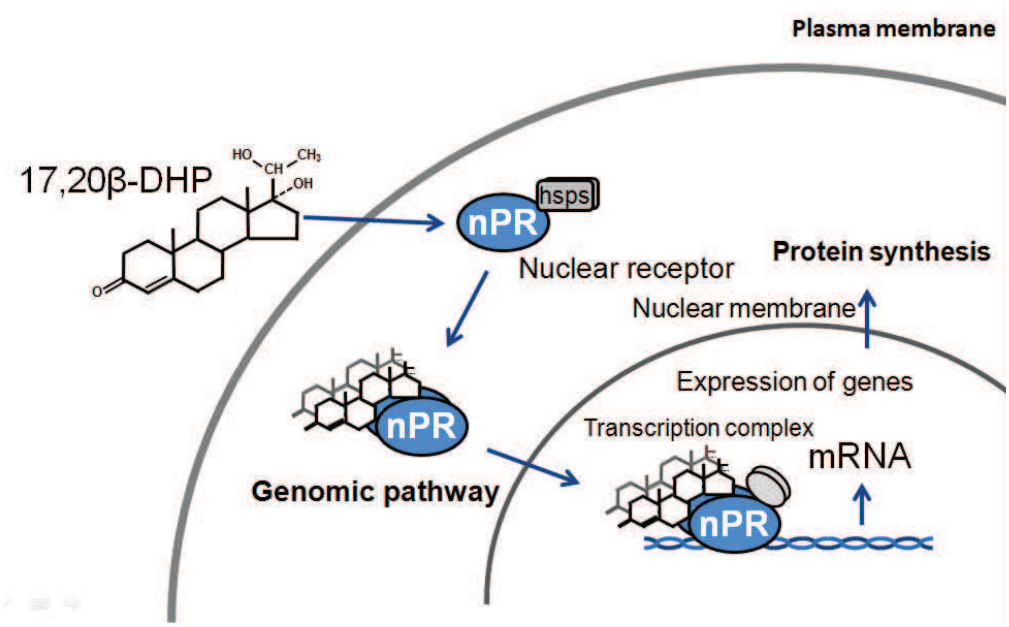


Fig. 4. Genomic pathway on the mechanism of steroid hormone actions through nuclear progesterone receptor. The heat shock proteins (hsps) are associated to inactivate the receptor. When the steroids bind to nuclear receptor the hsps will be released and receptors form a dimer as make a complex. The complex enter to nucleus, acts as a transcription factor to enhance the transcription of particular genes by its action on DNA. Finally protein synthesis will be induced and protein will be produced.

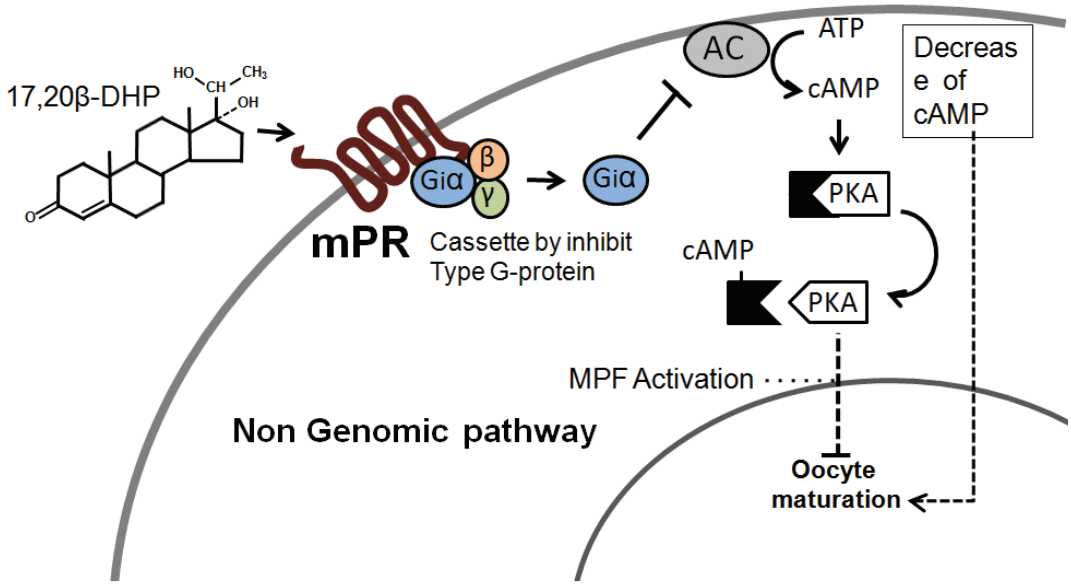


Fig. 5. The mechanism of steroid hormone actions for the induction of oocyte maturation through membrane progestin receptors through nongenomic pathway. MIH (17,20β-DHP) secreted from follicle cells, acts on mPR on the plasma membrane of oocytes and induce oocyte maturation through a decrease of intracellular cyclic AMP (cAMP) levels within a few minutes. Intracellular pathway is started by release of inhibitory G-proteins (Gi) from mPR.

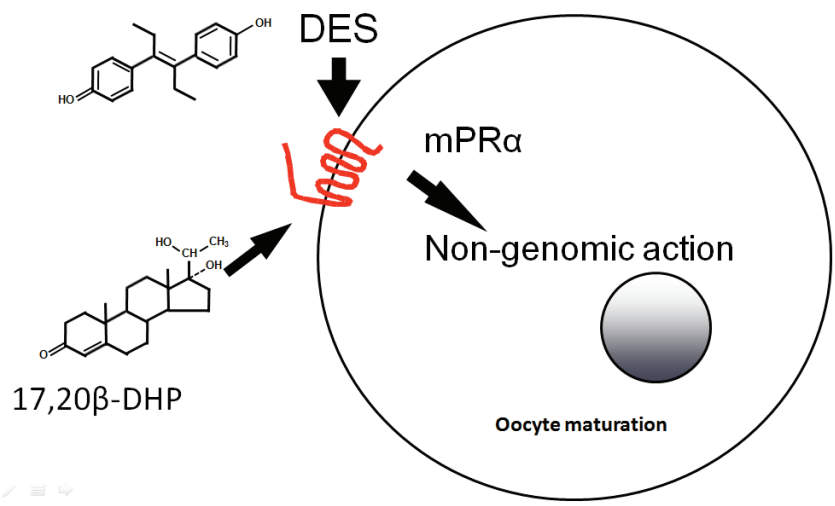


Fig. 6. Effect of endocrine-disrupting chemical (DES) on mPRα. This figure demonstrates that DHP and DES (EDC) induce oocyte maturation of goldfish through mPRα (Tokumoto *et al*., 2008).

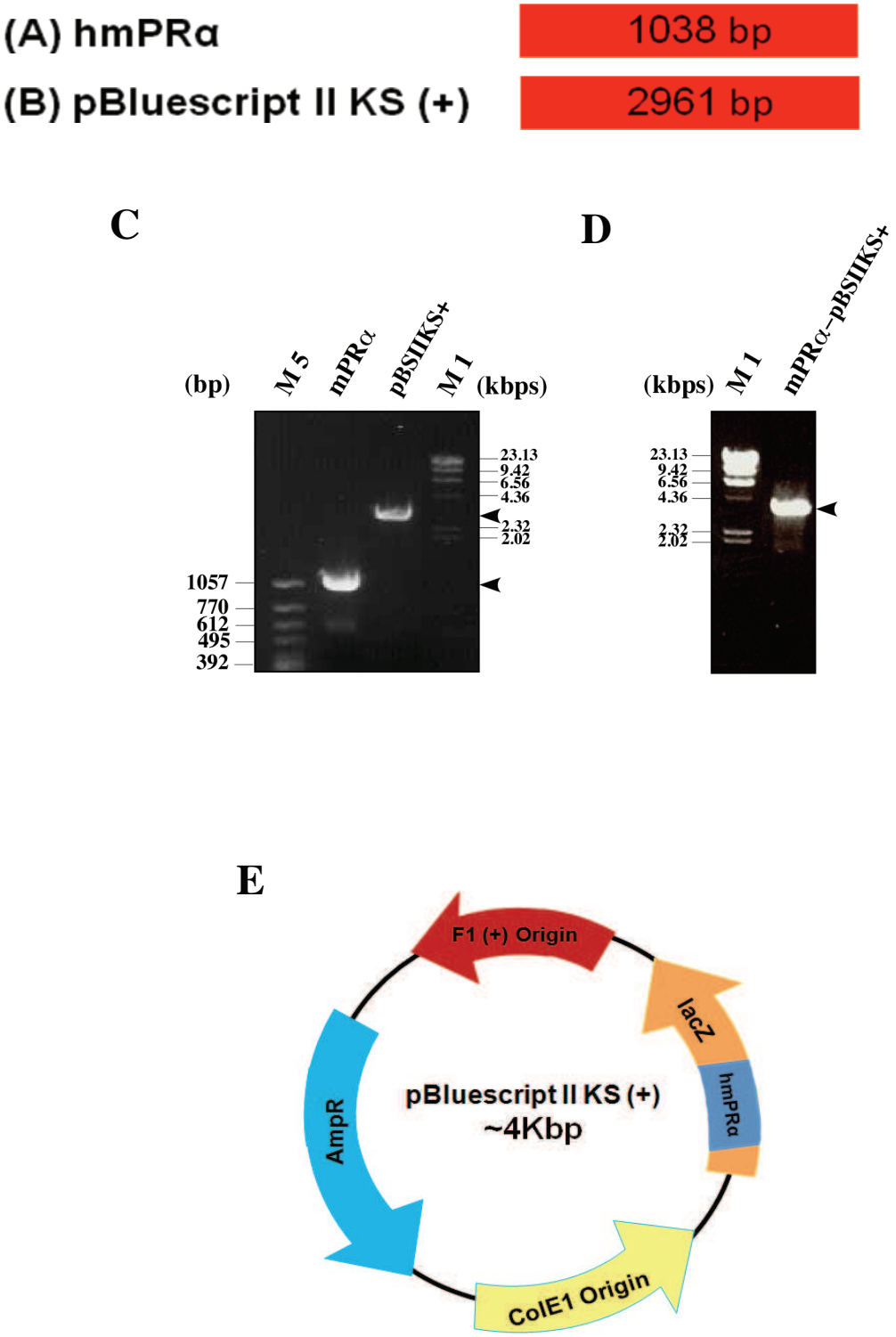


Fig. 7. Construction of recombinant plasmid. (A) Length of the hmPRα nucleotide sequence. (B) Length of pBluescript II KS (+) plasmid. (C) Electrophoresis of cDNA for hmPRα and pBSIIKS (+), (D) Electrophoresis of recombinant plasmid shows the size of hmPRα gene and pBSIIKS (+). Size of recombinant vector is ~4Kbp. M1, marker 1 and M5, marker 5. (E) Schematic presentation of recombinant plasmid.

