Development of the production process for a recombinant hormone and evaluation of its biological activity

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CERTIFIQUEM:

Que el Llicenciat en Biotecnologia Albert Font Ingles ha dut a terme sota la nostra direcció el treball amb el títol "Development of the production process for a recombinant hormone and evaluation of its biological activity" que es presenta en aquesta memòria i que constitueix la seva Tesi per optar al Grau de Doctor per la Universitat Autònoma de Barcelona.

I perquè es prengui coneixement i consti als efectes oportuns, presentem a l'Escola Tècnica Superior d'Enginyers de la Universitat Autònoma de Barcelona l'esmentada Tesi, signant el present certificat a

Barcelona, Maig 2012-05-16

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OBJECTIVES

After thirty years since first recombinant product for humans was launched to the market, still there is not any commercially available recombinant product for veterinary industry. Probably, narrow profit margins and still an excessive lax regulation on veterinary products lead to a lack of concern for the manufacturers. Nevertheless, new more secure and restrictive trends on regulatory affairs in veterinary products adumbrate a change of tendency to recombinant products free of virus, prions or other contaminant proteins.

Inside this framework, the main objective of this thesis is to obtain a recombinant PMSG expressed in *Pichia pastoris* with FSH activity as describe the European Pharmacopeia (Ph. Eur.).

In order to achieve this objective, different milestones or secondary objectives must be aimed;

1. First, to evaluate the use of novel strategies to increase the eventual half-life of the recombinant protein into the bloodstream.
2. Second, to clone the genes into the expression vectors and to obtain *P. pastoris* transformants.
3. Third, to select the best productive clone of *P. pastoris* X-33.
4. Fourth, to set up a purification process of rPMSG.
5. Fifth, to develop an *in vitro* cell-based assay to demonstrate a good folding of rPMSG
6. Sixth, to obtain enough quantities of product to perform efficacy test in vivo.
7. And finally, to evaluate the efficacy of the product *in vivo*.

CHAPTER 1

Introduction

1.1 PREGNANT MARE SERUM GONADOTROPIN

Pregnant mare serum gonadotropin (PMSG) is the formal name of equine Chorionic Gonadotropin (eCG) a molecule that belongs to the family of glycoproteins hormones. PMSG is produced by trophoblast cells of endometrial cups in pregnant mares and plays a major role in the maintenance of early gestation (during first three months) indirectly stimulating the production of progesterone by corpus luteum until the placenta can secrete this steroid for itself. Trophoblast cells secrete PMSG constitutively and reach its maximum around the day 50 of pregnancy and decrease progressively.

Only primates, equids and humans express and secrete this placental hormone which has two interesting characteristics. From an endocrinological view, the ability to work as FSH and LH in species other than horse, and the second unusual property consists in its high carbohydrate content, the highest among the glycoproteins resulting in 45% of its total weight. It is known that this high degree of glycosylation is the responsible of its large half-life in blood circulation, arriving to 6 days in geldings or mares, and 24-26 hours in rabbits[1].

Cole *et al.,* 1967 reported that PMSG is poorly eliminated by renal filtration, urine (1%) and in milk (0.2%), and led him to conclude that the majority of PMSG was metabolized and degraded (85%).

1.2 Chorionic Gonadotropins historical landmarks

Eighty years ago, Harold Cole and George Hart from the University of California found that injecting serum taken from pregnant mares could stimulate the ovarian growth of immature rats and mice. This effect could only be observed when serum was collected between days 37 and 210 of mare gestation.

The responsible hormone was called Pregnant Mare Serum Gonadotropin (PMSG) by Catchpole and Lyons in 1934. Despite initially was assumed to be produced by the pituitary gland, similarly to others glycoproteins hormones of the same family, PMSG was determined that it was secreted by the chorion and stored in the endometrium,

Later, it was described that hCG (human Chorionic Gonadotropin) can elicit responses characteristics of LH (Luteinizing hormone) a pituitary glycoprotein, in contrast, PMSG could mimetize the activity of other pituitary hormone of the same family, Follicle Stimulating Hormone (FSH), but also exhibits LH activity[2].

Due to its similarity to human Chorionic Gonadotropin (hCG) in 1978 Harold Papkoff suggest to change the name to equine Chorionic Gonadotropin.

1.3 Glycoprotein hormones family

Chorionic Gonadotropin (CG), Luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are essential for normal reproductive functions. In contrast to CG, LH and FSH are both synthesized in the pituitary and stimulate gametogenesis and steroidogenesis in the ovary and testis. On the other hand, Thyroid Stimulating Hormone (TSH) belongs to the same family and is produced by the anterior pituitary, but in this case regulates thyroid functions. All together form the glycoprotein hormone family, which are noncovalent heterodimers of α and β subunits.

Within a given organism, α subunit is common to all four hormones, whereas the β subunit confers the biological specificity for each hormone. In human and equine CG, subunit β contain a large C-terminal peptide (CTP) within O-linked oligosaccharides. Additionally, Asn-linked carbohydrates are present into α and β subunits.

In equids, in contrast to primates, both LH and CG β-subunits are encoded by the same gene[3]. Combarnous *et al.,* 1984 described that both placental eCG (PMSG) and

pitutitary eLH exhibit dual LH and FSH in non equine species[4]. Although eCG and eLH have identical polypeptide chains, they exhibit different oligosaccharides content in theirs chains.

On the other hand, both subunits have multiple intramolecular disulfide bonds and the assembly of the subunits occurs primarily in the endoplasmic reticulum, being the rate-limiting step in the process of dimer secretion[5].

Remarkably, α and β subunits, despite displaying completely different aminoacid sequences, have similar three-dimensional structures, including two β hairpin loops on one side of a central cystine knot and a single larger loop on the opposite side. Finally, it seems that the structure of heterodimer is stabilized by a 'seat-belt' structure formed by the C-terminal amino acids of the β subunit wrapping around α subunit[6] (see Figure 1).

The β-subunit contains fully conserved 12 disulfide bonds and the segment between Cys10th and Cys12th is considered the 'seat-belt'. This segment is composed by two sub-segments. Determinant loop comprised between the Cys10th and Cys11th, and 'C-terminal loop' comprehended between Cys11th and Cys12th (Figure 1).

The cristallization of hCG in 1994 by Lapthorn *et al*., revealed that glycoproteins hormones belongs to the superfamily of cysteine knot growth factors[7].

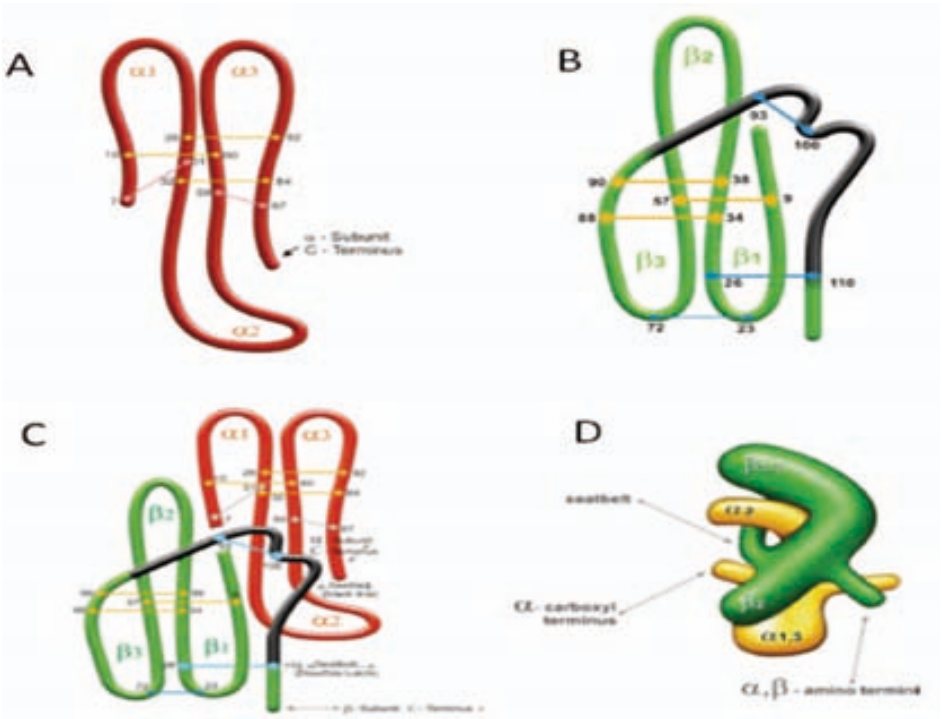

FIGURE 1. Diagram on hCG crystal structure. A) α-subunit. B) β-subunit. C) hCG heterodimer. D) Position of subunits forming the heterodimer[6]

1.3.1 Primary and secondary structure

In 1982, after several temptatives Ward D.N. defined horse α-subunit (P01220) as a glycosylated chain of 96 aminoacids (mature protein, without the signal peptide), consistent with the known structure of α-subunits of other glycoproteins. This subunit displays 70-80% of homology with other mammalian α-subunits. Along with nonconservative substitutions at positions 33, 70 and 96, there is a unique transposition of tyrosine and histidine at position 87 and 93 respectively[1]. All together alter the hidrophobicity of the subunit.

Horse CG β-subunit (P08751), is composed of 149 amino acid, and human CGβ share a 66% of homology between the first 110 aminoacids. However, despite having a similar C-terminal extension (CTP), only three amino acids of this structure are identical.

Relative to its primary structure some authors reported the heterogeneity of horse CG according to its origin and this heterogeneity may play a role in their final activity[1].

The common α-subunit have five disulfide bonds bridging Cys 35-59, Cys 38-88, Cys 56-110, Cys 60-112 and Cys 87-115. In turn, β-subunit contains six disulfide bonds between Cys 29-77, Cys 43-92, Cys 46-130, Cys 54-108, Cys 58-110, Cys 113-120. The positions of all these disulfide bonds are highly conserved and are identical to hCG and human LH, providing the proper folding for the generation of the 'seat-belt' in β-subunit, as well as the 'determinant loop'. Both structures, together with the 'wrapped' region α-subunit seem to bind to the LH/CG receptor.

1.4 Glycosylation of PMSG and its role

Pregnant mare serum gonadotropin is the most heavily glycosylated of the mammalian pituitary and placental glycoproteins, with approximately 45% of its mass attributable to carbohydrate moieties[8].

Both subunits contain N-glycosylations attached to asparagine and O-glycosylations attached to serine or threonine. Relative to the composition of these carbohydrates moieties the major difference is found in the increases of N-acetyl neuraminic (sialic) acid. However, the quantity of sialic acid per mg of PMSG varies between mares and between the stage of gestation in the mare serum[9].

The α-subunit bears two complex-type N-linked oligosaccharide chains located at asparagines 56 and 82, whereas β-subunit has only one at Asn 13. The β-subunit posses a carboxy-terminal peptide (CTP) of 28 amino acids (residues 122-149), which is O-glycosylated at the same twelve serine or threonine residues than eLH[10]. Both hormones strongly differ by their N-glycan termination with sialic acid (Siaα2,3 Gal) on PMSG and sulphated N-acetylgalactosamine (SO_4-4-GalNAc) on eLH[11,12]. Differences in their

molecular weight are essentially due to the presence of longer disialylated poly-N-acetyllactosaminyl O-glycans on PMSG. These structural differences explain why PMSG has such an exceptional half-life.

The disparity between the carbohydrate components are probably due to the site-specific occurrences of the glycosyltransferase enzymes, which have been shown to vary between the pituitary and placenta in other species.

1.5 Folding and heterodimer formation

Due to the similarity between hCG and PMSG, it may be possible to assume similar subunits folding and interaction. Moreover, there are extensive reviews of hCG folding steps from which almost all information have been used for summarizing this section[6,13].

The association of hCG subunits is made when they still contain a high content of mannose. Each subunit is structurally quite similar between them, with two hairpin loops at one end and a single loop at the other[14]. Xing *et al.,* 2004 described the assembly of hCG in the reticulum endoplasmic by threading the glycosylated end of hCGα loop 2 beneath a hole formed in a disulfide strand of β-subunit, the seat-belt, which stabilize the dimer[15] (Figure 2). The final closing of the β26-110 bridge locks the seat-belt preventing disassembly[13].

It seems that the carbohydrate moieties play an important role during the folding and assembly of subunits. In the alpha subunit formation the carbohydrates are not essential, but they may prevent the formation of non-active disulfide bonds and ensure that certain portions of the polypeptide chain remain on the surface during folding[16]. N-linked glycosylation at positions Asn-52 and Asn-78 seems to assure normal secretion of hCGα and its removal reduces αβ correct dimerization proving the importance of N-

linked oligosaccharides[17–19]. By contrast, O-glycosylation does affect neither the assembly nor secretion. On the other hand, the Asn-30 on the hCGβ is important for the secretion but not assembly, and the Asn-13 influences mainly the assembly[20].

1.6 Interaction with the receptor. The specificity.

The glycoprotein hormone receptor (GpHR) formed a family of receptors associated to its ligands, the glycoproteins hormones. The GpHRs are composed of a large N-terminal ectodomain (ECD) organized in leucine-rich repeats (LRRs) involved in affinity and selectivity, seven transmembrane helices and a cytoplasmic α-helix parallel to the cell membrane, characteristic of the rhodopsin-like G protein-coupled receptor (GPCR) family, responsible for the signal transduction[21].

The ECD adopts the horseshoe-like curvature of LRR proteins in order to adapt the hormone (Figure 3A). Downstream of the LRR region, the ECD has a cysteine cluster domain (hinge region), involved in receptor inhibition/activation.

In this case, once again, through the crystal structure of hCG it may be assume many similarities between human and horse.