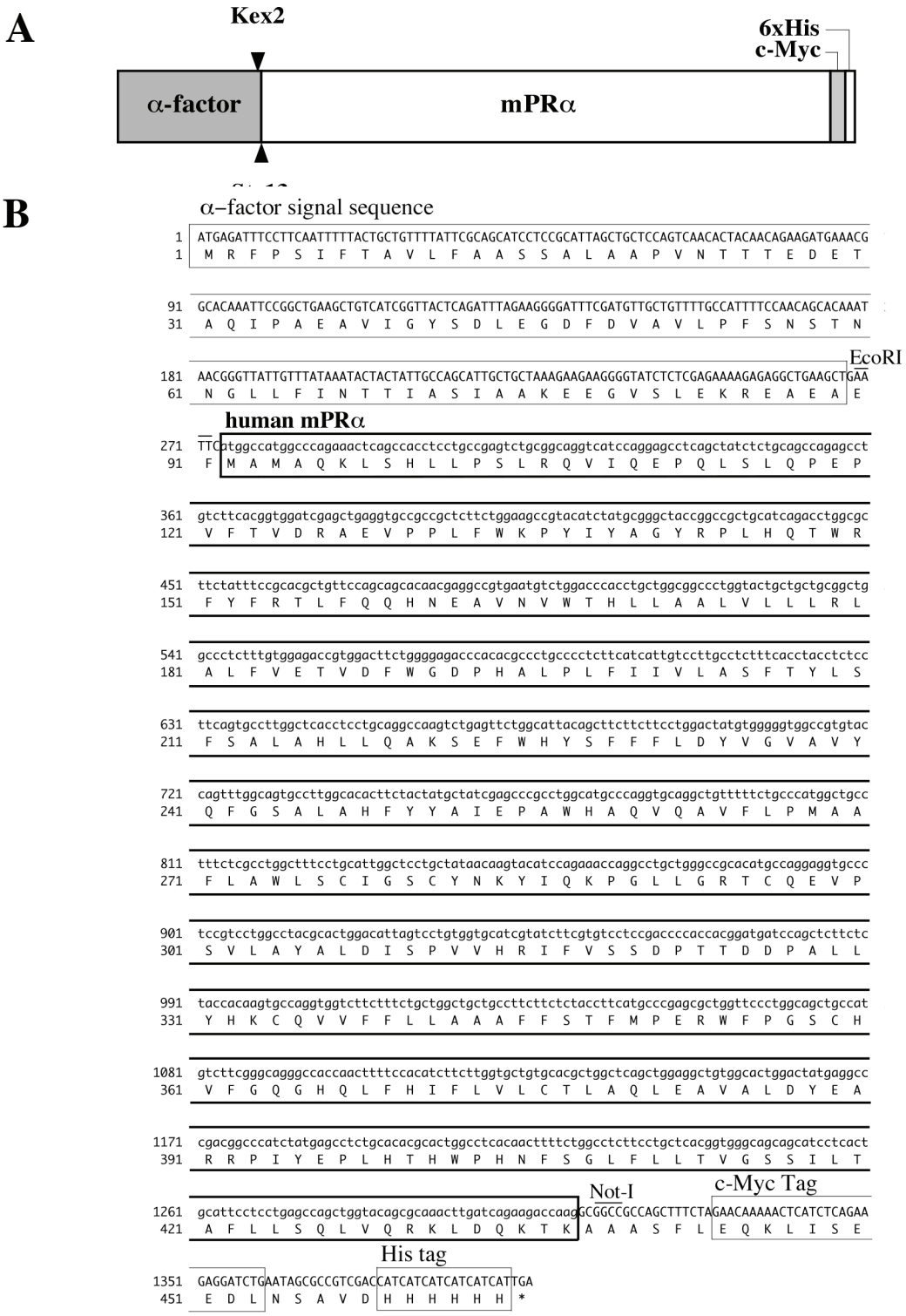


Fig. 8. (A) Schematic diagram of recombinant human mPRα sequence. (B) Nucleotide and amino acid sequences of recombinant human mPRα gene. The upper and lower letters correspond to the nucleic acid and amino acid sequences of recombinant hmPRα accordingly. The α-factor signal sequence, c-Myc-tag and His-tag sequences are indicated in box. The EcoRI and NotI restriction sites are upper lined.

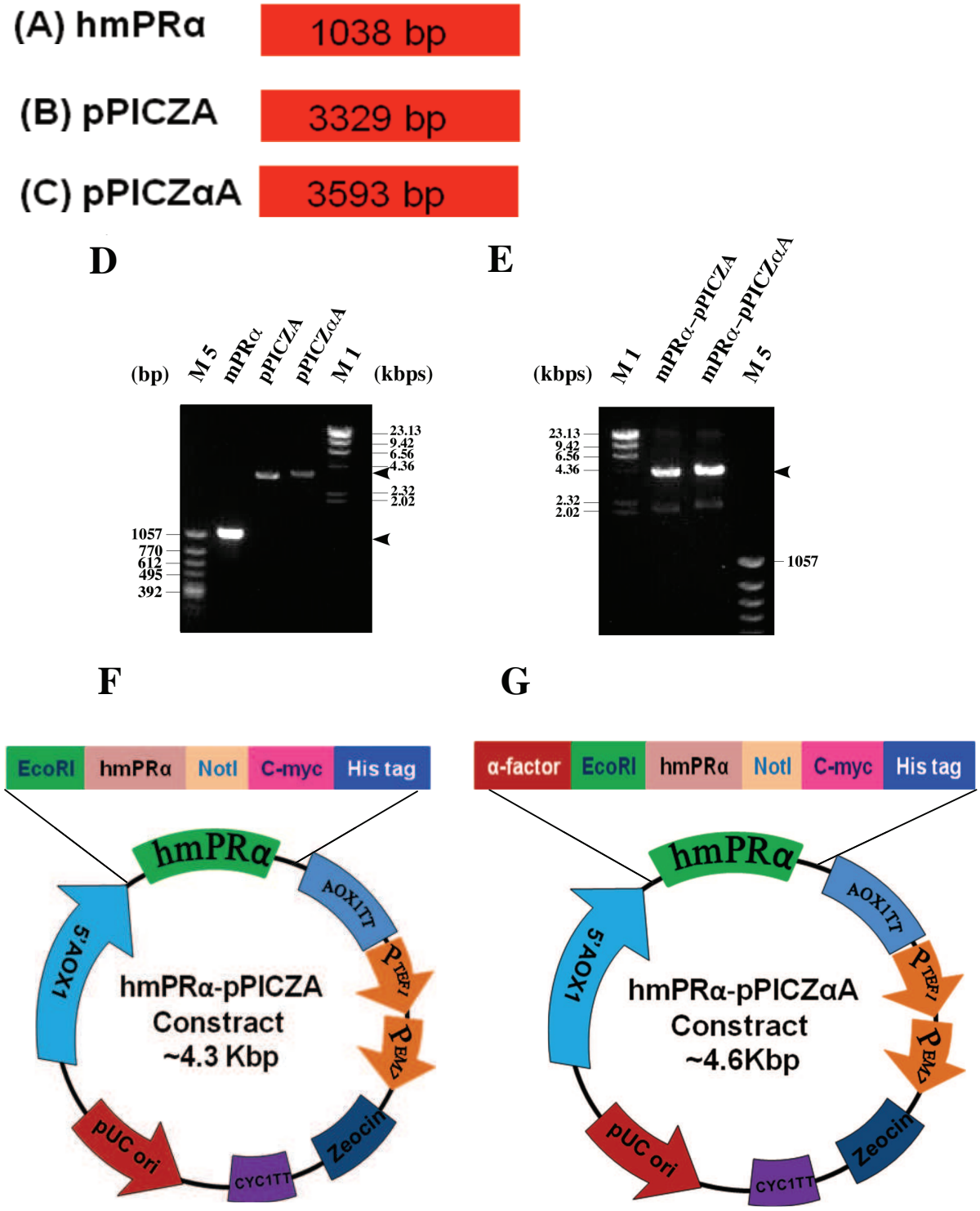


Fig. 9. Construction of recombinant plasmids, (A) Size of the hmPRα nucleotide sequence. (B and C) Size of plasmid nucleotides. (D) Electrophoresis of PCR product of hmPRα and plasmid pPICZA and pPICZαA (E) Electrophoresis of recombinant plasmid from recombinant bacterium colonies shows the conformation of hmPRα gene, mPRα-pPICZA and mPRα-pPICZαA clones, and size of recombinant vector is ~4.3 and ~4.6 Kbp accordingly. M1, marker 1 and M5, marker 5. (F and G) The schematic presentation of recombinant plasmids.

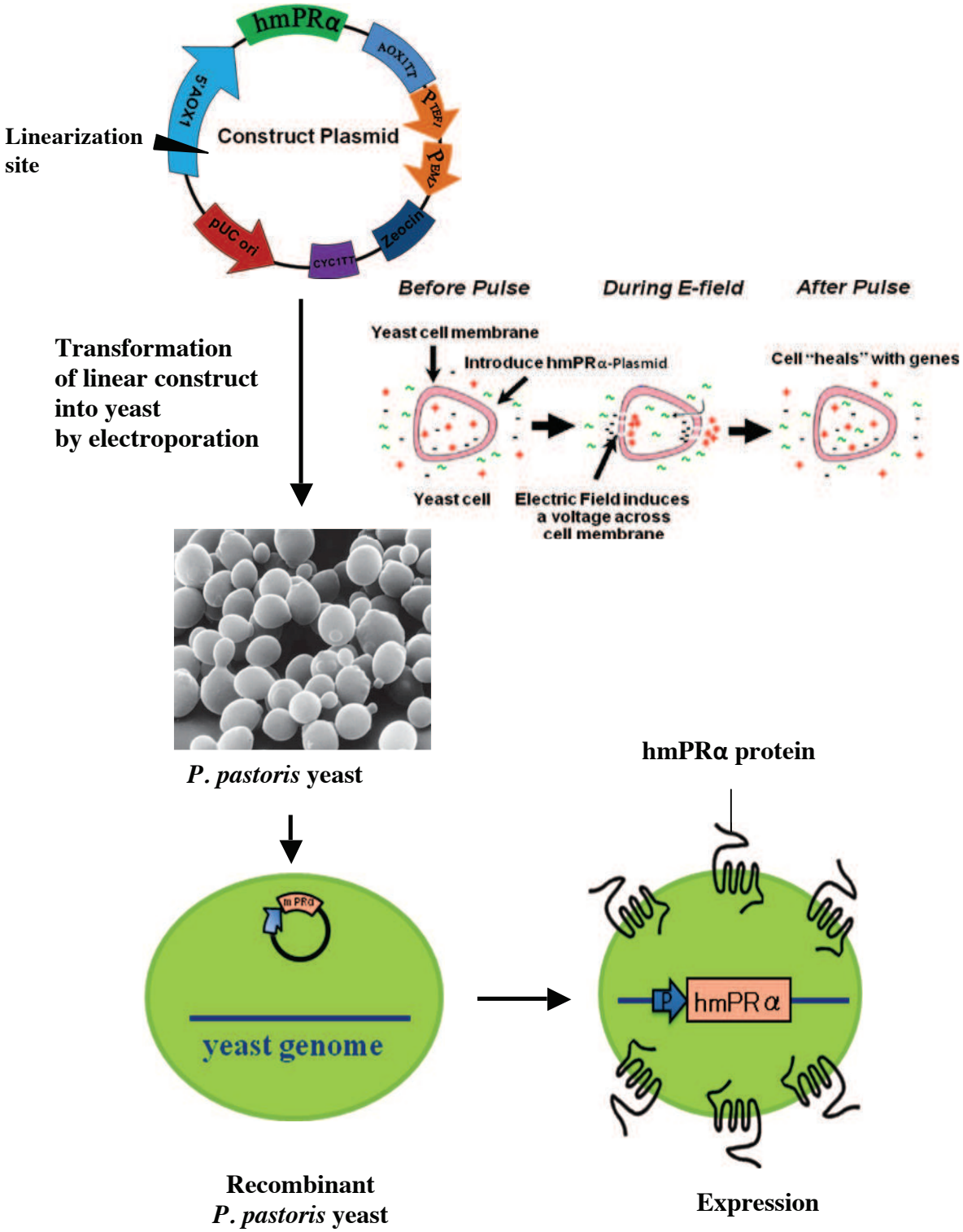


Fig. 10. The schematic representation for the transformation of recombinant plasmids (hmPRα gene contained) into the yeast, *P. pastoris* genome by electroporation for the production of hmPRα protein