It has been reported that specifically it is the 'determinant loop' of the hCG which determines the specificity of binding. Moreover, the 'determinant loop' of hCG has two basic arginines (Arg 94 and 95), while the FSH has two acidic residues. Appropriately, the electrostatic surface of LH/CG receptor in this area is negative and the FSH receptor is positive (Figure 3C and 3D).

In contrast to the 'determinant loop', the interactions of the 'C-terminal loop' of hCG and its receptor are very similar to those observed in the FSH-FSHr interaction[21] (Figure 3A).

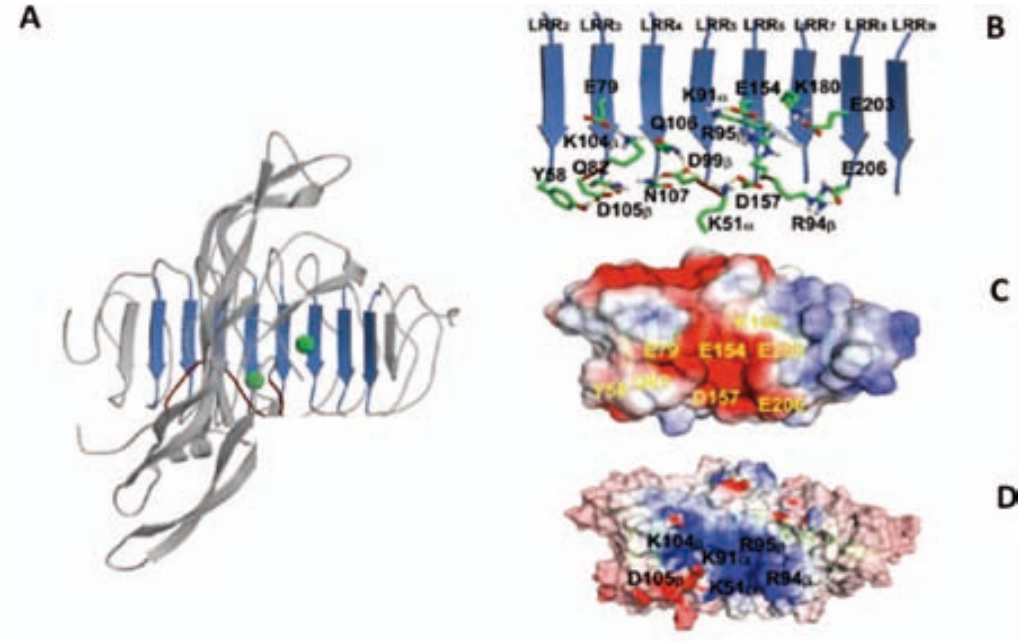

FIGURE 2. A) Crystal structure of the complex human (FSHr)-FSH. B) Detailed view of the interaction between LRR region and C-terminal of α subunit and seat-belt of β subunit. C) Molecular electrostatic potential at the concave inner of the hCG receptor. D) Molecular electrostatic potential of hCG hormone. The red zone represents negative net charge, and the blue zone represents the positive net charge.[21]

1.7 Biological activity of PMSG in equids

In 1974, Harold Papkoff reported the necessity of heterodimer formation for a full biological activity, since the dissociated subunits alone have much less activity than the dimer.

The similarity between PMSG and eLH explain the ability of hCG to bind LH receptors in equine testis. However, this occurred at about one-tenth or less the affinity of eLH[22]. Probably this difference may be made by their dissimilar carbohydrate content.

In horses, PMSG is primarily an LH-like hormone, since has been described its inability to bind to FSH receptors in horse follicles or testis. Surprisingly, PMSG binds to donkey FSH receptors.

1.8 Biological activity of PMSG in nonequids species

Undoubtedly the most interesting feature of the PMSG is its unique ability to express both FSH-like and LH-like biologic activities when they are administered to other mammalian species. In contrast, hCG and other CGs of other primates species only can induce LH-like responses.

With the discovery of radio receptor assay appeared the first indication that donkey CG exhibits also FSH-like activity in non-equine species, but considerably less than PMSG. Something similar was discovered for zebra CG.

Still now, the exact structural basis of dual biologic activities remains unclear. Probably it lies within the β subunit, which except the C-terminal region, is identical to LH-like (sharing 70% of homology with other LH β of other species)[23]. Therefore, the result of minor amino acid differences within the β subunit seems to the responsible of the dual activity. Although the carbohydrate moieties contribute to biologic half-life, receptor binding affinity and signal transduction there is not evidences to support their involvement in receptor binding specificity.

Nowadays, the FSH-like properties of PMSG have been extensively used for stimulate the follicular growth and ovulation in laboratory animals and in farm animals species. The main source is partially purified extracts of the hormone as the cheapest and most readily available. It can be used at lower doses as an adjunct to progestagen withdrawal or other forms of estrous synchronization to induce normal rates of ovulation in adult or prepubertal animals. Alternatively, when it is administered in higher doses, its FSH-like components stimulates the development of multiple follicles, which are then ovulated by the LH-like component to give the superovulation that is required in embryo recovery and transfer programs[23].

1.9 PREGNANT MARE SERUM GONADOTROPIN MARKETS

In Spain, products sales containing PMSG raised 3.05 M of Euros in 2007, 1.85 M of which were products where PMSG was the only active hormone[24]. It was reported that worldwide PMSG market worth 93 M Euros/year in 2008[25].

1.9.1 Reproductive sows market

It was estimated on 30.3 millions the number of reproductive sows in Europe, U.S.A, Russia and Brazil together and more of 44 millions in China (Table 1)[25]. Considering that artificial insemination is the normal procedure in developed countries and in emerging countries is indeed increasing considerably, worldwide market may be ciphered in 74.4 Million Euros, with realistic predictions to reach 126.6 Million Euros if only 50% of Chinese sows were inseminated artificially in the future.

TABLE 1. Reproductive sows in principal producer countries[25]

Country	Sows number (x1000)
China	44.099
UE-27	16.573
USA	6.605
Russia	4.109
Brazil	3.050
The Philippines	2.203
Canada	1.517

1.9.2 Ovine market

Despite of ovine artificial insemination is not as developed as sow's trade; this is presented as an interesting niche market due to its simple applicability and the necessity to create tools to improve livestock productivities.

In 2006 the worldwide niche market was 545.7 Millions of reproductive ewes, 134.7 Millions of which were in Europe, Oceania and USA (Table2). This can be traduced to a potential market of 139.4 Millions of Euros[25].

TABLE 2. Worldwide reproductive ewes in principal producer countries [25]

Country	Ewes number (x1000)
Europe	**69.500**
Oceania	**66.240**
North America	**3.640**
South America	**41.600**
Asia	**232.960**
Africa	**131.760**

In conclusion, adding previous section, a potential market over 210 M of Euros annually may be considered for PMSG. Considering new trends in regulatory policies of either EMEA or FDA promoting those drugs obtained recombinantly over those obtained from animal sources, this hormone represents a good opportunity to become the first recombinant drug launched to the veterinary market.

1.10 State-of-the-art of commercial recombinant gonadotropins for humans.

Originally gonadotropins were prepared from animal extracts, the original sources included pig pituitary glands, mare serum or pituitary glands from human cadavers, but

nowadays they are no longer used for clinical uses because of possible transmission of slow viruses and due to the demand for larger amounts of product. Advances on the purifying process let to obtain a highly purified urine-derived FSH (Metrodin HP, Serono Inc.) which allowed the subcutaneous injection. More recently, biotechnology has made available recombinant preparations of gonadotropins produced in Chinese hamster ovary cell lines.

The use of recombinant gonadotropins allows avoiding undesirable contaminations from urine which may cause local reactions in the tissue and may require intramuscular injection. Moreover, usually some LH contamination remains in purified forms and significant variability is present batch-to-batch.

The recombinant production process of gonadotropins allows absolute source control as well as batch-to-batch consistency and preparations free of LH activity. Additionally, recombinant products also have low immunogenicity and the amount of protein administered is lower allowing the subcutaneous self-administration. Furthermore, these recombinant preparations reduce the risk of viral contamination as well as drugs being coextracted from the urine[26].

At the time being, several recombinant products have been launched to the market, the following are the marketed products for human use.

Recombinant FSH came out in the mid nineties as Puregon® or later Follistim® (Organon, Schering-Plough), both are the brand name of the generic follitropin beta[27].

Serono Labs launch in 2000 Gonal-F® which was the brand name of follitropin alfa. Both follitropin alfa and follitropin beta are indistinguishable.

At the beginning of 2010, was approved Corifollitropin alfa (Elonva®, Merck & Co.Inc), a long lasting quimera formed with β-subunit of FSH and the C-terminal peptide

of the hCG β-subunit. With Elonva®, patients only need a single injection during the first seven days of treatment[28].

Recombinant LH receives the name of Luveris® (lutropin alfa, Merck & Co.Inc) which usually is used in combination with Gonal-F®.

Finally, Ovidrel® is the name of choriogonadotropin alfa for injection (Serono Labs) approved in 2000. It is the recombinant product of hCG, and it is almost identical with small variations on their carbohydrate content that do not affect its bioactivity.

1.11 RECOMBINANT PROTEINS. A GROWING MARKET

In 1982 the first recombinant-DNA-sourced protein insulin was launch to the market by Eli Lilly (Humulin)[29]. Since then, therapeutic protein market has shown a very healthy growth of 15-19%[30], with a continuous global expansion, business formation and technological diversification. Only the human medical biotechnology industry, worth over 32$ billion in 2003[31] with a global sales of $61 billion in 2009 (excluding antibodies), yielding an overall 2009 global market value of $99 billion[32].

Nevertheless, the rate of approval of new biological entities (NBE) has slowed over the past four years (see Figure 3). Among the 58 approved biopharmaceuticals since 2006, only 25 (40%) can be considered as strictly new. This data reflects a change of trend in the market, since 28 of approvals are considered biosimilars or 'me-too' products[32].

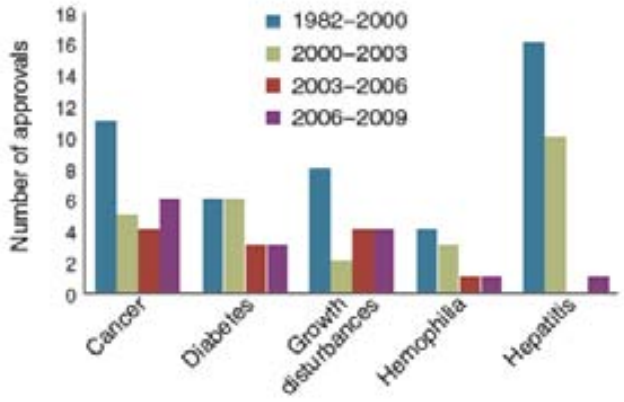

FIGURE 3. Number of biopharmaceuticals approved in five major markets[32]

Between these reformulated versions of pre-existing products, it stands out some gonadotropins such as Fertavid (Schering-Plough) which contains follitropin-β (rhFSH). The same substance is found in Puregon, approved in 1996. Serono's Pergoveris simply contains a fixed-dose combination of follitropin alpha (rhFSH) and lutropin alpha (rhLH), which have been individually marketed for years as Gonal F and Luveris, respectively.

During the early 2000s, European Union developed legislative and regulatory provisions for the approval of biosimilars. These regulations necessitate the generation of comparative data between the proposed new biosimilar product and the reference product. The application dossier must contain details of manufacture and analysis, as well as abbreviated clinical and non-clinical modules. However, the robustness of European guidelines has been validated by the approval of 14 biosimilar. European regulators are updating guidelines specific for biosimilar follicle-stimulating hormone or EPO products[32].

But certainly, the relevance of therapeutic biotechnology is not restricted to the human domain. Veterinary industry, since ancient times has been interested on breed selection for higher livestock productivities. Nowadays several biotech platforms, such as

genomics or proteomics, have been applied not only in animal health, but considering animals as models for in vivo studies of genetic diseases or new drug pharmacodynamic studies as well. Over the past 20 years, global socio-economic changes such as: i) the control of epidemic diseases in developed countries (being evident the benefits of their control), ii) the lower importance of agriculture in the national economy resulting in stronger competition for funds or iii) the increasing transfer of more responsibilities from the public to the private sector which is more concerned with the visible returns of investment, can explain the growing importance of animal health economics which have affected decisions on animal health measures[33]. Despite of this growing interest, biopharmaceutical industry has a limited presence in veterinary affairs. Mainly recombinant vaccines, which is perhaps to be expected, given that vaccination is essential for prevention of the rapid spread of communicable diseases through the high-density populations of domestic animals in modern veterinary practice (Table 3).

TABLE 3. RECOMBINANT VETERINARY MEDICINAL PRODUCTS APPROVED IN THE EU UNTIL 2002[29]

Product	Company	Therapeutic indication	Approved
Porcilis Porcoli (combination vaccine containing r *E.coli* adhesins)	Intervet	Active immunization of sows	1996
Fevaxyn Pentofel (combination vaccine containing r Feline leukemia viral antigen as one component)	Fort Dodge Laboratories	Immunization of cats against various feline pathogens	1997
Neocolipor (vaccine containing four inactivated *E.coli* strains;two wild type strains expressing *E coli* adhesions F6 and F41 and two recombinant strains, engineered to express F4 and F5 adhesins)	Merial	Reduction of neonatal enterotoxicosis of young piglets caused by *E. coli* straisn expressing F4, F5, F6 and F41 adhesins	1998
Porcilis AR-T DF (combination vaccine containing a modified toxin from *Pasteurella multocida* expressed in *E. coli*)	Intervet	Reduction in clinical signs of progressive atrophic rhinitis in piglets:oral administration	2000
Porcilis pesti (vaccine containing r Classical swine fever virus E_2 subunit antigen produced in an insect cell baculovirus expression system)	Intervet	Immunization of pigs against classical swine fever	2000

Ibraxion (vaccine consisting of an inactivated, bovine herpes virus type 1 engineered by removal of the viral glycoprotein *gE* gene)	Merial	Active immunization of cattle against infectious bovine rhinotracheitis	2000
Bayovac CSF E2 (vaccine consisting of r Classical swine fever virus E2 subunit antigen produced using baculovirus vector system)	Bayer	Immunization of pigs against classical swine fever virus	2001
Eurifel FELV (Vaccine consisting of an engineered canarypox virus into which the *gag*, *env* and a partial *pol* gene of feline leukemia virus have been inserted	Merial	Immunization of cats against feline leukemia virus (FeLV)	2000
Vibragen Omega (r feline interferon omega)	Virbac	Reduce mortality/clinical signs of canine parvovirosis	2001
Eurifel RCPFEVL (multicomponent vaccine containing as one component an engineered canarypox virus into which the *gag*, *env* and a partial *pol* gene of feline leukemia virus have been inserted	Merial	Active immunization of cats against viral pathogens, including feline leukemia virus	2002
Gallivac HVT IBD (live multicomponent vaccine containing as one component an engineered herpes virus of turkeys (HVT) housing a gene coding for the protective VP2 antigen of the infectious bursal disease virus (IBDV)	Merial	Active immunization of chickens against, among others, the viral causative agent of infectious Bursal disease	2002

Interestingly, several products listed above are produced in insect-based system, whereby insect cells can be grown inexpensively and subsequently infected by baculovirus carrying the gene coding the protein of interest.

1.12 OVERVIEW OF MAIN EXPRESSION SYSTEMS

Over the last few decades, microbiology and genetics have merged creating a large path where to run together identifying and expressing genes into a variety of organisms that are quite different from the source organism. Mainly these enlarging list of recombinant organisms are used to produce proteins since many of them have an immense commercial value[34].

Currently, six different expression platforms are used for manufacture of biopharmaceuticals; bacteria, yeast, insect cells, mammalian cells, transgenic animals and transgenic plants. But the selection of the right system is not a trivial problem since every platform presents several advantages and drawbacks for a given protein. In figure 4 are displayed main five different expression systems requested by Lonza's customers from 1998 to 2008.

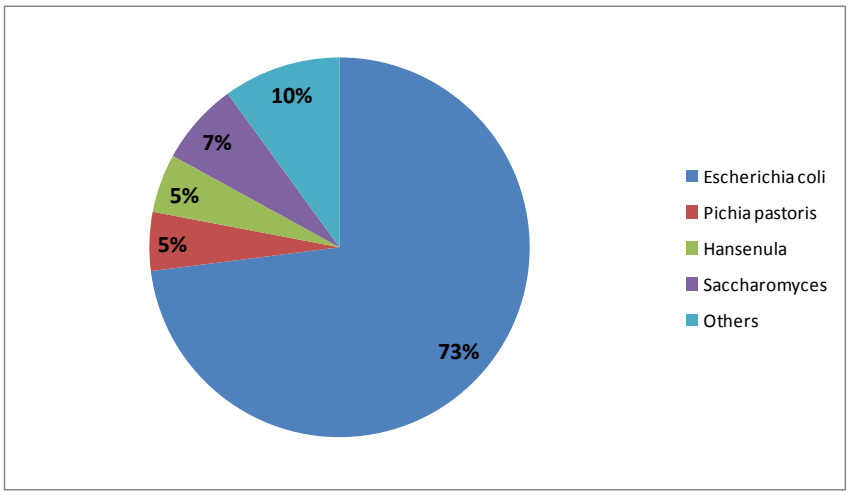

FIGURE 4. Distribution of expression systems requested by Lonza's customers from 1998 to 2008[1]

Nowadays, CHO cells and *E.coli* stand out over the others in biotechnology industry. However other expression systems, such as *Bacillus subtillis*, *Saccharomyces cerevisiae* or *Pichia pastoris* have been used with success.

Hereafter principal expression systems are briefly described in a table 4.

TABLE 4. Advantages and disadvantages of main expression system[35]

Organism	Advantages	Disavantages
E.coli	Genetically well-known Easy to scale up Short doubling time Established regulatory track record Cheap media	Endotoxin presence No post-translational modifications Intracellular expression Subsequent folding steps
Yeast	Few host proteins secreted GRAS status Short doubling time High yields/High cell-densities Post-translational modifications Cheap large scale cultures Genetically well-known	Over glycosylation of proteins Hipermannosylation Usual proteolysis of proteins
Insect cells	Post-translational modifications Protein secreted to the medium Innocuous vectors for infection	Cells are killed during infection No complex N-glycosylation Sensitive to shear forces Instability of cell lines Immunogenic host cell proteins Risk of infection with mammalian viruses
Mammalian cells	High-complex proteins Proteins secreted to the medium Good regulatory track record	Low-cell densities/Low productivity Low doubling time Expensive media Presence of virus Sensible to shear forces Characterization of transformed lines
Transgenic animals	High expression levels Low cost production High-complex proteins Easy to scale-up	Risk of viral contamination Risk of prion contamination Long period of time to obtain the protein No regulatory experiences

1.13 METHYLOTROPHIC YEASTS. A BRIEF HISTORY

A small portion of yeast genera with the ability to utilize methanol as a sole carbon source was first described for Koichi Ogata. Only six genera were identified as methylotrophic yeast: *Hansenula, Pichia, Candida, Turolopsis, Kloeckera and Rhodotorula*[36] .

By the year 1970, the company Phillips Petroleum was interested in the production of biomass (single-cell protein) from the methanol, leading to the isolation of *Pichia pastoris*. Efficient cultivation techniques were developed for *P. pastoris*, reaching cell densities higher than 100 g L^{-1} of DCW (Dry Cell Weight). However, because of the oil crisis during 1970's, the feed stocks production from methanol no longer was financially interesting.

After that, The Phillips Petroleum Co. contracted the Salk Institute Biotechnology/Industrial Associates, Inc. (SIBIA, La Jolla, CA, USA) to develop *P. pastoris* for heterologous protein expression. Specifically, a strain was genetically engineered to produce proteins on a cost efficient minimal defined medium.

In 1993 Phillips Petroleum Co. sold the system patent to RCT (Research Corporation Technologies) who is still the current patent holder and subsequently also licensed Invitrogen Corporation (Carlsbad, CA, USA) to sell components of the system.

1.14 *Pichia pastoris* AS PROTEIN EXPRESSION SYSTEM.

Pichia pastoris combines several unique characteristics which make it one of the most promising expression systems, especially during last decade when several glycosylation-modified strains have been developed to produce biopharmaceuticals, a field which so far was almost exclusive for mammalian cells[37].

Although its recent success in biopharmaceuticals, *P. pastoris* has been used extensively to produce successfully more than 1000 proteins[38].

The preference for respiratory growth (mandatory in the case of methanol), in contrast to the fermentative growth, allows the microorganism reach very high cell densities, up to 100 g·L^{-1} of DCW, in a minimal synthetic and cheap media. Likewise, using defined media, toxins or pyrogens sources are avoided, making it compatible for

biopharmaceutical manufacturing. Respiratory growth preference turn out to be a major advantage in comparison with the well-known *Saccharomyces cerevisiae*, making it ideal for large scale productions, without yielding overflow metabolites. Fermentative growth would produce rapidly enough amounts of ethanol which would limitate not only yeasts cells growth, but also foreign protein expression.

Additionally, its similitude to the *S. cerevisiae* regarding genetic manipulation techniques has supported the adaptability of several tags, sequences or signals such as those regarding protein secretion (e.g. α-factor), simplifying enormously the downstream processing as cells do not have to be disrupted in order to recover the protein. Moreover, the recovery and purification of recombinant proteins expressed in *P. pastoris* are facilitated by the very low levels of endogenous proteins secreted to the medium[39].

The expression vectors can be integrated directly into the *P. pastoris* genome maximizing the stability of the expression systems and obtaining single- and multiple-copy stable integrants.

Another important characteristic of *Pichia pastoris* is the presence of tightly regulated and highly methanol-induced alcohol oxidase promoter AOX1, that controls the expression of enzyme alcohol oxidase 1 in the wild type strain. This promoter is, by far, the most used for heterologous expression in this yeast[34]. Furthermore this tightly regulation allows to prevent the selection of non-expressing mutants during biomass generation using repressing carbon sources in those cases where the expressing protein is toxic for the yeast.

As eukaryotic organism, *Pichia pastoris* has the ability to perform many of the post-translational modifications usually carried out in higher eukaryotes. These modifications, such as disulphide bond formation, certain types of lipid addition, N- and O-glycosylation or processing signal sequences are usually essentials for the integrity of

pharmaceutical product. Unlike *S. cerevisiae*, *Pichia pastoris* normally does not hyperglycosilate the recombinant proteins which turns out in an important advantage, however, in contrast to mammal's glycosylation profile, yeasts prone to add highly immunogenic mannose residues.

1.14.1 Methanol utilisation pathway

The facultative methylotrophic yeast species are able to growth on methanol due to a system based on the expression of some enzymes implicated on methanol metabolism, but virtually absent in cells growing on glucose, glycerol or ethanol.

Oxidation of methanol is carried out in a highly specialized organelle called peroxisomes where the alcohol oxidase AOX catalyzes the oxidation of methanol into formaldehyde and hydrogen peroxide. Peroxisomes encapsulates the hydrogen peroxide, sequestering it away from the rest of the cell, herein a catalase (CAT) is the responsible of its elimination to water and molecular oxygen (Figure 5), whereas formaldehyde can enter in either the assimilatory pathway or the dissimilatory pathway. In the dissimilatory pathway, formaldehyde is oxidized in the cytoplasm by two different NAD dependent dehydrogenases; formaldehyde dehydrogenase (FLD) and the formate dehydrogenase (FDH). Lee *et al.,* 2002 suggested that this pathway constitute an important role in the detoxification of formaldehyde in methylotrophic yeasts[40].

Glutathione-dependent formaldehyde dehydrogenase catalyzes the production of formate, from which carbon dioxide is produced by the action of formate dehydrogenase.

In the assimilatory pathway, formaldehyde will form cell constituents. Growing in methanol, these yeasts synthesize cell biomass through via xilulose-5-phosphate (Xu_5P). After a transkelotase reaction, two trioses are synthesized in the peroxisome by a dihydroxiacetone synthase (DAS), glyceraldehyde-3-phosphate (GAP) and

dihydroxiacetone (DHA). Subsequently, in the cytosol DHA is phosphorilated by a dihydroxiacetone kinase[2] and in an aldolase reaction condensate to fructose-1,6-bisphosphate ($F_{1,6}BP$) and later to fructose-6-phosphate (F_6P), which will enter to pentose phosphate pathway regenerating xylulose-5-phosphate. After 3 cycles a molecule of glyceraldehyde-3-phosphate will be generated in order to form biomass.[41]

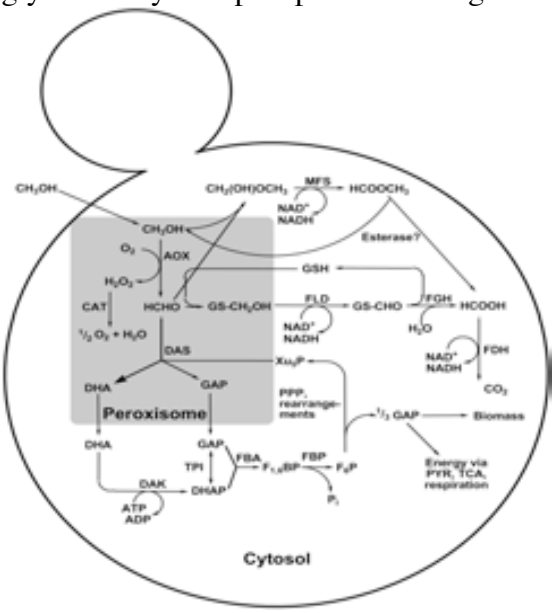

FIGURE 5. Methanol utilisation pathway in methylotrophic yeasts: The main pathways and the respective enzymes working in the methanol metabolism in methylotrophic yeasts are shown. AOX: alcohol oxidase (EC 1.1.3.13), CAT: catalase (EC 1.11.1.6), FLD: formaldehyde dehydrogenase (EC 1.2.1.1), FGH: S-formylglutathione hydrolase (EC 3.1.2.12), FDH: formate dehydrogenase (EC 1.2.1.2), DAS: dihydroxyacetone synthase (EC 2.2.1.3), TPI: triosephosphate isomerase (EC 5.3.1.1), DAK: dihydroxyacetone kinase (EC 2.7.1.29), FBA: fructose 1,6-bisphosphate aldolase (EC 4.1.21.13), FBP: fructose 1,6-bisphosphatase (EC 3.1.3.11), MFS: methylformate synthase; DHA: dihydroxyacetone, GAP: glyceraldehyde 3-phosphate, DHAP: dihydroxyacetone phosphate, $F_{1,6}BP$: fructose 1,6-bisphosphate, F_6P: fructose 6-phosphate, P_i: phosphate, Xu_5P: xylulose 5-phosphate, GSH: glutathione, PYR: pyruvate; PPP: pentose phosphate pathway, TCA: tricarboxylic acid cycle[41]

1.14.2 The AOX1 and another promoters

The genome of *P. pastoris* contains two copies of the AOX gene[42]. Around the 85% of oxidase activity in the cell is regulated by the AOX1, isolated by Ellis in 1985, whereas AOX2 regulates the other 15%[43]. The AOX1 can account over 30% of the total cell protein when is growing on methanol, these large amounts of the AOX1 protein are produced because of its low affinity for oxygen. The AOX1 protein transcription is tightly regulated by two different mechanisms: a repression/derepression mechanism plus an induction mechanism, in which the presence of methanol seems to be an indispensable

element[44], unlike *Hansenula polymorpha* where the AOX gene can be induced by limiting concentrations of glycerol or glucose[45]. The repression of transcription by the initial carbon source guarantees a good cell growth before over expression of recombinant product.