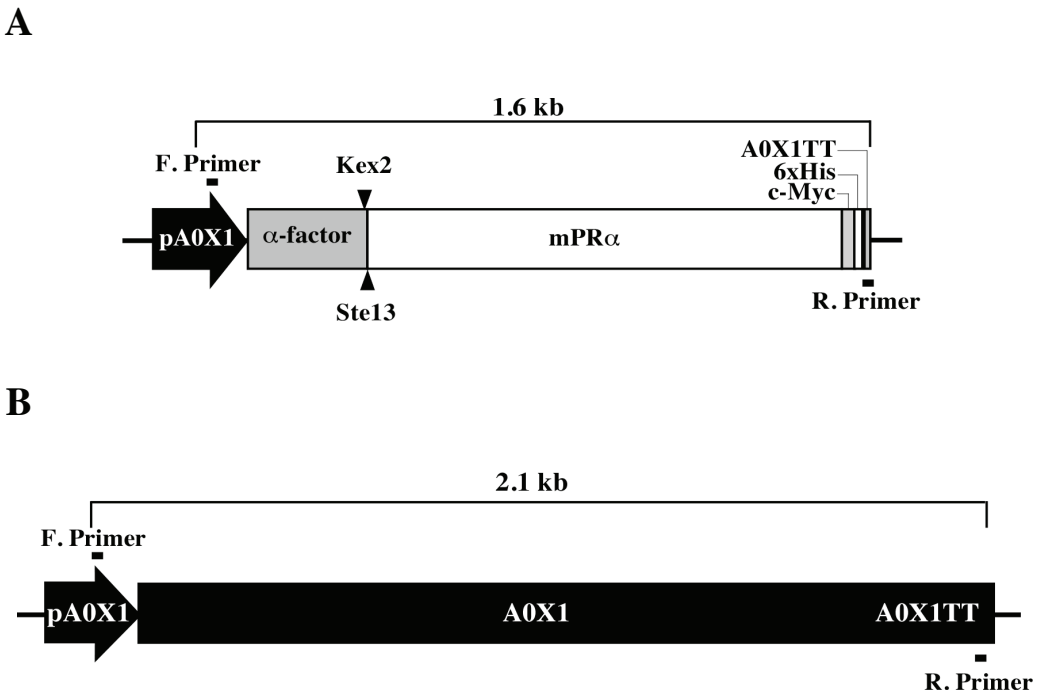


Fig. 11. Expression of human mPRα in *Pichia pastoris*. (A) Schematic representation of the mPRα expression cassette that was inserted into the yeast cell for producing mPRα protein. The fusion peptide consisted of hmPRα with a α-factor signal sequence, a C-terminal histidine (6X His) and a c-Myc epitopes controlled by the methanol-inducible AOX1 promoter (pAOX1) and the AOX1 transcription termination region (AOX1 TT), size of about 1.6 kbp. The black bars above and below the cassette indicate the 5'AOX1 (F. primer) and 3′ AOX1 (R. primer) binding site, respectively. (B) *Pichia* AOX1 gene which is remaining after insertion of transformed gene, size about 2.1 kbp is also shown.

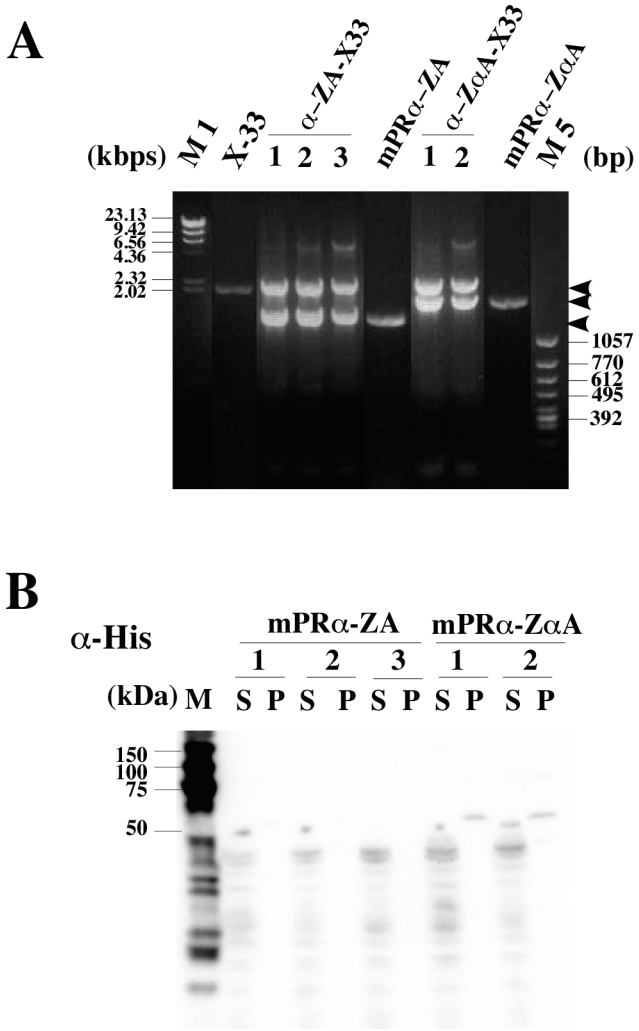


Fig. 12. Detection of the recombinant hmPRα protein producing *Pichia pastoris* colonies, where BstX1 linearized construct was transformed. (A) Electrophoresis for the determination of hmPRα gene insertion from yeast colonies. By PCR analysis, it is demonstrated that hmPRα gene is inserted into all colonies from hmPRα-pPICZA and hmPRα-pPICZαA vector inserted clones (α-ZA-X33 and α-ZαA-X33) accordingly. (B) The western blot result demonstrated that all the colonies were failed to induce for the production of recombinant hmPRα protein. Here it is mentioned that the molecular weight of recombinant hmPRα protein was 50 kDa. 1, 2, 3 mentioned the colony number; S, supernatant; P, pellet of disrupted yeast cells.

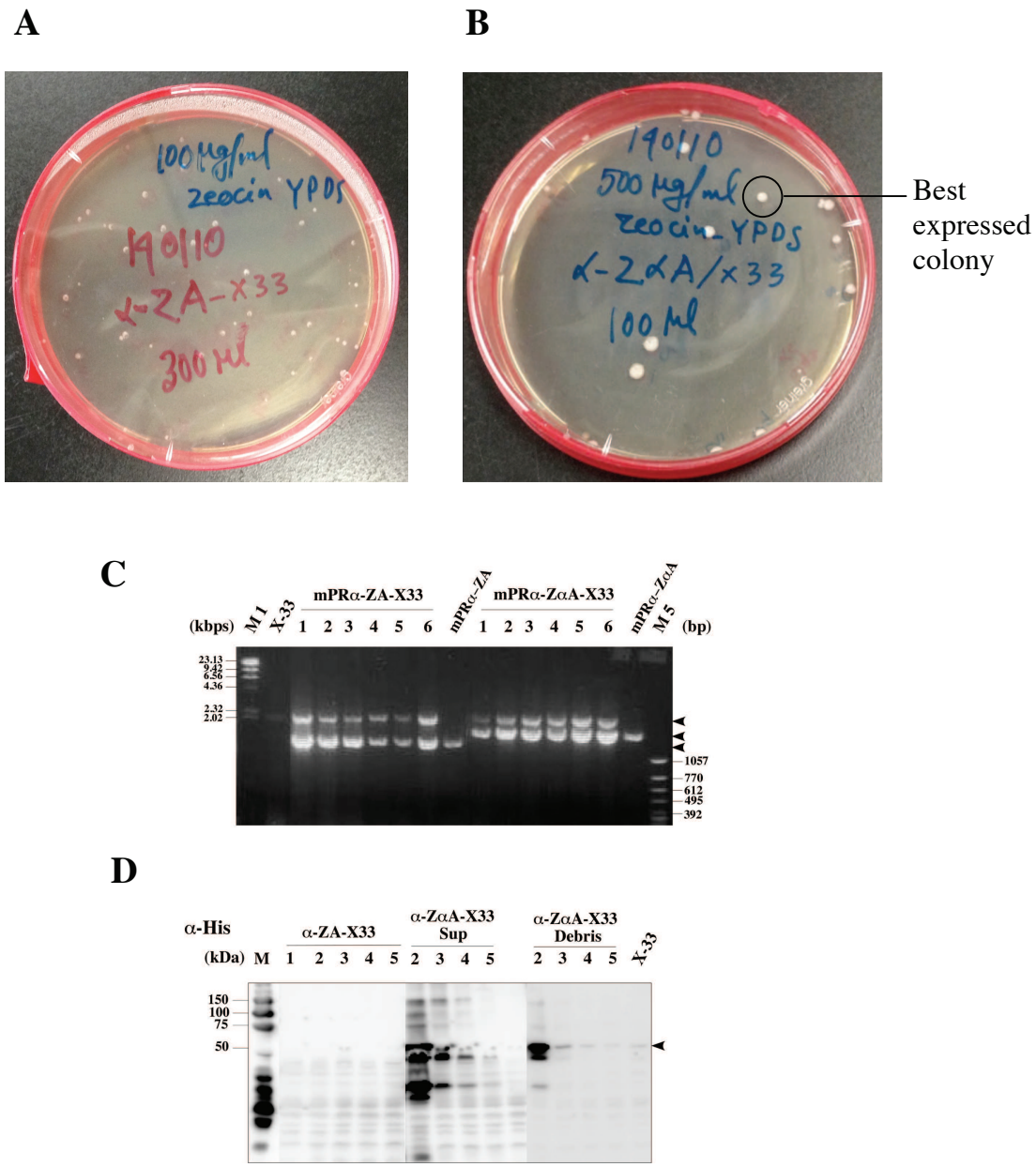


Fig. 13. Determination of the recombinant hmPRα protein producing *Pichia pastoris* colonies, where PmeI linearized construct was transformed. (A and B) Colonies from hmPRα-pPICZA (α-ZA-X33) and hmPRα-pPICZαA (α-ZαA-X33) vector inserted clones accordingly. (C) Electrophoresis for the determination of hmPRα gene insertion from yeast colonies. PCR analysis demonstrated that hmPRα gene is inserted into all colonies. (D) The western blot result shows that only colony 2 from hmPRα-pPICZαA construct inserted X33 clones (α-ZαA-X33) were produced the hmPRα protein. The hmPRα protein is mentioned by arrow mark. These colonies were grown on YPD plates containing 500 µg/mL Zeocin and the expressed colony is circled. 1, 2, 3, 4, 5 & 6 mentioned the colonies number; sup, supernatant of yeast cells disruption.