However, due to inaccuracy of online probes and the difficulties of offline measurements, monitoring methanol during a process usually represents an important handicap. Another important disadvantage of AOX1 in large scale productions is the necessity to store huge amounts of methanol which constitute a not inconsiderable fire hazard. Furthermore since methanol is mainly derived from petrochemical sources, may require critical purification steps for production of certain foods and additives[34].

There are several alternatives to AOX1 promoter for the expression of heterologous proteins in *P. pastoris*. In 1997 Waterham and co-workers proposed the use of constitutive glyceraldehydes-3-phosphate dehydrogenase (GAP) promoter as a practical alternative to the AOX1 in some cases. They concluded that in glucose-grown shakeflask cultures, GAP promoter appeared to be significantly stronger than AOX1, furthermore they also underline that its strength varies depending on the carbon source[46].

The formaldehyde dehydrogenase (FLD1) promoter is a strongly and independently induced either by methanol as a carbon source or methylamine as nitrogen source[47]. The formaldehyde dehydrogenase plays an important role in the methanol catabolism, as well as in the methylated amines metabolism as nitrogen source. Shen e*t al*., 1998 found that induction levels of the FLD1 promoter by methanol were dependent on the carbon/nitrogen source combination used, with co-induction using methanol and methylamine resulting in higher expression levels of *Rhizopus oryzae* lipase than induction of the FLD1 promoter with methanol and ammonium[47].

Recently other promoters have been described successfully for recombinant expression on *P. pastoris*; 3-phosphoglycerate kinase (PGK1)[48], isocitrate liase (ICL1) promoter[49], or translational elongation factor 1-α (TEF1) promoter[50], constitute valuables alternatives to AOX1.

1.14.3 Strains and phenotypes

The majority of *P. pastoris* strains are derivatives of the wild-type strain Y-11430 from Northern Regional Research Laboratories (NRRL; Peoria, IL). Strain X-33 is a GS115 transformed with the wild type *P. pastoris his4* gene, therefore no requiring supplementation for growth on minimal media[51,52].

There are available several auxotrophic strains with multiple selection markers that allow the concomitant expression of several heterologous genes. Many of these strains are derived from *his4* auxotrophic strain GS115. Similarly different combinations of auxotrophic mutations in *arg4, ade1, ura3* and *ura5* are accessible[51].

Regarding with methanol metabolism and its capability to growth on this carbon source exists three methanol-utilizing phenotypes (Mut) of *P. pastoris*; Mut^+, Mut^s and Mut^- strains. The Methanol utilisation plus (Mut^+) phenotype contains both AOX1 and AOX2 and grows on methanol at the wild-type rate and requires high feeding rates of methanol in large-scale fermentations. Mut^s (slow) has a disruption in the AOX1 gene, affecting its ability to growth on methanol and protein production since only AOX2 gene remains intact. Mut^- strain lacks both AOX1 and AOX2 genes and resulting cells are unable to grow on methanol.

Both Mut^s and Mut^- retain the ability to induce high-level expression from the AOX1 promoter. In fact, in some cases Mut^s has been described as ideal strain for difficult expressions or certain proteins which may require large processing into the cell.

Additionally, these strains do not require large amounts of methanol routinely used for large-scale fermentations of Mut^+.

A reiterative problem associate with *P. pastoris* expression is the protein degradation. In order to reduce this drawback a group of protease deficient strains; SMD1163 (*his4 pep4 prb1*), SMD1165 (*his4 prb1*) and SMD1168 (*his4 pep4*) are available. These strains are particularly useful in high-cell densities fermentation where the lysis of small amounts of cells provoke a high concentration of vacuolar proteases such as proteinase A (pep4) and proteinase B (prb1) in the medium that affect the final protein yield. It should be underline that protease-deficient strains exhibit a slower growth rates, lower transformation efficiencies and lower viability.

Differences in protein-linked carbohydrate synthesis between *P. pastoris* and humans have made the proteins expressed in *P. pastoris* unsuitable for therapeutic uses, mainly due to the recognition of high-immunogenic terminal mannoses by mannose specific receptors in macrophages which finally lead to a dramatic decrease of protein half-life.

Such a reason drove to develop by two different laboratories different strains capable to express therapeutic proteins with a partial- or fully-humanized glycosylation. Jacobs *et al.,* reviewed in 2009 the Glycoswitch technology, which can reach hybrid type ($GalGnM_3$) and complex type ($Gal_2Gn_2M_3$) oligosaccharides with terminal galactoses[53]. On the other hand, the company Glycofi (Lebanon, NH) has developed an engineered a strain with fully humanized sialylated glycoproteins, opening the therapeutic glycoprotein market to *Pichia pastoris* expression platform[37].

On the other hand, specific characteristics of some strains such as overexpressing protein disulfide isomerise (PDI) or *fld1* mutants as selectable marker have been

established in order to facilitate the recombinant protein expressions. Table 5 shows principal strains described so far.

TABLE 5. List of Pichia pastoris strains[51]

Strain	Genotype
Y-11430	Wild type
X-33	Wild type
Auxotrophic strains	
GS115	*his4*
GS190	*arg4*
JC220	*ade1*
JC254	*ura3*
GS200	*arg4 his4*
JC227	*ade1 arg4*
JC304	*ade1 his4*
JC305	*ade1 ura3*
JC306	*arg4 ura3*
JC307	*his4 ura3*
JC300	*ade1 arg4 his4*
JC301	*ade1 his4 ura3*
JC302	*ade1 arg4 ura3*
JC303	*arg4 his4 ura3*
JC308	*ade1 arg4 his4 ura3*
YJN165	*ura5*
Protease-deficient strains	
KM71	*his4 arg4 aox1Δ::ScARG4*
MC100-3	*his4 arg4 aox1Δ::ScARG aox2 Δ::Pphis4*
SMD1168	*his4 Δpep4::URA3 ura3*
SMD1165	*prb1 his4*
SMD1163	*pep4 prb1 his4*
SMD1168 kex1::SUC2	*Δpep4::URA3 Δkex1::SUC2 his4 ura3*
Other strains	
GS241	*fld1*
MS105	*his4 fld1*

1.14.4 Vectors

A wide variety of commercial vectors are available for *P. pastoris*, most of them are covered by patents by the company Research Corporate Technologies RCT (Tucson, AZ) and commercialise by Invitrogen (Carlsbad, CA). The current situation is that some patents for the original *P. pastoris* vectors, mainly created in the 80's, are already expired or they are just about to expire. For that reason some companies have started to develop its own system free of licenses, fitting pieces of interest like a puzzle.

All the expression vectors available are *E. coli*/*P. pastoris* shuttle vectors, containing markers in one or both organism and an origin of replication for plasmid maintenance in *E .coli*. In addition, these vectors have a multiple cloning site (MCS) for insertion of the expression sequence, flanked by promoter and termination sequences derived from AOX1.

Vectors can be used either for intracellular or extracellular expression. Extracellular expression requires a secretion signal sequence before the MCS, as the alpha-mating factor pre-pro leader (α-MF) from *S.cerevisiase* or *P. pastoris* acid phosphatase (PHO) signal.

Another important part is the selectable markers. In bacteria the stability of plasmid into the cell needs the presence of a selectable antibiotic resistance gene. However, several plasmids rely on a single resistance marker for selection in both bacteria and yeast, reducing by almost half the size of the expression vectors. These two genes are: *Sh ble* gene that confers resistance to Zeocin and the *blasticidin S deaminase*, *bsd* gene, which confers resistance to blasticidin S HCl. Furthermore, multi-copy integration events of the expression cassette can be selected using any of them.

Currently vectors containing Zeocin resistance such as the pPICZαA, B, C series are the most used in comparison with the others (Figure 6).

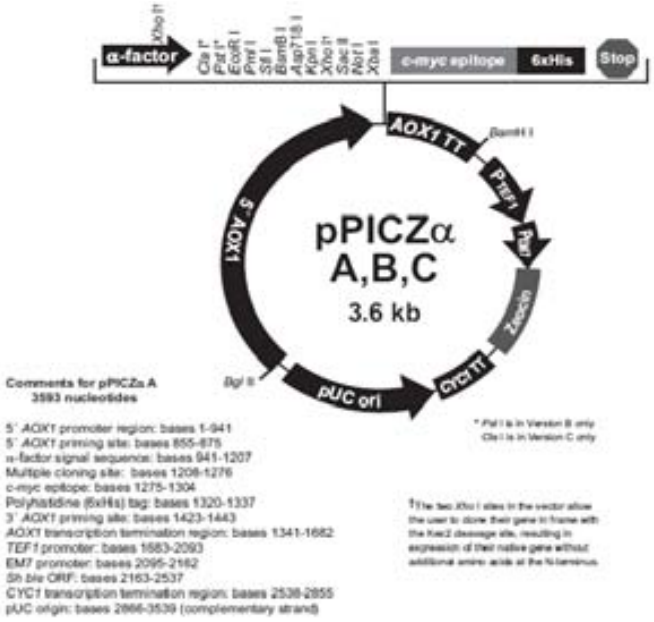

FIGURE 6. *Pichia pastoris* vector pPICZαA, B, C. MCS is flanked by *S. cerevisiae* α-MF, c-myc, and 6xHIS tags for immunological detection and purification. The TEF1 promoter and CYC1TT are from *S. cerevisiae*. EM7 promoter is from *E. coli*. BamHI and BglII sites allow for cloning of multiple head-to-tail expression cassettes[52]

1.14.5 Integration into the genome

One of the advantage of yeast as expression system underlies in the integration of expression cassette into the genome when it is internalized into the cell, maximizing the stability of expression strain.

There can be two different integration events; the simplest way consist in restrict the vector at unique site in either the marker gene or the AOX1, GAP or FLD1 promoter fragment, and subsequently transform it into the appropriate strain. The linear vector will integrate into the host genome by a single crossover insertion event[52].

Otherwise, some vectors in *P. pastoris* allow a gene replacement by double recombination crossover event between the AOX1 promoter and the 3' AOX1 regions of

the vector and genome. The vector must be digested previously in such a way that the expression cassette and marker gene are released. This event results in AOX1 gene deletion.

1.15 POST-TRANSLATIONAL MODIFICATIONS IN YEAST

The majority of approved therapeutic proteins, as well as those in development, carry some kind of post-translational modifications (PTMs). Moreover, glycosylation which represents the most widespread PTM found in natural and biopharmaceutical proteins (~50% of human proteins are glycosylated) can be found in 40% of the approved therapeutics today, excluding monoclonal antibodies[54,55]

A broad range of different PTMs can be attached to proteins, the most common of which are; Acetylation, acylation, ADP-ribosylation, amidation, γ-carboxylation, β-hydroxylation, disulfide bond formation, glycosylation, phosphorylation, proteolytic processing and sulfation[56].

Nowadays approximately 70% of approved therapeutic proteins have been expressed in CHO cells[55], probably because of its ability to perform with success the majority of PTMs. However, yeasts have the potential to perform many of the PTMs typically associated with higher eukaryotes. This represents one of the major advantages of yeast over bacterial expression system, which are not able to form disulfide bonds or add sugar chains in the right place.

1.15.1 Secretion signals

Purification steps usually represent the major cost of any biopharmaceutical production process. For this reason, being *Pichia pastoris* an organism which almost does not secrete endogenous proteins to the extracellular medium, it represents an excellent

system. More, for recombinant expression in *P. pastoris* there are largely used secretion signals which can be attached in frame to the protein to be exported out of the cell.

Several different secretion signal sequences, including the native secretion signal present on heterologous proteins, have been used successfully, α-factor prepro peptide of *S. cerevisiae* and *P. pastoris* acid phosphatase are the most applied.

The pre-pro sequence of the *S. cerevisiae* α-mating factor consists of 19-amino acid signal (pre) sequence followed by a 66-residue (pro) sequence containing three consensus N-linked glycosylation sites and a dibasic Kex2 endopeptidase processing site. Brake *et al.,* 1984 reported the specific proceed which follows this signal, which begins with an early removal of pre signal in endoplasmic reticulum, followed by a cleavage between Arg-Lys of the pro leader sequence by Kex2 endopeptidase and finalized by a rapid cleavage of Glu-Ala repeats by the Ste13 protein[57].

1.15.2 O- and N-linked glycosylation

The amino acid sequence of a protein does not determine how that protein is going to be glycosylated. The mix of glycosyltransferases and even the conditions under which those cells are cultured will conclude what oligosaccharides structures are added to the molecule. Moreover, cells do not add identical oligosaccharides to each protein despite having the same glycosyltransferases, which means that the same protein may have different variants[55], obtaining an extremely heterogeneous mixture of glycoforms that is both difficult to purify and to characterize in detail.

The presence of these variants or glycoforms may affect the glycoprotein folding, stability, trafficking and immunogenicity as well as its primary functional activity (Table 6)[56]. Furthermore, in the same organism the glycoforms profile and activity can be different depending on the tissue in which it is expressed.

TABLE 6. The potential role and effect of the glycocomponent of glycoproteins[56]

Role/effect	Comment
Protein folding	Glycosylation can affect local protein secondary structure and help direct folding of the polypeptide chain
Protein targeting/trafficking	The glycocomponent can participate in the sorting/directing of a protein to its final destination
Ligand recognition/binding	The carbohydrate content of antibodies function in antibody binding to monocyte Fc receptors and interaction with complement component $C1_q$
Biological activity	The carbohydrate side chain of gonadotropins (specially the α-subunit N^{52} side chain) is essential to the activation of gonadotropin signal transduction
Stability	Sugar side chains can potentially stabilize a glycoprotein in a number of ways including enhancing its solubility, shielding hydrophobic patches on its surface, protecting from proteolysis and directing participation in interchain stabilizing interactions
Regulates protein half-life	Large amounts of sialic acid can increase a glycoprotein's half-life. Exposure of galactose residues can decrease plasma half-life by promoting uptake through hepatic galactose residues. Yeast N-glycosylation is of high-mannose type, driving rapid removal from circulating through mannose receptors
Immunogenicity	Some glycosylation motifs characteristic of plant-derived glycoproteins (often containing fucose and xylose residues) are highly immunogenics in mammals.