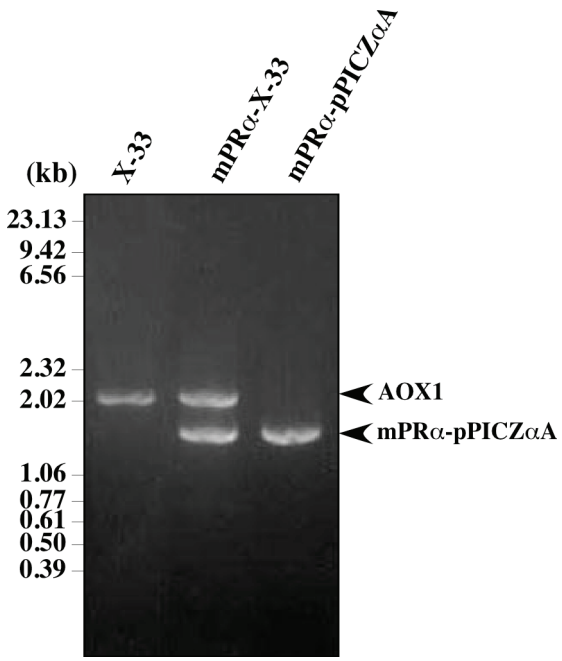


Fig. 14. Gene insertion into *Pichia* was verified by PCR amplification. DNA fragments were amplified using genome DNA from untransformed yeast cells (X-33), genome DNA from hmPRα-transformed cells (mPRα-X-33) and transformed vector DNA (mPRα-pPICZαA) as templates.

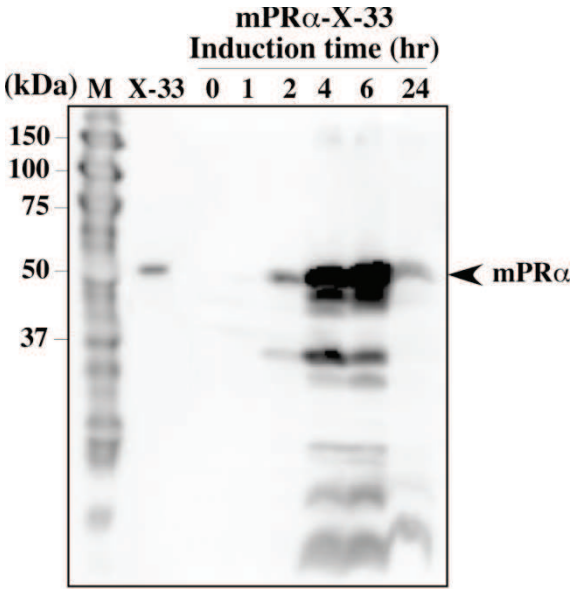


Fig. 15. Determination of the optimal conditions for the expression of hmPRα. After protein expression induction in culture with methanol, samples were taken at 0, 1, 2, 4, 6 and 24 hours. Expression was checked by western blot analysis. A 50 kDa protein band was reacted with anti-His-tag antibody in the extract prepared from human mPRα transformed cells (mPRα-X-33).

A

B

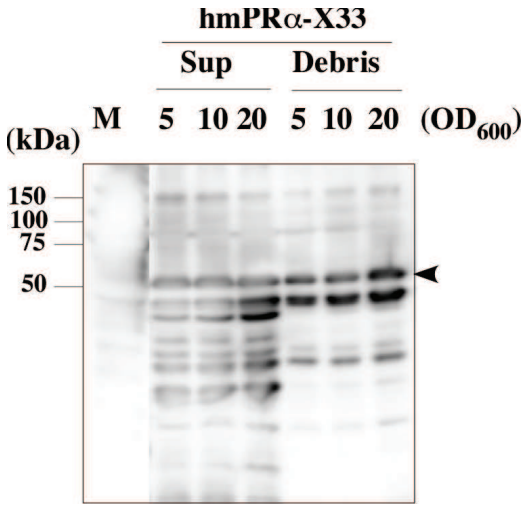
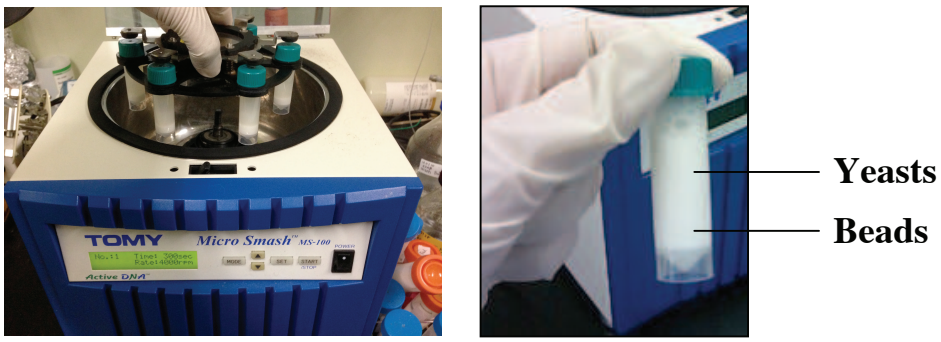


Fig. 16. hmPRα production by culturing of yeast cells. (A) Yeast cells culture shaker and flask with induction medium. (B) A comparison of induction yeast cell densities for the hmPRα protein production. The western blot result shows the higher cell densities (OD_{600}, 20) were produced the high amount of hmPRα protein.

A



B

C

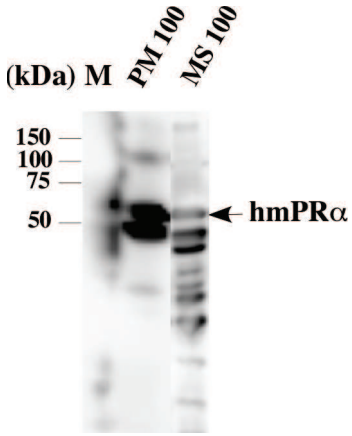


Fig. 17. Yeast cells breaking. (A) Beads crusher Micro Smash MS100 (TOMY Seiko) (B) Ball Mill PM100 (Verder scientific Co., Ltd., Haan, Germany), by which cells were disrupted with five iron balls in a chilling condition. (C) The comparison of hmpRα protein quality, after disrupted the cells using Ball mill PM100 and Micro Smash MS100. The western blot result shows that the hmPRα protein was degraded using MS 100.

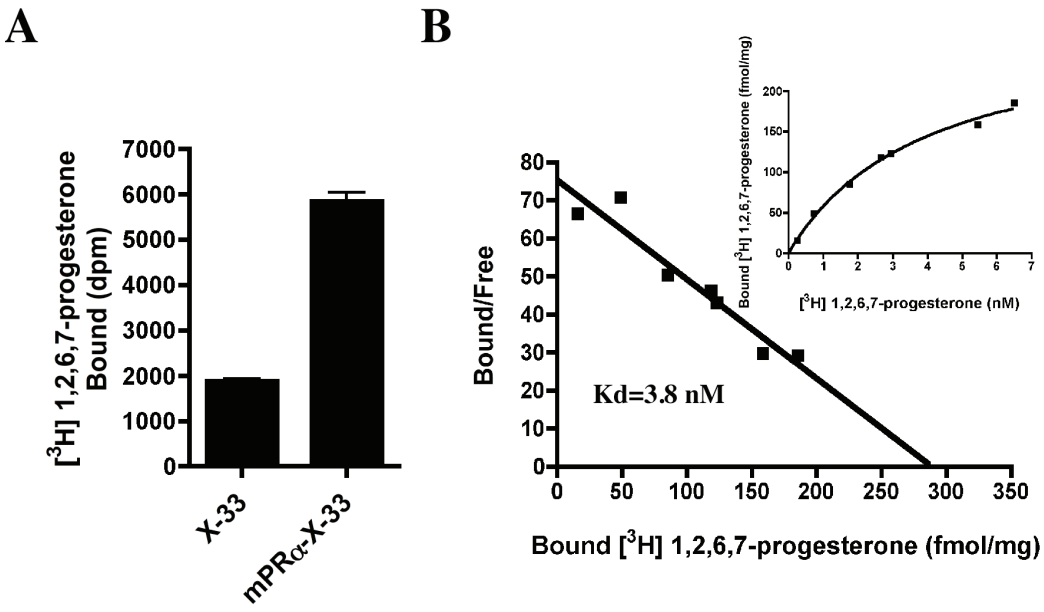


Fig. 18. Characterization of binding activity in membrane fractions. (A) Specific binding activity of [^{3}H]1,2,6,7- progesterone to membrane preparations from untransformed (X-33) and hmPRα- producing cells (mPRα-X33). (B) Saturation curves and Scatchard plots of specific [^{3}H]1,2,6,7-progesterone binding to membrane preparations from hmPRα-producing cells (mPRα-X33).

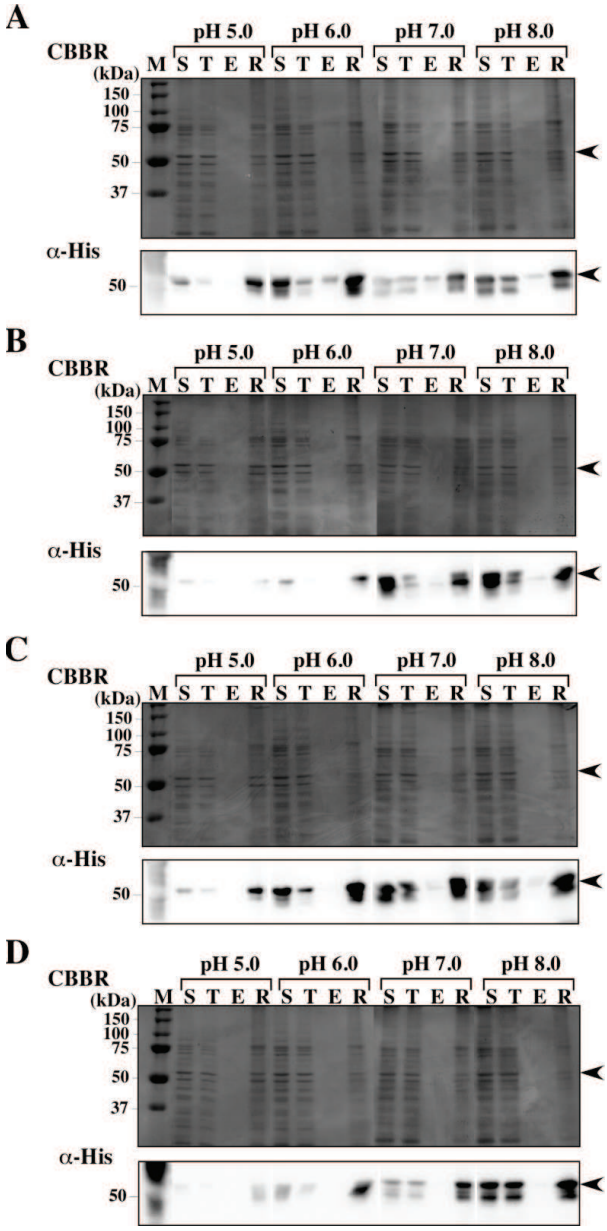


Fig. 19. Optimization of the conditions for Ni-NTA affinity chromatography. Binding presentation of solubilized hmPRα onto the Ni-NTA resin was examined with 16 lysis buffers using different concentrations of imidazole (10, 20, 40 or 80mM) and pH values (pH 5.0, 6.0, 7.0, or 8.0) in Ni-NTA binding buffer (50mM NaH_2PO_4, 300mM NaCl). Samples for each lane are following, M, marker; S, solubilized hmPRα protein fractions; T, flow through protein after Ni-NTA binding; E, eluted proteins with elution buffer (50 mM NaH_2PO_4, 300mM NaCl, 250mM imidazole pH 8.0); R, remained on Ni-NTA resin after elution. The proteins were detected by CBBR staining (upper panel in each set) and western blotting (lower panel in each set). The panels showing the results obtained using (A) 10 (B) 20 (C) 40 and (D) 80 mM imidazole- containing buffer of various pH levels.