Therefore, it seems difficult to know which the proper glycosylation for therapeutic proteins are, since there are no rules for what is the appropriate addition of carbohydrates. Furthermore, the recombinant protein is usually administered via the blood-stream, which might not be where it is normally found in the body. Sometimes the ideal glycosylation for efficacy might not be ideal for a long half-life in the bloodstream. This is one of the main reasons that expensive mammalian cell culture techniques are still the mainstay for the production of glycoprotein therapeutics, although in these systems, homogeneity and level of syalylation are often sub-optimal[55].

Alternatives are being sought in insect cells, where glycan engineering efforts have result on strains able to generate sialylated glycans[58]. However, insect cells carry a pack of common problems associated to mammalian cells cultures[59].

Transgenic plants and animals are also areas of intensive search, but has been widely reported that plants usually add substituents that are not present in humans being potentially immunogenic/allergenic. Similarly, glycoproteins produced in the milk of transgenic animals contain substantially more high-mannose glycan structures that their natural counterpart[60].

For this reason re-engineering yeast N-glycosylation pathway seems a promising alternative[61,62]. Common yeast used in recombinant expression share with vertebrates the basic biosynthetic machinery to construct N-glycans efficiently and transfer to the nascent protein. The main problem of yeast rely on their lack to construct fully modified complex-type N-glycans of the human type, instead create a large and heavy chain of mannose. This hypermannosylated glycans are recognized by mannose receptors present in the liver and reticulo-endhotelial system.

1. Biosynthesis of Oligosaccharides

To synthesize a specific oligosaccharide structure for a growing polypeptide, the monosaccharides must be added properly, following an order which will require specific and complex machinery composed for glycosyltransferases and sugar donors as well as signals which localize this machinery in the correct place throughout the ER and Golgi.

Yeast and mammals share the first part of N-glycosylation. This co- or posttranslational modification takes place in the endoplasmic reticulum (ER) and/or Golgi apparatus. N-glycans are attached to the amide nitrogen of an asparagine (Asn) residue through an N-glycosyl linkage within the consensus sequence Asn-X-Ser/Thr

where the X is any aminoacid except proline. Gemmill and Trimble summarize these processes in an excellent review[63].

The initial steps of N-glycosylation take place at the cytoplasmatic side of the endoplasmic reticulum where a dolichol-linked glycan precursor is synthesized with the addition of N-acetylglucosamine (GlcNAc) and mannose (Man). A $Man_5GlcNAc_2$-PP-Dol precursor is then internalized into the lumen of ER where it is further elongated by mannosyltransferases until $Man_9GlcNAc_2$. After addition of 2 more glucose (Glc) by glucosyltransferases, the resulting $Glc_3Man_9GlcNAc_2$ oligosaccharide is transferred to asparagine residues of the nascent polypeptide chain and trimmed to $Man_8GlcNAc_2$ by certain glucosidases and mannosidases. This new glycoconjugate is released to the Golgi apparatus for further processing where the pathways of higher eukaryotes and yeasts split up. Mammalian cells generally continue the trimming process in the Golgi giving $Man_5GlcNAc_2$-Asn or $Man_3GlcNAc_2$-Asn structures which are precursors for the generation of hybrid and complex types of N-glycans (Figure 7).

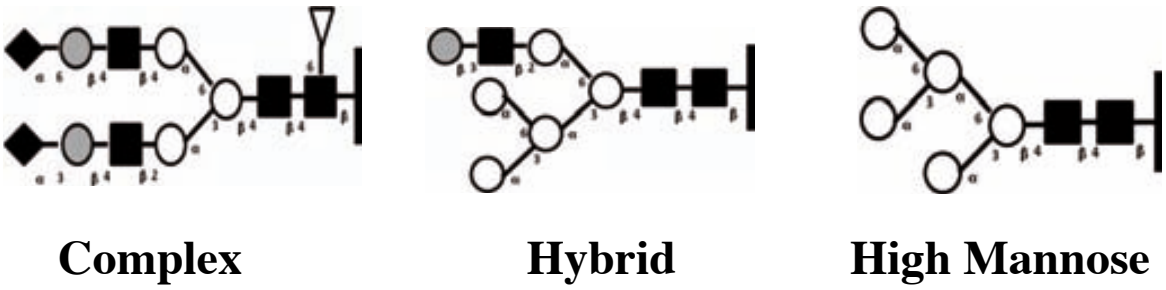

FIGURE 7. *N-glycan subtypes.* In complex and hybrid subtypes the end of structures change among Sia, Gal or GlcNAc outside the core $Man_3GlcNAc_2$

In yeast, the Golgi apparatus can be divided into at least three biochemically different subcompartments called 'cis/medial/trans' with their own glycosyltransferases[64]. When the N-glycan arrives to the cis-Golgi is modified by the Och1p α-1,6-mannosyltransferase which transfer α-1,6-linked mannose residue. This residue is the first piece to create a polymer of oligomannose by the action of polymerizing complexes. These N-glycans are said to be 'hyperglycosylated'. The α-1,2-mannosyltransferase Mnn2p add side branches on the α-1,6-linked polymer, and extended with two or three α-1,2-mannose residues by Mnn5p[65,66]. Usually these side branches are phosphorilated by the action of Mnn6p helped by Mnn4p. This phosphorylation is the responsible of the anionic charge of the yeast cell wall. All this process can be followed in Figure 8.

In *Saccharomyces cerevisiae* an α-1,3-linked mannose residue are added on some α-1,2-mannose by Mnn1p. These residues are immunogenic and frustrate the use of therapeuthic glycoproteins produced in wild type *S. cerevisiae*. In consequence, the addition of all these residues leads to N-glycans larger than 150-200 residues which mean an extensive heterogeneity. On the other hand, *Pichia pastoris* do not add highly immunogenic α-1,3-linked terminal mannose[67,68], and hyperglycosylation does not occur as often as in baker's yeast. One can expect N-glycans in the size range from $Man_8GlcNAc_2$ to $Man_{14}GlcNAc_2$ (Figure 8).

By contrast, O-glycan biosynthesis is completely different between lower eukaryotes and vertebrates from the initial step. O-glycan is attached to the –OH of a serine (Ser) or threonine (Thr) with its anomeric carbon through a glycosidic linkage and there are not known consensus sequences for this glycans. In yeast, O-glycosylation begins in the ER by the addition of a single mannose from a dolichol linked mannose donor after which the protein is transferred to the Golgi for further processing. In

mammalian cells, biosynthesis begins in the Golgi by the addition of GalNAc from a nucleotide sugar donor.

However, O-glycosylation still remains quite unclear in yeast, specifically in *P. pastoris*. Willer and co-workers reported in that protein O-mannosylation is essential for cell wall integrity, septation and viability of fission yeast[69]. Logically, O-glycosylated modifications had not been attempted in yeast because of the lethality of the deletion of O-mannosylation, but some groups are working on this field and first publications have began to appear[70,71].

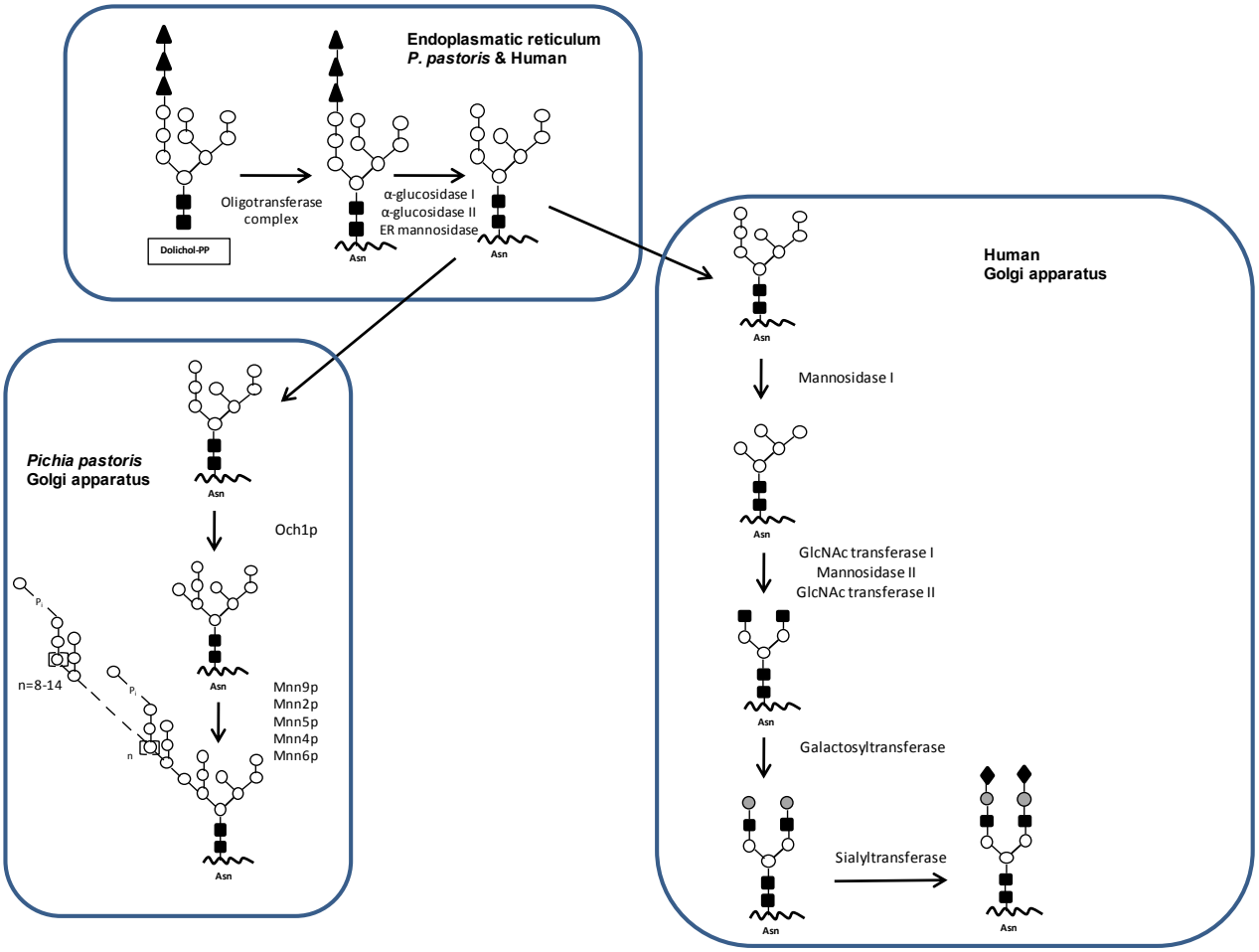

FIGURE 8. N-linked glycosylation pathways in humans and yeast.

1.16 PREVIOUS WORKS IN RECOMBINANT GONADOTROPINS

Up to date, recombinant gonadotropins have been produced in mammalian cell lines due to the complexity of their carbohydrates chains; also they are used only for treatment of infertility in humans.

Several attempts of producing gonadotropins in other expression systems different of mammalian cells have been unsuccessful.

Pichia pastoris have been one the most used expression system for the expression of human CG with the intent of sorting out the high costs associated with mammalian cells productions.

In 1999, C. Sen Gupta and R.R Dighe reported the expression of recombinant hCG expressed in *P. pastoris* achieving 12-16mg/L of hormone capable of binding to the LH receptors and stimulating response *in vitro*[72]. Later, the same group described the hyperexpression and purification of hLH and hCG in the same yeast, once again these hormones were active *in vitro*, but not information about their activity *in vivo* was described[73].

Véronique Blanchard and co-workers published in 2006 the composition of N-linked oligosaccharides from hCG expressed in *P. pastoris*[74]. Neutral oligosaccharide structures in the range of $Man_8GlcNAc_2$ to $Man_{11}GlcNAc_2$ were established, and the phosphate containing oligosaccharides ranged from $Man_9PGlcNAc_2$ to $Man_{13}PGlcNAc_2$. Surprisingly, they did not find Mannosyl O-glycans, and the amounts of carbohydrates varied with the culturing media. Afterward, they elucidated the preference of GS115 strain over X-33 for the study of ^{15}N-labeled hCG because of its lower content of mannoses into the carbohydrate chains[75].

Currently, there is not any non-vaccine product for animal health expressed recombinantly. The narrow profit margins of veterinary products disqualify mammalian

cells as expression system because of their expensive productions. Therefore, setting up a feasible recombinant system would be a good point, and glycoprotein hormones represent a good opportunity because of their large market, not only in veterinary, but in human therapeutics.

Pichia pastoris satisfy most of the necessities described above; consequently several groups have been trying to express some members of this family.

In this way, Fidler AE[76,77], expressed a single-chain polypeptide ovine FSH (both subunits fused genetically) active *in vitro*. Samaddar M *et al*., 1997 expressed bovine FSH, and Boze H. achieved up to 187 $mg \cdot l^{-1}$ of porcine FSH optimizing the composition of culture medium using a Mut^s in 2001[78].

Recently, it should be noticed an article published in 2009, where Qian and co-workers expressed a recombinant bovine FSH with *in vivo* activity. In this case, they use *H. polymorpha* as expression system instead of *P. pastoris*. In this article, the co-expression with a calnexin from *S. cerevisiae* and lower degrees of glycosylation in *Hansenula* are the major causes of this landmark[79].