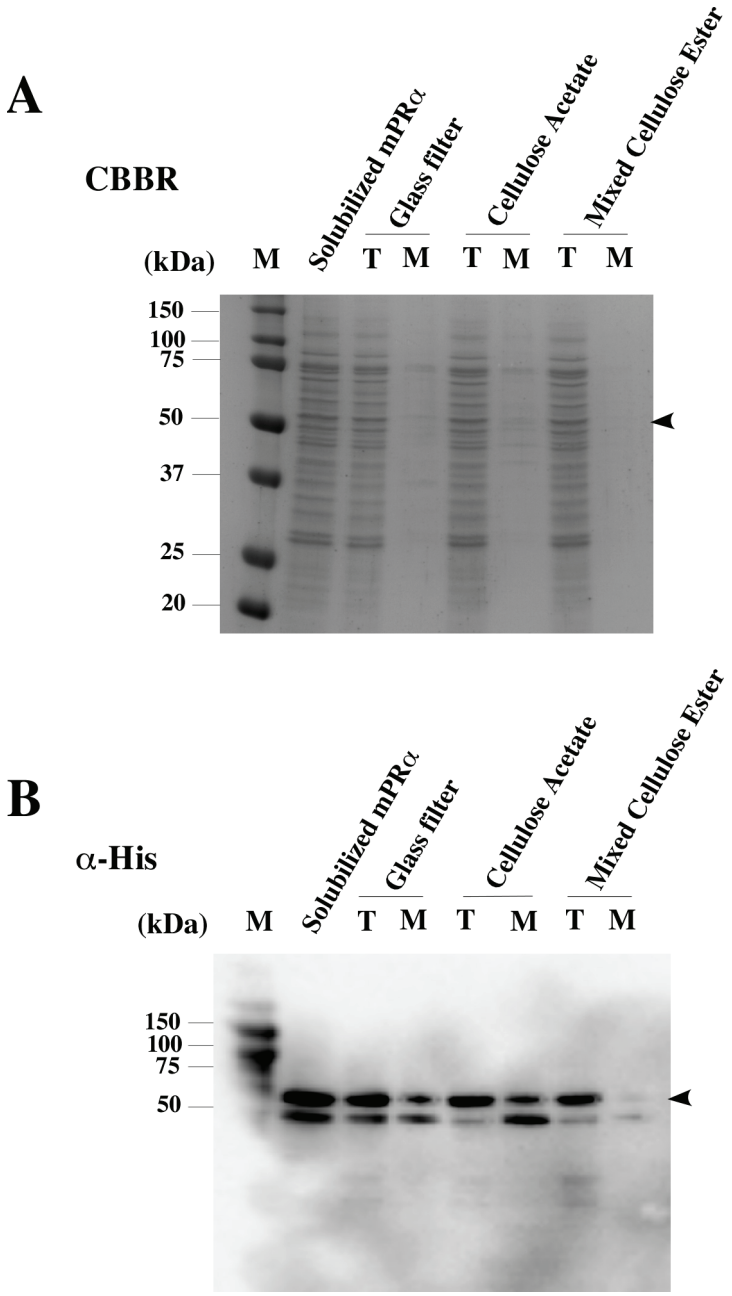


Fig. 20. Filter selection for the separation of debris from solubilized hmPRα solution. (A) CBBR staining and (B) western blot results filtrated with three filters. Mixed Cellulose Ester can filtrated the debris from solubilized hmPRα protein mixtures. Here mentioned T, throughout samples after filtration and M, membrane bound samples.

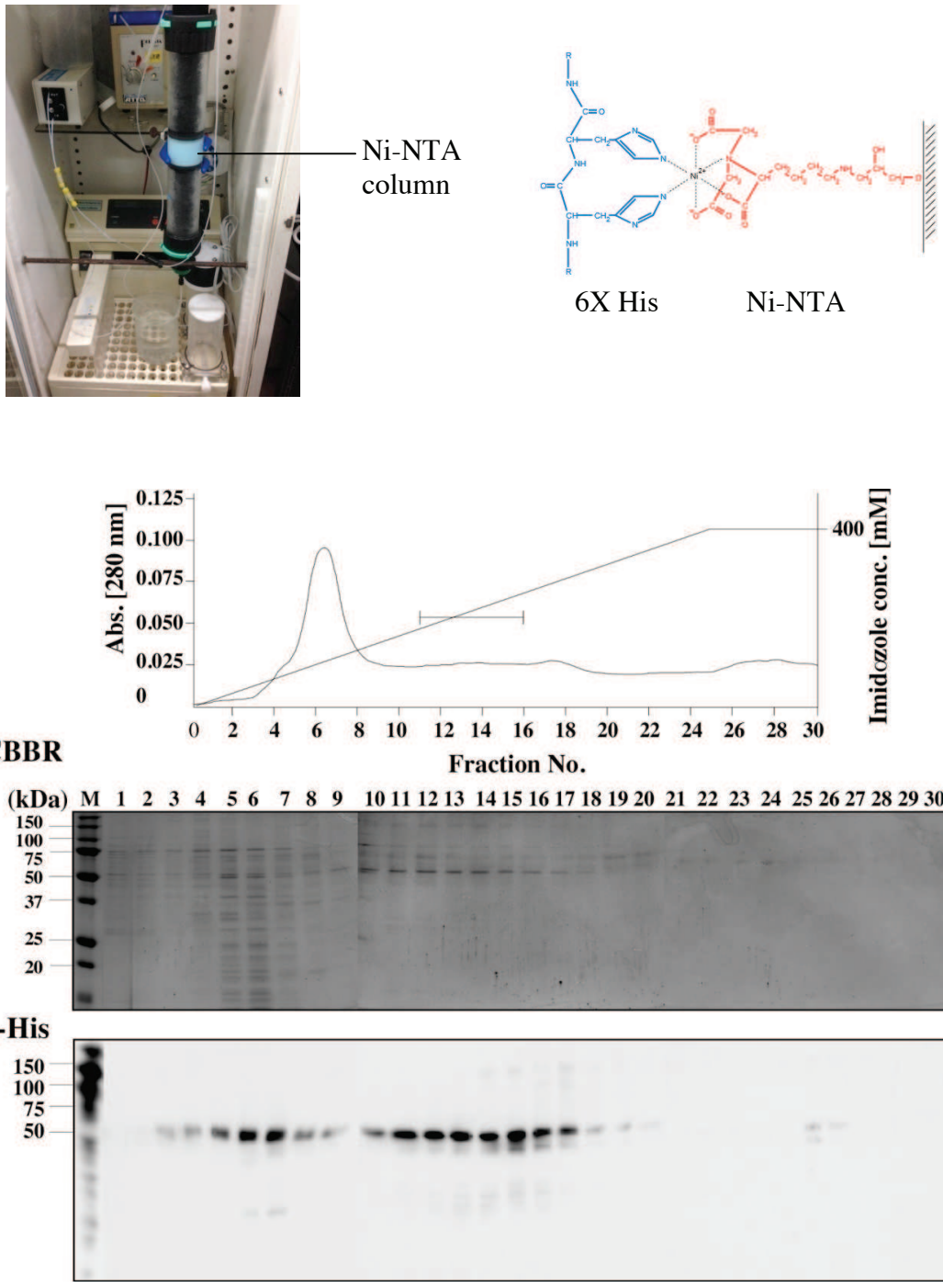


Fig. 21. Purification of hmPRα protein by Ni-NTA column chromatography. Chromatogram, SDS-PAGE and the western blot analysis results of the Ni-NTA column chromatography. Elution profiles were monitored by absorbance at 280 nM. The horizontal bar in the chromatogram represents the fractions collected and used in subsequent purification steps.

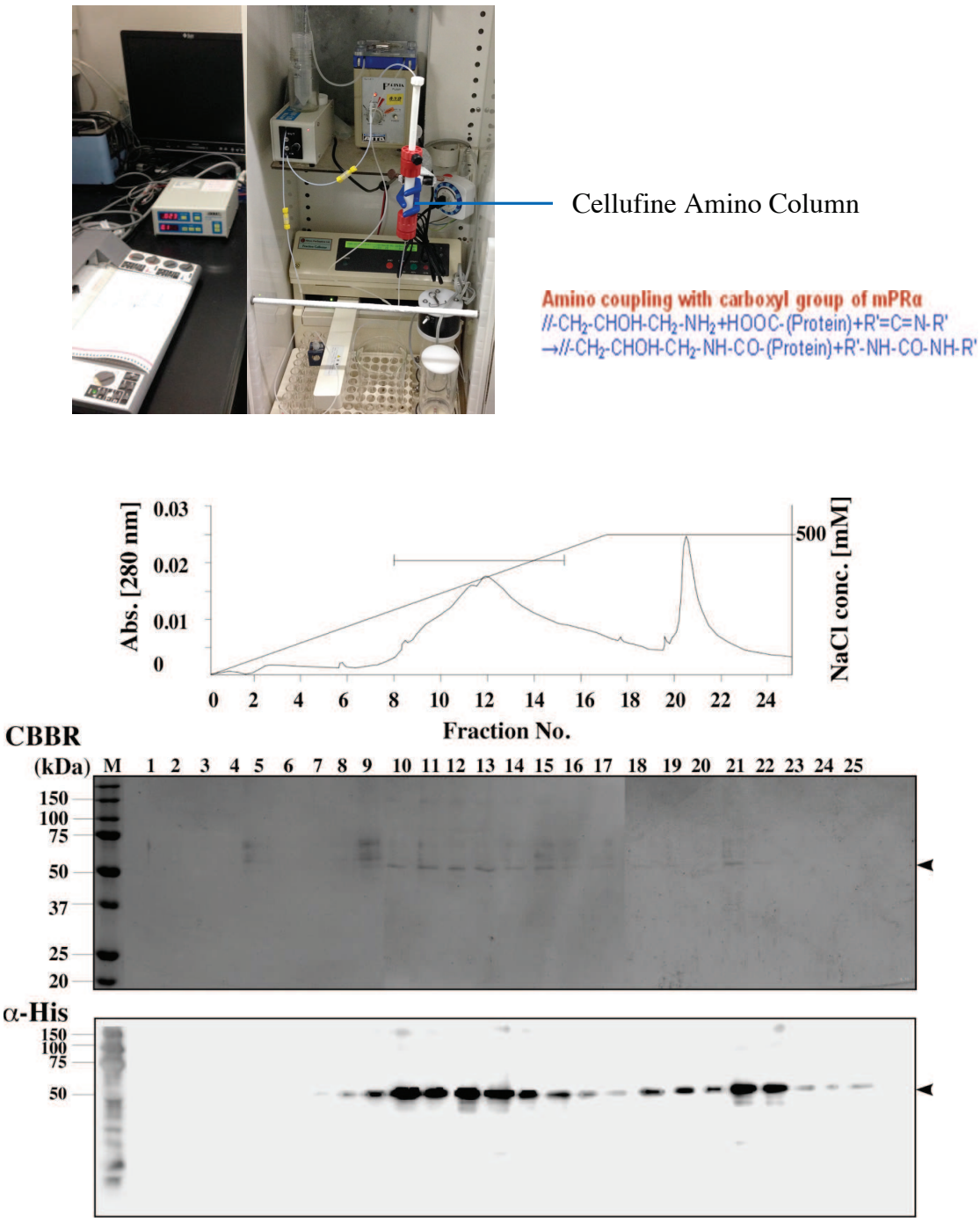


Fig. 22. Purification of hmPRα protein by Cellufine Amino column chromatography. Chromatogram, SDS-PAGE and the western blot analysis results from the Cellufine Amino column chromatography fractions. Elution profiles were monitored by absorbance at 280 nM. The horizontal bar in the chromatogram represents the fractions collected for further steps.