Relative to equine chorionic gonadotropin, members of *Unité de Physiologie de la reproduction et des Comportaments* (Université François Rabelais de Tours, Nouzilly, France) have published during the last ten years several articles describing the expression of PMSG in insect cells Sf9 and Mimic™ insect cell line. These cells are stably-transformed Sf9 cells expressing five mammalian glycosyltransferases in order to express glycoproteins using the mammalian pattern.

The hormone expressed using this system was unable to give activity using *in vivo* models. They explained that probably this lack of activity is because of its short half-life. The rapid clearance of protein from bloodstream seems to be caused for the absence of sialic acid at terminal position of complex oligosaccharide chains[58,80,81].

In 2009, Anna Ubach described in her PhD Thesis the synthesis by PCR of α and β subunits of PMSG and its expression in *P. pastoris* under control of AOX1 promoter, achieving production levels of 100 $mg \cdot l^{-1}$ in high-cell densities cultures. More, a purification process was implemented and basic characterizations of recombinant hormone were carried out. Unsuccessfully, *in vivo* assays in female rats reported negative results and pointed out a potential short half-life of the recombinant hormone expressed in the methylotrophic yeast[2].

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CHAPTER 2

General Material and Methods

The most common material and methods used throughout this work are detailed in this chapter. Specific material and methods are described in detail at the correspondent chapters.

2.1 STRAINS

The wild type *P. pastoris* X-33 strain was used throughout this work. For subcloning tasks *Escherichia coli* DH5α (Invitrogen), genotype F- ϕ80dlacZΔM15Δ(lacZYA-argF)U169 recA1 endA1 tonA hsdR17(rK-, mK+) phoA supE44 λ -thi-1 gyrA96 relA1 was employed.

2.2 STRAIN MAINTENANCE

Long term stocks were prepared as recommended by Invitrogen and stored frozen at -80ºC. *P. pastoris* strains were grown at 30ºC on YPD plates containing 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) agar and stored at 4ºC.

2.3 MOLECULAR BIOLOGY METHODS

2.3.1 Reagents

DNA modifying enzymes utilized in molecular biology were purchased at Fermentas.

2.3.2 DNA extractions

Plasmidic DNA extractions were performed using the Illustra PlasmidPrep Mini Kit (GE Healthcare, 28-9042-69) according to the manufacturer instructions. DNA fragments were recovered from agarose gels applying the Illustra GFX PCR DNA and

gel band purification kit (GE Healthcare, 28-9034-71). Genomic DNA from *P. pastoris* was extracted using the DNeasy Tissue Kit (Qiagen) following the manufacturer's instructions.

2.3.3 Agarose gel electrophoresis

Agarose gel electrophoresis were carried out according to the protocol of Sambrook *et al*., 1989. Photographs of the gels were obtained by the Bioimaging system GeneGenius (Syngenne). To determine the DNA fragments size the GeneRuler DNA Ladder Mix, ready-to-use (#R0491) was used. Agarose was purchased from Sigma.

2.4 SDS-PAGE AND WESTERN BLOTTING ANALYSES

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) 12% was carried out in a Mini-PROTEAN II (BioRad) apparatus following the standard procedures recommended by the manufacturer and according to the protocol of Laemmli *et al,*. 1970. Prestained protein molecular weight marker (Fermentas, SM0441) or Spectra Multicolor Broad Range protein ladder (Fermentas, SM1841) were used to molecular weight determination. Gels were stained using silver stain procedure (Fermentas, PAGESILVERTM Silver Staining Kit, K0681) or Coomasie Blue (Sigma, Brilliant Blue R Concentrate, B8647).

Western blot were performed as follows: after running SDS-PAGE gels and cellulose Blotting paper (Sigma, P7796) were incubated for 30 minutes in Towbin buffer (0.025 M Tris (hydroxymethyl) aminomethane, 0.192 M Glycine and 20% Methanol). Immobilon-P PVDF membranes were incubated 30 minutes in pure methanol. After incubation , transference was carried out using a semi-dry transfer cell system (Trans-Blot SD, Biorad). Once transference was completed, membranes were incubated in

blocking buffer (Na2HPO4 1.42 g·L-1, KCl 0.20 g·L-1, KH2PO4 0.20 g·L-1, NaCl 8.00 g·L-1, Tween 20 1 mL·L-1, plus 5% skimmed milk) overnight at room temperature and gentle agitation at 4ºC. After blocking, the membranes were incubated for two hours at room temperature in blocking buffer plus primary antibody Anti-6xHis diluted 1:1000 (Roche, 04905318001). Once incubated with primary antibody, the membranes were washed with wash buffer (Na2HPO4 1.42 g·L-1, KCl 0.20 g·L-1, KH2PO4 0.20 g·L-1, NaCl 8.00 g·L-1, Tween 20 1 mL·L-1) three times during 10 minutes. Incubation with secondary antibody was performed under the same conditions mentioned above with a dilution (1:20000). Finally, the membrane was washed three times for 10 minutes using wash buffer and incubated for five minutes with the chemiluminescent substrate Supersignal West Pico (Pierce) and the signal was collected by a photographic film.

2.5 PROTEASE ACTIVITY DETERMINATION

Protease activity in the culture supernatant was determined using the Quanticleave Protease Assay Kit (Pierce) according to the manufacturer's instructions.

2.6 TOTAL PROTEIN DETERMINATION

Bradford assay (Pierce) was used for total protein quantification. Absorbance was measured in 96-wells plates using the spectrophotometer Powerwave XS (Biotek).

2.7 BIOMASS DETERMINATION

Biomass was expressed in optical density units (OD) measured at a wavelength of 600 nm. The spectrophotometer used was WPA colourwave (CO7500).

2.8 TRANSFORMATION

2.8.1 E. coli transformation

Competent cells were prepared according to Sambrook *et al*., 1989 as follows: A 10 mL LB (Luria-Bertani medium containing 10% yeast extract, 10% peptone and 5% NaCl) culture was inoculated from a cryostock and incubated overnight at 37ºC. 1-5 mL of the overnight culture were used to inoculate a 1000 mL LB culture. This culture was incubated at 37ºC until reachan optical density of 0.5-1.0. Once achieving the desired optical density, cells were chilled in ice for 20 minutes and subsequently centrifuged at 4000 rpm and 4ºC for 20 minutes. Supernatant was discarded and the cells resuspended in 500 mL of cold 10% glycerol solution. This washing step was then repeated resuspending in 250, 20 and finally 2 mL 10% glycerol. Cells were frozen at -80ºC in 40 μL aliquots or used for electroporation.

Electrocompetent *E. coli* DH5α cells were transformed with a Gene Pulser (BioRad) AT 2000 V, 25 μF and 200 Ω. Cells were thawed in ice and 40 μL were mix 1 to 5 μL of DNA. The mixed solution was transferred to a 0.2 cm gap electroporation cuvette (Sigma, Z706086), previously chilled on ice. Then, cells were pulsed and 500 μL of LB was added immediately. The solution was transferred to a sterile tube and incubated for one hour at 37ºC. After time was elapsed, aliquots were spread on corresponding agar plates.

2.8.2 P. pastoris transformation

Competent *P. pastoris* were prepared following the protocol by Higgins *et al*., (1998) as follows: A 10 mL YPD (20% glucose, 20% peptone, 10% yeast extract) were inoculated from a cryostock and grown overnight at 30ºC. The overnight culture was transferred to a new 500 mL YPD culture and incubated at 30ºC until an optical density

of about 1,5. Then, culture was chilled in ice for 15-20 minutes and centrifuged at 4000 rpm and 4ºC for 20 minutes. Supernatant was discarded and cells were resuspended in 100 mL of a new medium containing 100 mL YPD plus 20 mL of 1 M HEPES pH 8.0, and 2,5 mL DTT 1M. Cells were incubated for 30 minutes at 30ºC and subsequently centrifuged at 4000 rpm 15 minutes. The cell pellet was resuspended with 250 mL of cold water and centrifugued again under the same conditions. Supernatant was discarded and the pellet resuspended with 20 mL of 1 M cold sorbitol, the centrifugation step was repeated once again and the pellet resuspended in an final approximately volume of 1,5 mL of 1M cold sorbitol. Aliquots of 80 μL were performed for subsequent electroporation or for storage at -80ºC.

Electrocompetent *P. pastoris* cells were transformed using a Gene Pulser (BioRad) at 1500 V, 25μF and 400 Ω. A volume of 80 μL of fresh cells and about 1 μg of DNA in no more than 10 μL total volume were transferred into a 0,2 cm gap electroporation cuvette (Sigma, Z706086) previously chilled on ice. Cells were pulsed and immediately 1 mL of cold sorbitol 1M was added. The cuvette content was transferred to a sterile tube and incubated for 1 hour at 30ºC without agitation. Selected aliquots were spread onto agar plates containing an appropriate selective medium. Plates were incubated at 30ºC for 2-4 days.

CHAPTER 3

Development of a cell-based assay for determination of rPMSG activity

3.1 INTRODUCTION

Being able to identify, quantify or validate either the conformation or activity of any biopharmaceutical product is a critical aspect along the development phase.

The development of cell-based assays has increased with the biotechnology expansion. The necessity for testing the product in a controlled environment, easily intelligible mechanism of action, cheap fungible, reproducible experiments and fast evaluations has lead to a vast development of cell culture procedures for this purpose. Moreover, for cell-based analyses are not required any animal manipulation course neither specific installations nor bioethical permissions.

However, the controlled conditions present in the *in vitro* system differ significantly from those *in vivo*, and may give misleading results. For this reason, *in vitro* studies come often with *in vivo* assays.

In vitro determination of bioactivity in hormones requires a measurable cell response such as the production of some metabolites susceptible to be analyzed. This response is caused after hormone and membrane-cell receptor bound and signal transduction into the cell.

Because of duality in PMSG activity, different in vitro assays have been largely used to prove the proper folding and its correct receptor bound. For FSH bioactivity measurement of estradiol or cAMP production in rat Sertoli cells seminiferous tubule homogenates[1] or estrogen production in cultures of granulose cells from inmature rat ovaries have been accepted, while for LH, testosterone/progesterone production by murine Leydig cells has been the assay most frequently used[2,3]. The FSH-dependent increase of plasminogen activator secretion by cultured Sertoli cells or granulose cells are used as well.

The main problem of these cells underlie on a specific receptor loss after several generations or subcultures, being interesting to work with transformed cells to avoid the use of primary cell cultures. Transformed or tumoral cells have the ability to be immortal and preserve the expression of the required receptors in the membrane.

In mammalians there are two different types of cells capable to express FSH receptors, and obviously qualified to be used for FSH bioactivity detection; in males Sertoli cells are found into seminiferous tubules and are involved in spermatozoids maturation. One of metabolites secreted during the activation by the FSH is the estradiol which is produced by aromatase activity (being citocrom P450 aromatase enzyme the main responsible). On the other hand, granulose cells produce estradiol in females as well, but in this case are part of ovarian follicles and lead the oocyte growth.

Figure 1 shows how the bound between ligand and receptor activates intracellular adenilate cyclase enzyme, generating high amounts of cyclic adenosine monophosphate (cAMP) as a second messenger. cAMP indirectly triggers several transcription factors, ending in an overexpression of the enzyme P450 aromatase, which interferes in different steroidogenic processes from androgens[4].

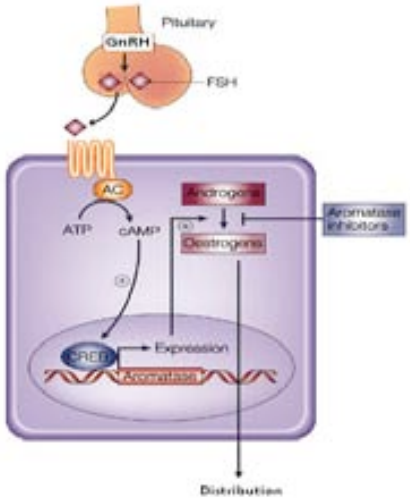

FIGURE 1. Activation of aromatase activity by FSH[4]

In another way, Leydig cells express specific LH receptors widely described as optimal to bind the chorionic gonadotropin. These cells produce either progesterone or testosterone, and both can be detected extracellularly.

When LH or CG binds to the receptor, the pathway AC/cAMP/PKA/CREB (Adenilate cyclase/cyclic AMP /protein kinase/cAMP response element binding) and different events take place (figure 2).

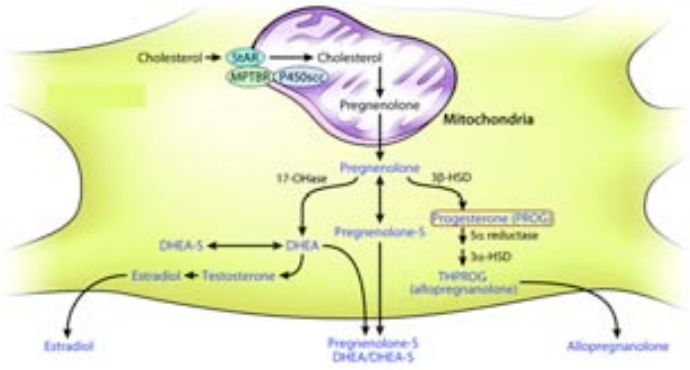

FIGURE 2. Conversion of cholesterol to progesterone after activation by LH bound.

First, the expression of the protein StAR (steroidogenic acute regulatory protein) is stimulated, internalizing the cholesterol from the cellular membrane to the internal mitochondrial membrane. Later, the P450scc (P450 cholesterol side chain cleavage enzyme) synthesizes pregnolone from the internalized cholesterol, and finally pregnolone exits from mitochondria and is converted to progesterone by 3β-HSD (3β-hydroxyesteroid dehydrogenase).