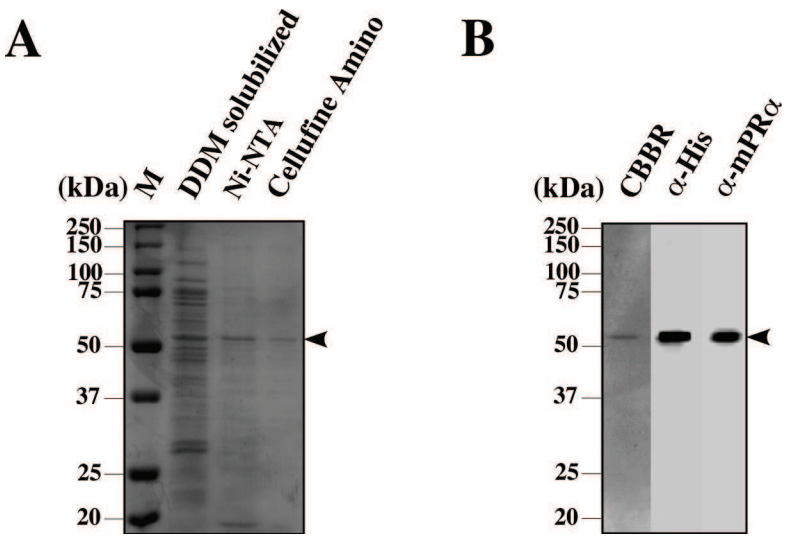


Fig. 23. Summarization of overall purification steps for hmPRα. (A) SDS–PAGE analysis of representative fractions after solubilization of the membrane preparation (DDM solubilized), column chromatography over Ni-NTA, amino cellulose (Cellufine Amino). Protein bands were detected by CBBR staining. (B) SDS –PAGE analysis of purified hmPRα. Protein bands were detected by CBBR staining (CBBR) or were immunostained by anti His-tag antibody (α-His) or anti hmPRα antibody (α-mPRα). An arrow indicates hmPRα protein bands.

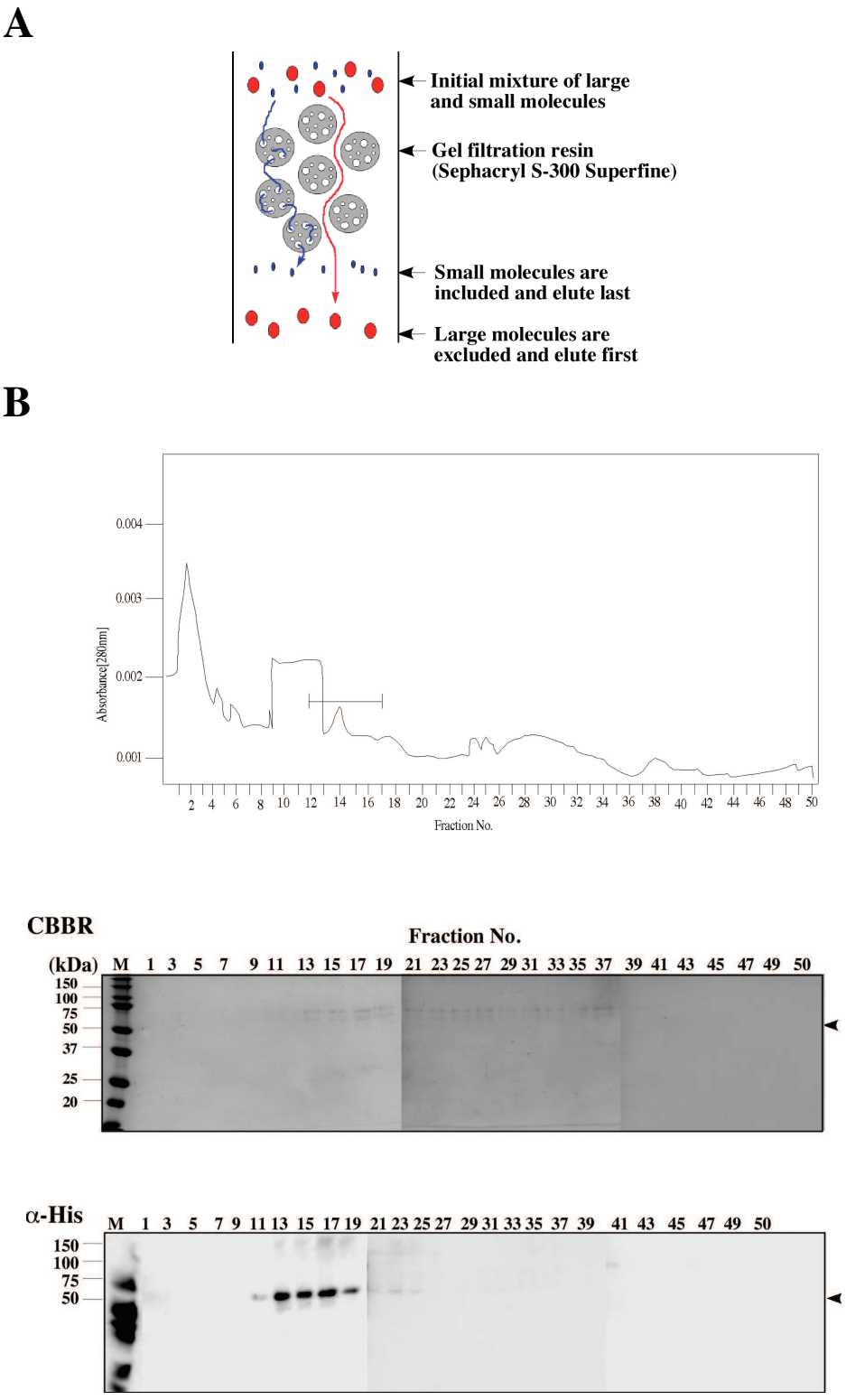


Fig. 24. Purification of hmPRα protein by Sephacryl S-300 gel column chromatography. The figures demonstrated the Chromatogram, SDS-PAGE and the western blot analysis results from the Sephacryl S-300 column chromatography fractions. Elution profiles were monitored by absorbance at 280 nM. The horizontal bar in the chromatogram represents the fractions collected for hmPRα protein (fractions 12-18).

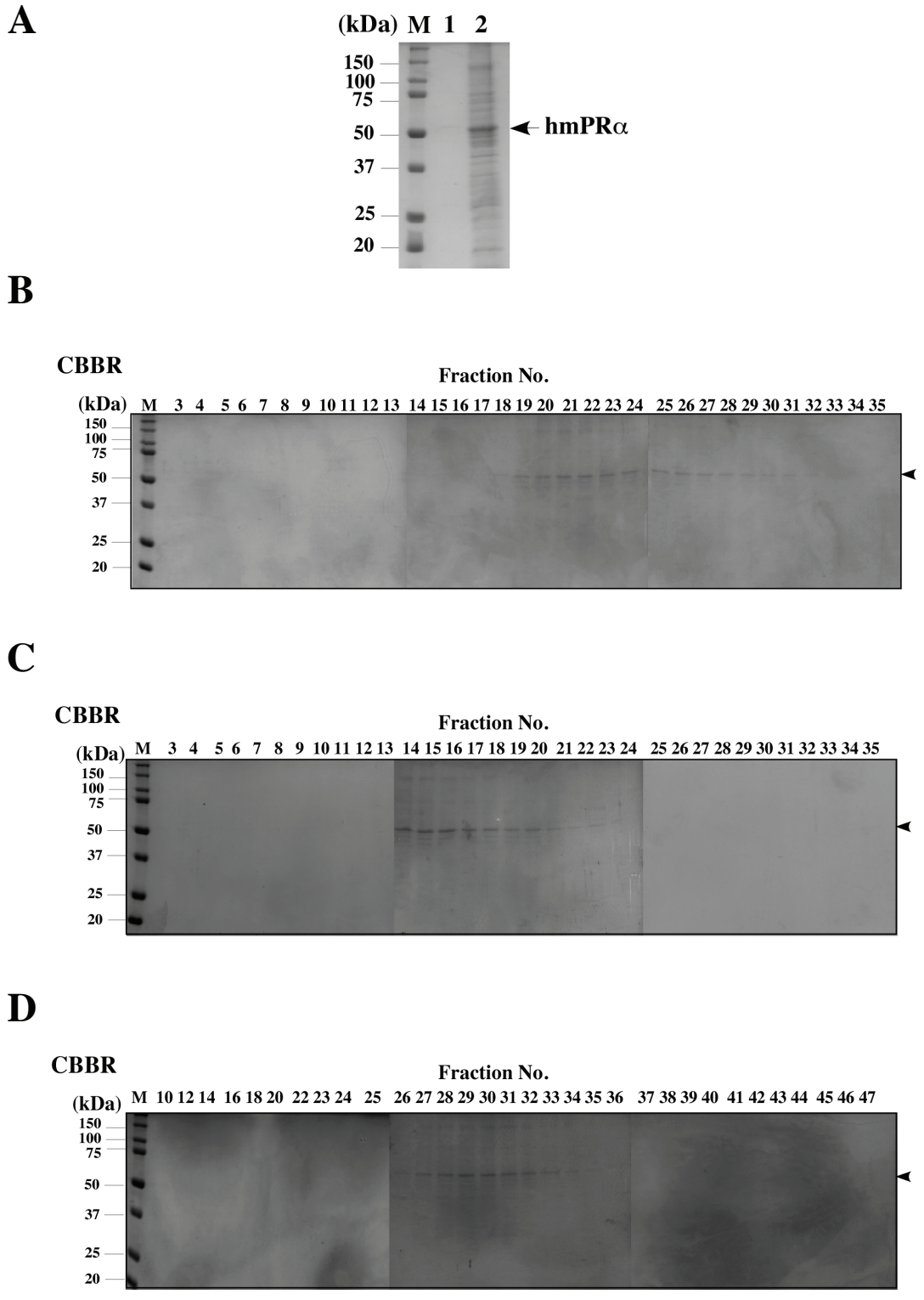


Fig. 25. Purification of Ni-NTA fractions of hmPRα using Sephadex G75, G150 and G200 gel chromatography. (A) M, marker; lane 1 indicated the Ni-NTA fractions and 2, concentrated Ni-NTA fractions that applied on gel column. (B) SDS-PAGE results from the Sephadex G-75 column chromatography fractions. (C) SDS-PAGE results from the Sephadex G-150 column chromatography fractions. (D) SDS-PAGE results from the Sephadex G-200 column chromatography fractions. The arrow mark mentioned the hmPRα protein band.

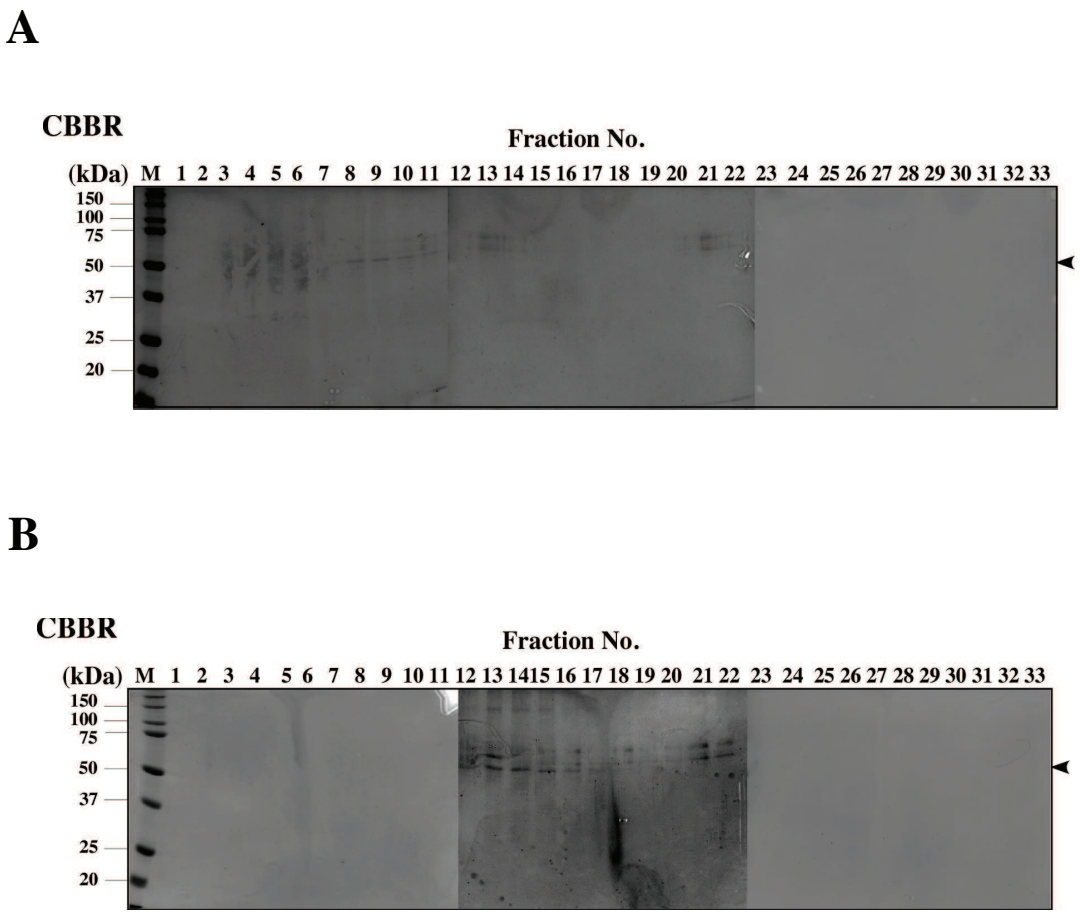


Fig. 26. SDS-PAGE analysis showed the purification of Ni-NTA fractions of hmPRα by Sephadex G-150 gel chromatography. Samples applied on column with the vortex or sonication in the presence of 0.1% DDM. (A) Fractionation result, 30 seconds vortex sample was used on column. (B) Fractionation result, 1 min sonicated sample was used on column. The arrow mark mentioned the hmPRα protein band.

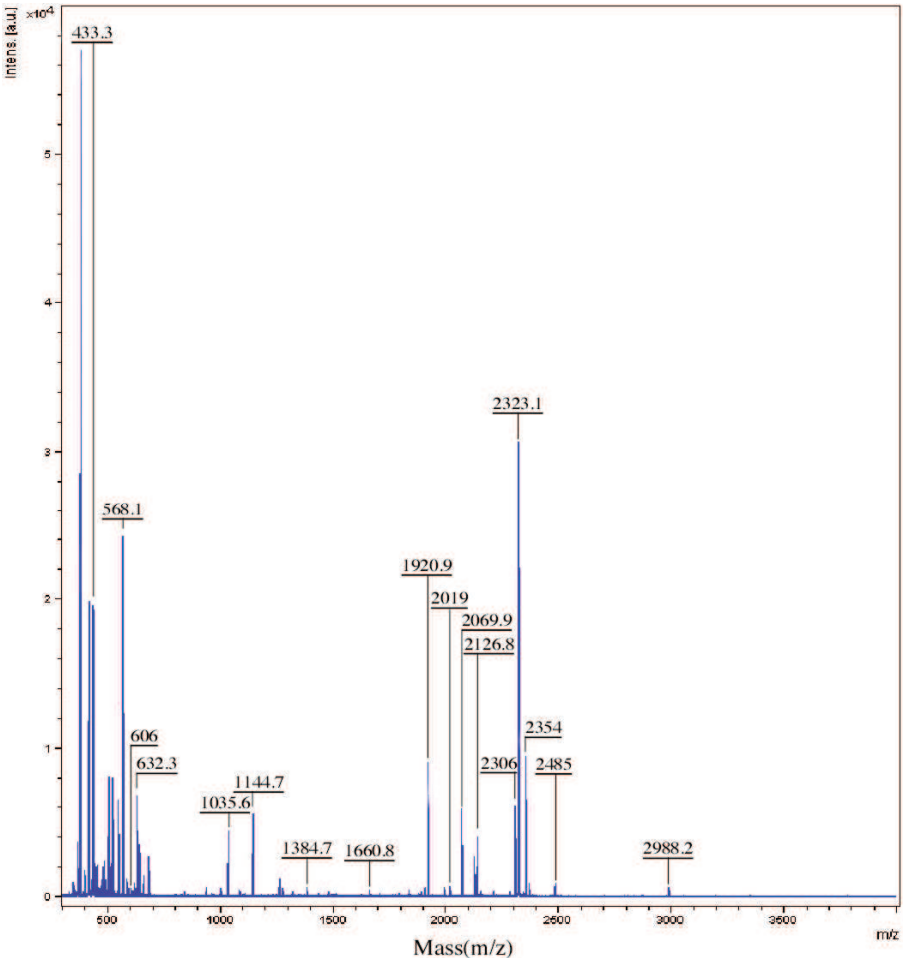


Fig. 27. Identification of purified recombinant protein as hmPRα. Result of MALDI-TOF mass spectrum of purified hmPRα. At least 300 laser shots were used to take the average result. Protein calibration standard mono was used to calibrate prior to analysis of the sample.

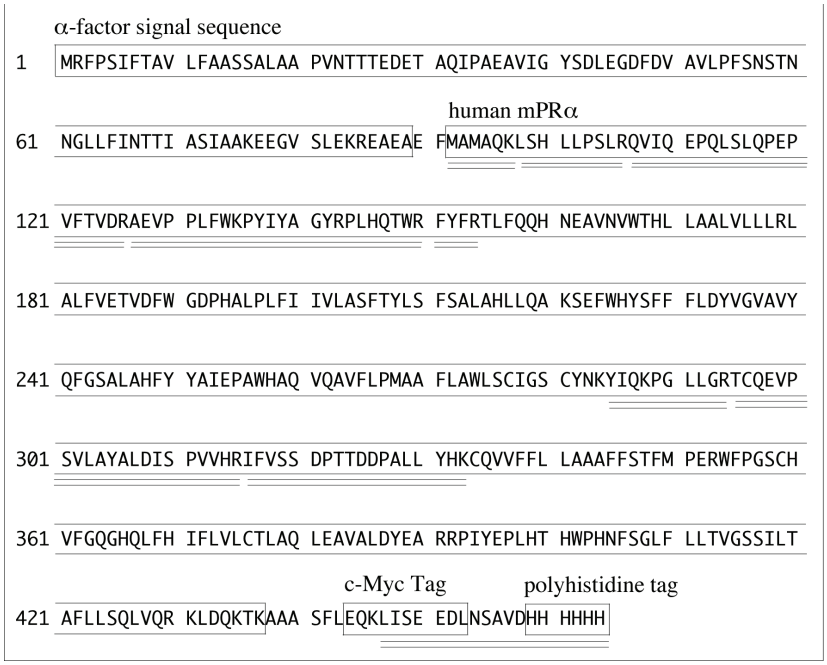


Fig. 28. Identification of purified recombinant protein as hmPRα by MALDI-TOF mass analysis. The matching peptide fragments of recombinant hmPRα are underlined, which is detected by the peptide mass fingerprint analysis.

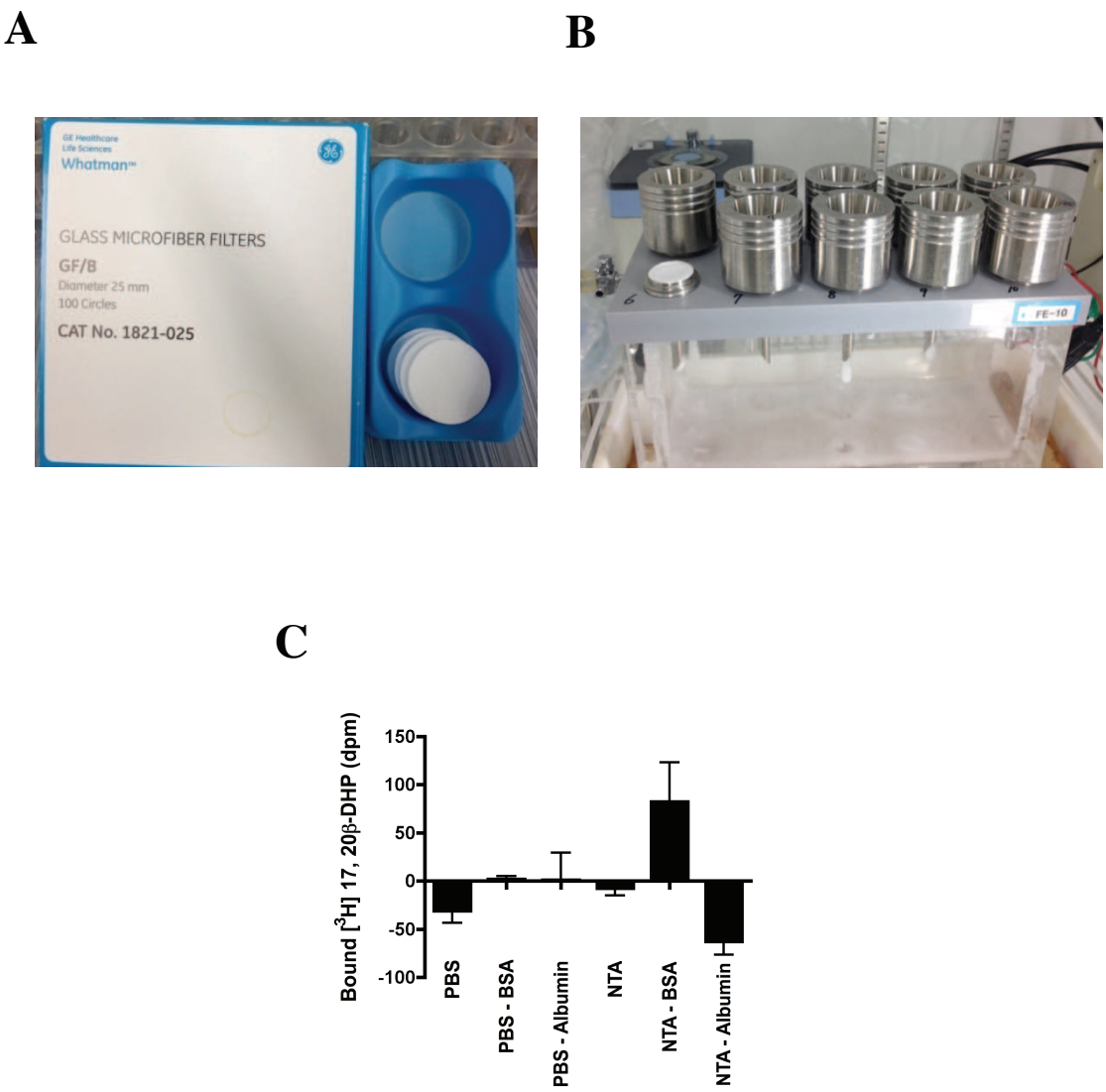


Fig. 29. Specific binding assay analysis for purified goldfish mPRα (gmPRα) for [^{3}H] 17, 20β-DHP. (A) Whatman UK GF/B filters were used for the binding assay analysis. (B) Membrane was washed after applying the binding reaction mixture on membrane. (C) Specific binding activity of purified gmPRα to [^{3}H] 17, 20β-DHP was measured using PBS and Ni-NTA buffers containing gmPRα or with albumin or BSA. In the figure it is demonstrated that the specific activity was seen in the Ni-NTA buffer containing gmPRα-BSA reaction mixture (NTA-BSA).