In the present study our aim was to demonstrate the bioactivity of the recombinant PMSG expressed in *P. pastoris*. First, Sertoli cells (TM4) were used to detect the FSH activity produced for the rPMSG, but a probable loss of FSH receptor expression on their cell membrane invalidated this cell line. Similarly, we investigated granulose cells with the same purpose, but although levels of estradiol increased significantly, the vast variability among assays provoked that these cells were rejected too.

Finally, Leydig cells (MLTC-1) has been tested. Although Leydig cells does not express FSH receptors, they present LH receptors and progesterone production can be stimulated by PMSG LH-activity.. With MLTC-1 cells, significant amounts of secreted progesterone induced by the action of rPMSG were successfully detected.

3.2 OBJECTIVES

The objective of this chapter was to develop an in vitro cell-based assay which demonstrates the bioactivity of rPMSG accurately. This assay had to be economically feasible, robust, reliable, repetitive and as accurate as possible.

This assay not only had to show us the presence of an active form, but should give us an accurate quantification of the activity and warrant a good comparison with the no-recombinant form.

3.3 MATERIALS AND METHODS

3.3.1 Cell lines

Three different mammalian cell lines were used during this work. Sertoli cells are called TM4 and were purchased from ECACC (*European Collection of Cell Cultures*). This cell line was established in 1980 by Mather from *Mus musculus* testicles[5]. Granulose cells were kindly given by T. Mogas (Faculty of veterinary, Universitat Autònoma de Barcelona), granulose cells were extracted by suction from calf ovary.

Leydig cells were a transformed cell line from testicles of *Mus musculus* called MLTC-1 and were also kindly given by Dr. Guinovart; originally purchased from ATCC (American Type Culture Collection).

3.3.2 Media composition for cell cultures

All reagents were purchased to Sigma-Aldrich®, unless other brand is specified.

Growth media (non-defined) for Sertoli (TM4) and granulose cells: Gentamicin 20 $\mu g \cdot mL^{-1}$ (G1272), Amfotericin 1 $\mu g \cdot mL^{-1}$ (A2942), Horse serum 5% (H1138), Fetal Bovine Serum 2.5% (F7524) and DMEM-F12 (D8437).

Growth medium (non-defined) for Leydig cells (MLTC-1): Gentamicin 50 $\mu g \cdot mL^{-1}$ (G1272), Horse serum 9% (H1138), Fetal Bovine Serum 4.5% (F7524) and RPMI-1640 (R7388).

Assay medium for Leydig cells (MLTC-1): Horse serum 9% (H1138), Fetal Bovine Serum 4.5% (F7524), Bovine serum albumin (BSA) 0.1% (B4287), Metil-isobutil-xantina (MIX) 0.2 mM (I7018), hCG (CG5) or Forskolin (F3917) variable concentration (see results) and RPMI-1640 (R7388).

Defined medium for sertoli cells (TM4): Gentamicin 20 $\mu g \cdot mL^{-1}$ (G1272), Amfotericin 1 $\mu g \cdot mL^{-1}$ (A2942), Insulin 1 $\mu g \cdot mL^{-1}$ (A2942), Transferrin 5 $\mu g \cdot mL^{-1}$

(T8158), EGF 10 ng·mL^{-1} (E1257), Tiroxin 20 pg·mL^{-1} (T1775), Hydrocortisone 3.62 ng·mL^{-1} (H0888), Retinoic acid 0.3 μg·mL^{-1} (R2625) and DMEM-F12 (D8437). Some *optimized assays* were performed without retinoic acid or EGF (further information is explained in detail in 2.3.4)

Assay medium for sertoli cells (TM4): Was exactly the same that defined media but supplemented with Metil-isobutil-xantina (MIX) 22.2 μg·mL^{-1} (I7018) and 19-hidroxi-androstenedione (H0379) or androstenedione (46033) 0.756 μg/mL and DMEM-F12 (D8437).

Defined medium for granulose cells: Gentamicin 20 μg·mL^{-1} (G1272), Amfotericin 1 μg·mL^{-1} (A2942), Insulin 1 μg·mL^{-1} (A2942), Transferrin 5 μg·mL^{-1} (T8158), EGF 10 ng·mL^{-1} (E1257), Tiroxin 20 pg·mL^{-1} (T1775), Hydrocortisone 3.62 ng·mL^{-1} (H0888) and DMEM-F12 (D8437).

Assay medium for granulose cells: Was exactly the same that defined media but supplemented with Metil-isobutil-xantina (MIX) 66.6 μg·mL^{-1} (I7018), Dietilestilbesterol (DES) 0.125 μM (46207), 19-hidroxi-androstenedione 0.756 μg·mL^{-1} (H0379) or androstenedione and DMEM-F12 (D8437).

3.3.3 Cell culture techniques

3.3.3.1 Estimation of cellular viability and cell count

A first estimation of cellular viability or cell count was carried out with a hemocytometer, specifically Neubauer hemocytometer (Z359629, Sigma). Adherent cells must be dissagregated first as is explained in section 2.3.3.2.

For cellular staining, 50 μL of cells were transferred into an eppendorf tube together with 50 μL of Trypan blue solution (93590, Fluka) (dilution 1:1). The solution was mixed by pipeting and 20 μL transferred to each hemocytometer grid. Looking at the

chamber through a microcospe (Eclipse TE200, Nikon), the number of cells in the chamber was determined by counting, and cell concentration was determined using following formula (eq. 1)

$$Cell\ concentration = \frac{Number\ of\ cells\ counted \times volume\ sample\ dilution}{Volume\ of\ chamber} \quad (1)$$

In a common hemocytometer or Neubauer chamber the volume between the surface area (1 mm^2) and the cover slip is 0.1 mm^3 (10^{-4} mL). The cells in four large squares are counted and cells over or touching the lines on top and on the left are counted, but cells over or touching the right or bottom lines are ignored.

Non viable cells are stained by the action of Trypan blue since this stain can go through the damaged membrane. Hence, dividing the non-stained cells by the total number of cells, the percentage of viability was elucidated.

3.3.3.2 Subculture of adherent cells

The cell lines used during this work were adherent cells. This characteristic required the use of trypsin (T4549, Sigma) in combination with EDTA (Etilenediaminotetraacetic acid) (131026, Panreac) for further manipulations in several processes. EDTA is a chelating agent that avoids the inhibitory effect on trypsin of certain elements presents in serum.

First of all, old medium was removed and 5 mL of EDTA 1mM diluted in PBS were added on the opposed side of the cells, then the cells were washed with. Subsequently, the necessary amount of trypsin at 0.1% were added on cells in order to cover completely the monolayer and incubate the flask at 37ºC for 3-10 minutes until monolayer began to unhitch (time of incubation varies in function of the cell line).

Later, 2,5 mL of growth medium previously preheated at 37ºC were added and pipeted up-and-down gently in order to disaggregate cells cumulus. Once the cells were completely disaggregated, a cellular count was carried out and the desired final cellular concentration was adjusted with growth medium. Then new cell cultures were incubated at 37ºC and 5% CO_2[6].

3.3.3.3 Cell freezing for stocks preparation

There are two main aspects to consider before proceeding with a cell cryopreservation. First, cells must be validated visually; they must be free of bacterial or fungal contamination as well as present a typical morphology of that cell line. Second, to choose the correct moment to begin with the freezing protocol. Usually, this moment is just before the plateau phase, at the end of log phase.

Based on the cell line, different protocols were applied in order to find the best one;

Sertoli cells (TM4)

The protocol used for Sertoli cells was described by Freshney *et al.,* 2005 and consists of in the following standard procedure[6].

Cultures were grown until the end of exponential phase. After a visual inspection of culture appearance by means of phase contrast microscope, cells were trypsinized and resuspended in 2 mL of growth medium. When the cellular suspension was homogenous, the number of cells was determined and cell concentration was adjusted to $1\text{-}2\cdot10^6$ cells·mL^{-1}. In parallel a solution 20% of DMSO (Dimethyl Sulfoxide) (D2650, Sigma) in growth medium was prepared. Cell suspension was diluted with the 20% DMSO solution in a proportion 1:1, mixing them gently, resulting in a final cell suspension of 10% DMSO and $5\cdot10^5\text{-}10^6$ cells·mL^{-1}. Subsequently cells were aliquoted in 1 mL tube and kept

into a Styrofoam box at -80ºC for 16 hours in order cool the cells slowly and avoid intracellular crystals formation. Finally cells were transferred into a liquid nitrogen tank (Forma Scientific, Cryoplus 2).

Granulose Cells

Is important to underlie that cell concentration reached in granulose cells cultures is around 10 times lower because its size (granulose cells are considerably bigger than Sertoli cells) and because of its capacity to pile up.

Since granulose cells came from a primary culture, three different protocols were assayed in order to improve the cell viability after thawing cells.

The first protocol was the same procedure used for Sertoli cells (standard protocol), but in this case the cell concentration after trypsinization was slightly lower, around 10^5 - $2 \cdot 10^5$ cells·mL^{-1}.

Second and third alternatives were very similar and only differed on medium composition used for cell resuspension. The detailed protocol is described below.

After a visual inspection of culture appearance by phase contrast microscope, cells were trypsinized and resuspended in 2-3 mL of growth medium (for small flask surface, e.g. 25 cm^2). The suspension was centrifugued for 10 minutes at 100 *g* and the resulting supernatant was dicarded. At this point the protocols differed. First alternative consisted in pellet resuspension in 7,5 mL of serum mix (65% Horse Serum and 35% Fetal Bovine Serum), by contrast, in the second alternative the pellet was resuspended in 7,5 mL of growth medium. In parallel a solution of 20% of DMSO (Dimethyl Sulfoxide) in growth medium was prepared and the cell suspension diluted with the 20% DMSO solution in a proportion 1:1, mixing them gently, resulting in a final cell suspension of 10% DMSO and $5 \cdot 10^5$-10^6 cells·mL^{-1}. Subsequently cells were aliquoted in 1 mL tube

and kept into a Styrofoam box at -80ºC for 16 hours in order cool the cells slowly and avoid intracellular crystals formation. Finally were transferred into a liquid nitrogen tank

Leydig Cells (MLTC-1)

The protocol for Leydig cells was exactly the same used for granulose cells, but in this case only one alternative was tested. The resuspension of pellet was carried on with growth medium instead of serum. Recommended cell concentration into the flask was $2.25 \cdot 10^6$ cells·mL^{-1}. The rest of steps were basically the same.

3.3.3.4 Cell thawing

The desired tube was moved to the laminar flow cabinet and it was slightly unscrewed in order to let the remaining nitrogen get away. Later, this tube was thawed in a bath at 37ºC and transferred into a new sterile tube. Subsequently, cells were diluted adding gently and very slowly 7 mL of pre-warmed growth medium in order to reduce the cryoprotectant concentration gradually. Take an aliquot and measure cell viability as well as a cell count. Based on the results, adjust the volume to reach the desired concentration, distribute the cellular suspension to flask and incubate into the incubator (Heraus, HERAcell) at 37ºC, 5% CO_2. After 24 hours, check the proper cell adhesion on the flask.

3.3.4 In vitro analysis of estradiol production by sertoli cells (TM4 cells) stimulated with native PMSG

Based on an initial protocol, different sequential experiments with some variables were assayed in order to improve the results. All the assays hereafter were based on a protocol described by Padmanabhan[7], but in this case because the difficulty to work with

a primary culture, all the experiments were performed with the established TM4 cell line[5].

3.3.4.1 Estradiol production by TM4 cells; Assay 1.

In this analysis both native and recombinant PMSG were assayed by triplicate at concentrations 50, 100, 200 and 400 $ng \cdot mL^{-1}$. Controls were also analysed by triplicate, controls were carried out with commercial PMSG hormone with and without substrate (19-hidroxi-androstenedione), and substrate without hormone.

Hormone concentrations were selected considering several bibliographic values. Combarnous *et al*., 1984 and Padmanabhan *et al*., 1987 reported that 100 $ng \cdot mL^{-1}$ of FSH gave the highest values of estradiol[8,9]. Additionally, Combarnous *et al*., 1984 informed that PMSG has approximately half of FSH activity that FSH for itself. The procedure is described hereafter.

Cryovials were thawed as was described in protocol 2.3.3.4 and a cellular suspension was carried out in growth medium with a concentration of $2.5 \cdot 10^4 - 5 \cdot 10^4$ $cells \cdot mL^{-1}$. Subsequently 1.5 mL of cell suspension was distributed into each well and cells grew at 37ºC and 5% CO_2 until 90% of confluency.

Once the cells reached this point, growth medium was discarded and the monolayer washed with sterile PBS with the aim to eliminate any serum trace still remaining in the well, since it may cause an undesirable inhibitory effect. Later the wells were filled with 1.5 mL of defined medium and the 12-wells plates incubated during 72 h at 37ºC and 5%CO_2 to allow the cells adapt to a minimal medium. After this time, medium was changed to the final assay medium and the induction lasted next 72 hours. Finally, the medium was extracted from wells and centrifugued at 100 *g* during 10 min to eliminate cellular debris which may interfere in following analysis. Samples may be

stored at -20ºC for two months. In this assay, samples were analysed by ELISA with the kit *Human Estradiol (E2)* (Biosource) or by RIA with the kit *Ultra-sensitive Estradiol RIA* (Diagnostic Systems Laboratories).

3.3.4.2 Estradiol production by TM4 cells; Assay 2.

This analysis was an adaptation of Assay 1. Variations are exposed next; the hormone FSH (100 $ng \cdot mL^{-1}$) was included as a positive control and the only negative control had the absence of hormone, but the presence of substrate. More, in order to assure a maximum stereidogenic response, the hormone concentration (both native and recombinant) were increased to 2.5 $\mu g \cdot mL^{-1}$. In this case, samples were collected at 0, 6, 24 and 48 hours and only were analysed in duplicate instead of triplicate. Finally, the chosen kit to analyze samples was *Estradiol EIA Kit* (Cayman). The procedure was exactly the same but the induction phase last 48 hours instead of 72 hours.