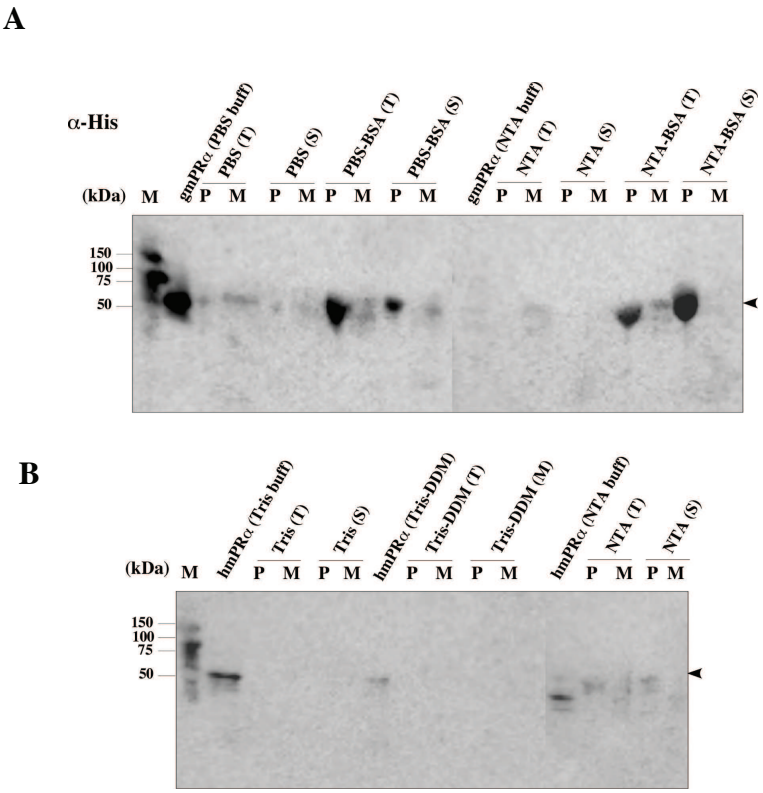


Fig. 30. Binding presentation of purified gmPRα and hmPRα onto the Whatman UK GF/B filters. The attaching proteins on membranes were measured by western blot analysis. (A) Western blot analysis demonstrated the absence of gmPRα with PBS or NTA buffer or PBS-BSA or NTA-BSA containing reaction mixture was used on membrane. (B) The western blotting result showing the absence of hmPRα on membrane when used the hmPRα with Tris or Tris-DDM or NTA buffer containing reaction mixture. Samples for each lane are following, M, marker; T, total binding reaction mixture, S, non specific binding reaction mixture, P, flow through proteins after membrane attaching, M, membrane attached proteins after wash.

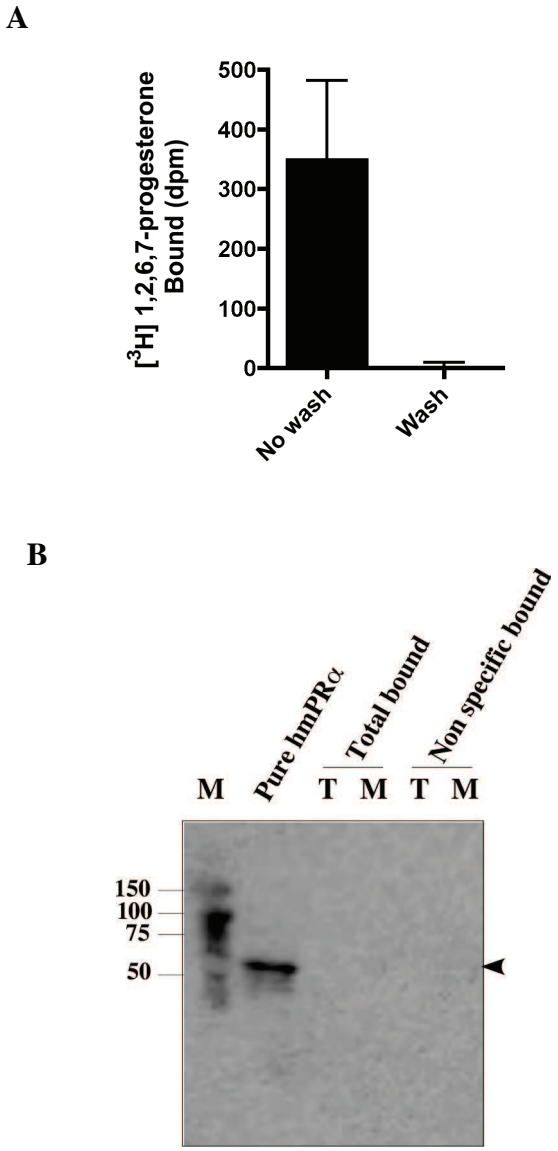


Fig. 31. Specific binding assay analysis of purified hmPRα for [^{3}H]1,2,6,7- progesterone. (A) Specific binding activity was detected in hmPRα, when measured without washing the membrane (No wash). On the other hand, when washed the membrane, the specific activity was not detected (Wash). (B) The western blot analysis was demonstrated the absence of hmPRα proteins on membrane. After washed, the membrane bound hmPRα protein sample was used for the western blot analysis.

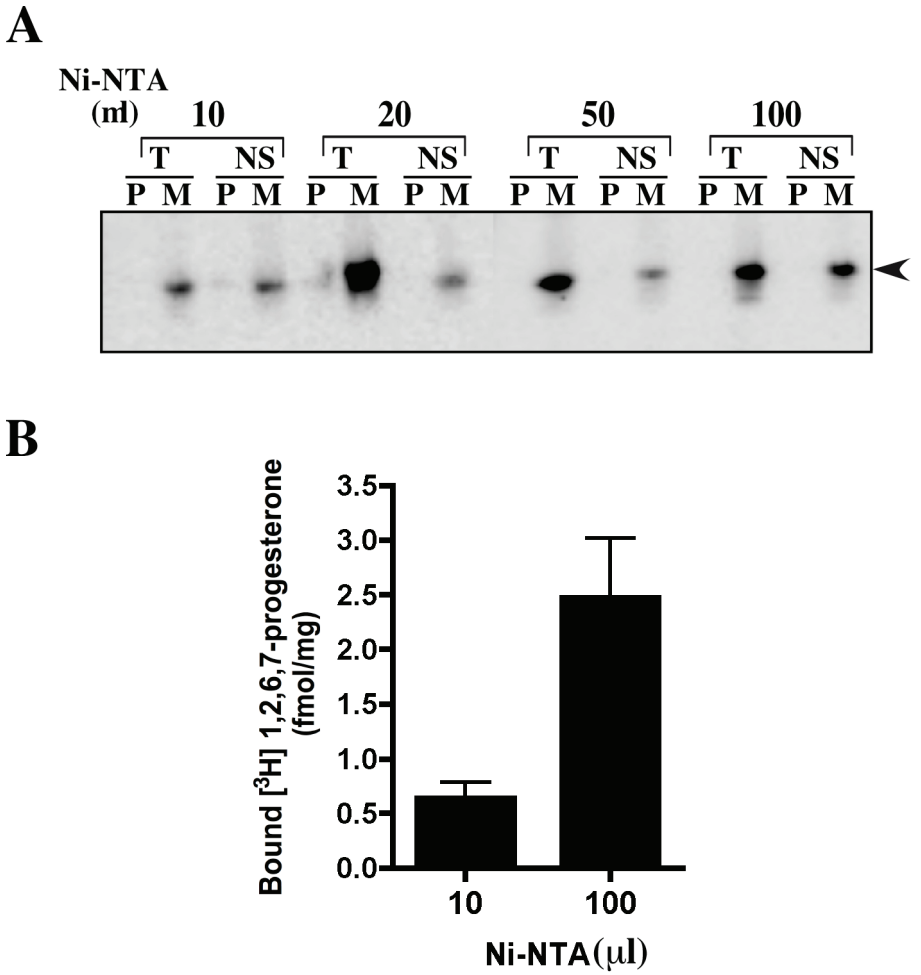


Fig. 32. Optimization of the attachment of purified hmPRα with whatman UK GF/B filters for [^{3}H]1,2,6,7- progesterone binding assay analysis. (A) The indicated amount of Ni-NTA resin (10, 20, 50 or 100µl) was supplemented into the reaction mixture for the steroid binding assay. After filtration, the hmPRα protein was remained on the filter or present in the flow-through was determined by western blot analysis using anti-His-tag antibody. (B) Specific binding activity of purified hmPRα to [^{3}H]1,2,6,7- progesterone with 10 and 100 µl Ni-NTA resin supplemented in the reaction mixture.

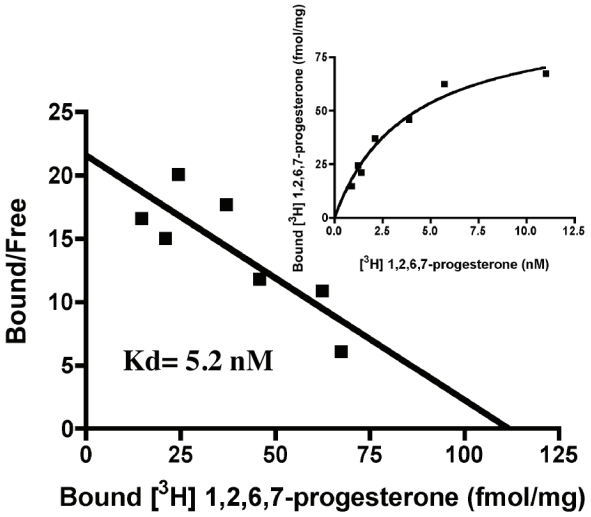


Fig. 33. Saturation curves and Scatchard plots of specific [^{3}H]1,2,6,7-progesterone binding to purified recombinant hmPRα.