3.3.4.3 Estradiol production by TM4 cells; Assay 3.

In this assay, mediums were slightly changed, but the procedure was the same and samples were analysed with *Estradiol EIA Kit* (Cayman). Retinoic acid and EGF were removed, but the rest of components were the same. The final cell concentration for induction was hardly reduced to 80% of confluence, instead of 100%. Moreover, the adaptation time to minimal medium was reduced to 48 hours, and the only hormone assayed in this case was the native PMSG (2.5 $\mu g \cdot mL^{-1}$). Samples were collected at 24 h, 48 h, 72 h and 96 h in duplicate.

3.3.4.4 Estradiol production by TM4 cells; Assay 4.

The substrate 19-hidroxi-androstenedione was replaced by androstenedione and the hormone concentration was expressed now in 0.1, 1 and 10 $IU \cdot mL^{-1}$ (International Units). 10 $IU \cdot mL^{-1}$ corresponded to the maximum concentration used in the previous assay (2.5 $\mu g \cdot mL^{-1}$). The adaptation time remained at 48 hours and the induction time was changed to 72 hours again.

The negative controls consisted of absence of hormone with lack or presence of substrate. Additionally, another control without native PMSG but with the old substrate (19-hidroxi-androstenedione) was included.

3.3.5 In vitro analysis of estradiol production by granulose cells stimulated with native PMSG

Despite the majority of assays based on the estradiol production in granulose use cells from immature rats or mice (8-20 days of age) and these cells usually lose this property after few subcultures[10], in this analysis, cells were extracted by suction from ovary of slaughtered calf.

The procedures followed in assays below were based on *Dahl et al., 1989*[11].

3.3.5.1 Estradiol production by granulose cells; Assay 1.

The Cells were cultivated in 6-wells plates with growth medium until 80-90% of surface was covered. Then, the monolayer was washed twice with sterile PBS and cells were incubated in defined medium for 48 hours. After this time, cells were cultured during 72 hours with the specific medium assay and subsequently centrifugued at 100 *g*. In this first assay 19-OH androstenedione was used as substrate for estradiol production.

Increasing concentrations of native PMSG were assayed ($2 \cdot 10^{-3}$ IU·mL^{-1}, $2 \cdot 10^{-2}$ IU·mL^{-1}, 0.2 IU·mL-1, 2 IU·mL^{-1}, 10 IU·mL^{-1}) in duplicate as well as negative control which did not contain hormone.

3.3.5.2 Estradiol production by granulose cells; Assay 2.

The substrate used in assay 1 was replaced by androstenedione (similarly to assay 4 in Sertoli cells). Additionally, three negative controls without hormone were combined; with substrate (androstenedione or 19-OH-androstenedione) or without substrate.

Increasing native PMSG concentrations were assayed in duplicate (10^{-3}, 10^{-2}, 10^{-1} IU·mL^{-1}).

3.3.6 In vitro analysis of progesterone production by Leydig cells (MLTC-1).

The MLTC-1 cells were cultivated in a flask to 80-90% of confluence. In this point, the cells were trypsinized and subcultivated to a final concentration of $1.5\text{-}2 \cdot 10^{5}$ cells·well^{-1} in 12-wells plates. The cells were incubated during 24 hours at 37ºC and 5% CO_2 allowing them to adhere to the well surface. Subsequently, they were visually evaluated (expected morphology, distribution and proper adherence) and washed with sterile PBS. The cells were changed to assay medium and after 8 hours of induction the supernatant was collected and centrifugued at 100 *g* during 10 minuts. Then samples were ready to be analysed or conveniently stored at -20ºC.[12]

3.3.6.1 Progesterone production by MLTC-1 cells; Assay 1.

The main objective of assay 1 was to evaluate the progesterone production of MLTC-1 cells after induction of different concentrations of hCG (human Chorionic Gonadotropin). Each hormone concentration, negative and positive control were assayed

in duplicate. Increasing hCG concentrations were 0.01, 0.1, 1 and 10 $IU \cdot mL^{-1}$, while negative control consists of 0 $IU \cdot mL^{-1}$ of hormone. A solution of 10 μM forskolin was used as positive control.

3.3.6.2 Progesterone production by MLTC-1 cells; Assay 2.

Once MLTC-1 cells were demonstrated that response to hCG induction, then was checked with native PMSG. Hormone concentrations assayed were 0.01, 0.1 and 100 $IU \cdot mL^{-1}$. Similarly to assay 1, negative control did not contain hormone (0 $IU \cdot mL^{-1}$), while positive controls in this assay was 10 $IU \cdot mL^{-1}$ of hCG. All samples were assayed in duplicate.

3.3.7 Sample analysis

Since granulose cells and sertoli cells produce estradiol after activation, this is the metabolite to analyze. There are two different commercial kits based on immunoaffinity; RIA (radioimmunoassay) and ELISA (Enzyme-linked Immunosorbent Asay). For radioimmunoassay was used *Ultra-Sensitive RIA* (Diagnostic Systems Laboratories, Inc., ref. DSL-4800) and for ELISA two different kits were used; *Human Estradiol (E2) Immunoassay Kit* (BioSource International Inc., ref. KAQ0621) and *Estradiol EIA Kit* (Cayman Chemical Company, ref. 582251).

To determine the amount of progesterone produced by MLTC-1 Leydig cells after induction, a Progesterone ELISA kit (Neogen, 402310) was used.

3.3.8 Determination of PDT (Population Doubling Time) for TM4 cell line

With the goal to define the appropriate strategies for *in vitro* assays with Sertoli cells a growth curve was plotted with cells growing on non-defined growth medium. In order to draw the curve, every 24 hours a cell count was carried out. Since was not possible to trypsinize the same cells every day (cell viability is usually affected during this procedure and cells need a re-adhesion time to the plate), a small independent cultures in 12-wells plate with an initially known cell concentration was accomplished. Ideally, was expected a homogeneous growth rates between wells as the conditions were exactly the same to each.

Two different cell concentrations were cultured initially ($1 \cdot 10^4$ and $2 \cdot 10^4$ cells·mL^{-1}), and each concentration was analysed by triplicate every day (Figure 3).

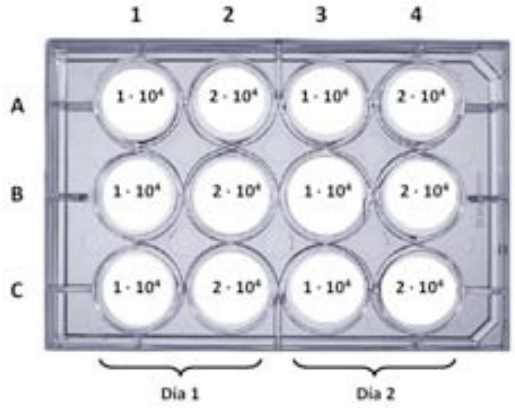

FIGURE 3. Example of well distribution for one plate. As the assay takes 8 days, four different plates are needed.

3.4 RESULTS AND DISCUSSION

3.4.1 PDT and other growth characteristics of TM4 cell line

To define de population doubling time, the sertoli cells were cultivated in non-defined growth media and every 24 hours three wells of each cellular concentration were count and represented in next graphic (figure 4).

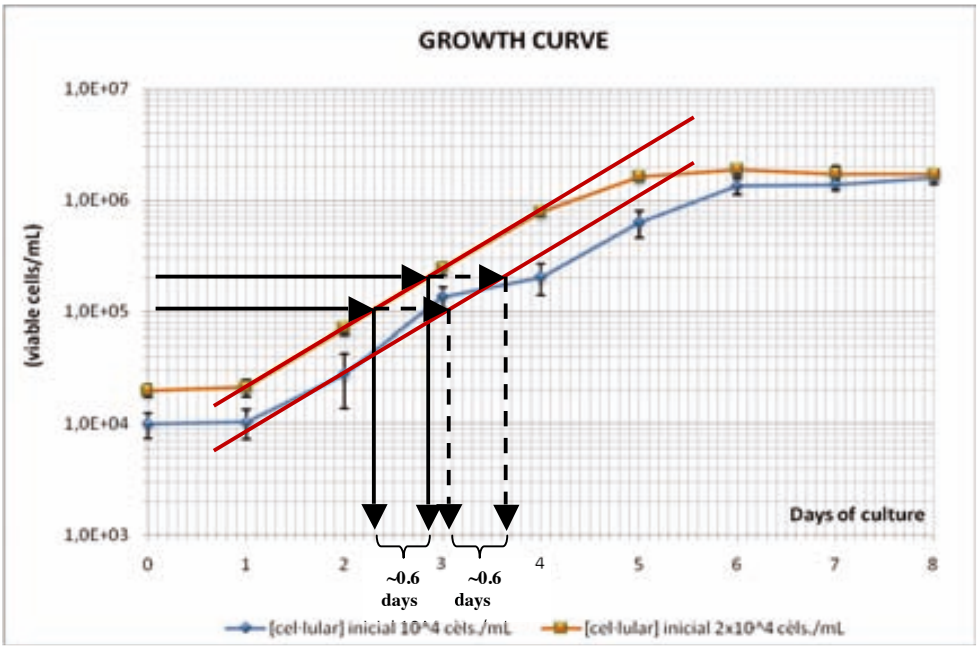

FIGURE 4. Cellular concentration (viable cells/mL) at different days of culture. Each growth curve have a regression line to get the PDT (Population doubling time).

To determine the PDT value, two fit curves (red lines) were characterized for each cell concentration. Subsequently, the determined concentration ($1 \cdot 10^5$ cells·mL^{-1}) and its double ($2 \cdot 10^5$ cells·mL^{-1}) were interpolated and the required time to reach the second value was called PDT. Logically, both times coincide for each cell concentration, and it

was estimated in 0.6 days (14.5 hours), which coincide with the data determined by Mather et al., 1980[5].

Additionally, with this experiment the maximum cell concentration was established to ~$2 \cdot 10^6$ cells·mL^{-1} and the lag phase for each culture coincide in 24 hours. These data provide valuable information for planning next cultivations.

3.4.2 *In vitro* analysis of estradiol production by sertoli cells (TM4) stimulated with native PMSG

Unfortunately, results obtained from samples of assay 1 (Materials and methods 2.3.4.1) were not significant and without concordance (data not shown), especially those results analyzed by RIA. Probably, kits used were not appropriated for this assay. Both kits *Ultra-sensitive Estradiol RIA* and *Human Estradiol (E2)*, based on RIA and ELISA respectively, were set up for small amounts of estradiol in serum, but not for estradiol in a culture medium. To demonstrate this point, a serial dilution of commercial estradiol (Calbiochem, 3301) diluted in H_2O miliQ was measured and likewise, the results were uneven.

In assay 2 (Materials and methods 2.3.4.2), the purpose was to demonstrate the steroidogenic activity of TM4 cells. Owing to this necessity, the ELISA kit was changed for another more appropriated (*Estradiol EIA Kit, Cayman)*. Moreover, other adjustments, such as the inclusion of FSH as positive control or the increase of hormone to assure the steroidogenic activity were assayed with satisfactory results. Figure 5 shows the apparent differences between cultures induced with commercial hormones, both native PMSG and the control FSH, and the recombinant PMSG, with those cultures without hormone used as a negative control (12 pg·mL^{-1}). The highest values were reached for those cultures stimulated with 100 ng·mL^{-1} of FSH after 48 hours, specifically

32 pg·mL^{-1}. Meanwhile native PMSG attained values slightly lower (25 pg·mL^{-1}), but higher than those reached for its recombinant homologue (18 pg·mL^{-1}).

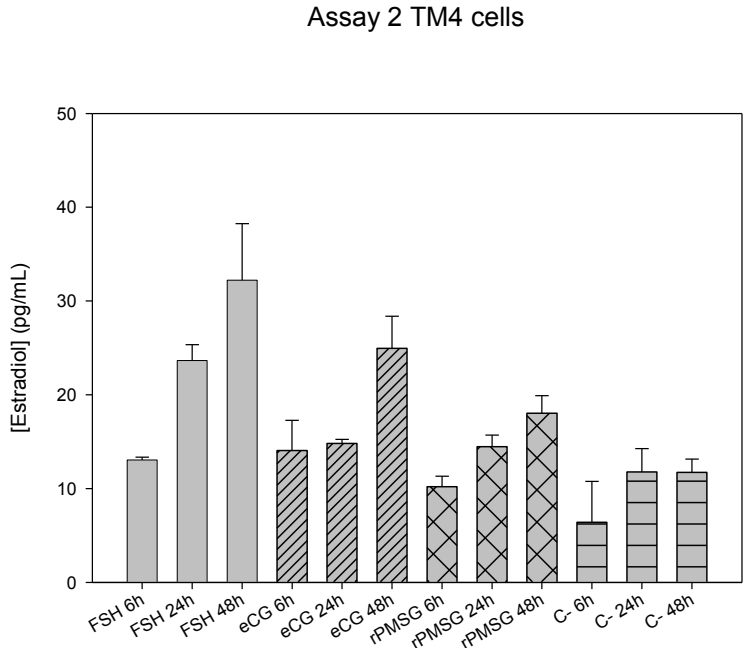

FIGURE 5. Graphical representation of estradiol production by sertoli cells (TM4) using new commercial ELISA provided by Cayman. Commercial eCG in the figure corresponded to the native PMSG mentioned in text.

In spite of the proved sterodoigenic activity, the levels of estradiol were lower than those reported in bibliography, where several reports published estradiol concentrations that oscillate between 1000 and 3000 pg·mL^{-1}.[7,13,14]

These values are almost 30-fold higher probably because of the cell line. All reports cited above use primary cultures of sertoli cells from immature mouse (between 8 and 22 days of life) for the assay. Primary cultures from these animals are not completely differentiated; allowing cells to attain the expression of big amounts of FSH receptors in the membrane (since they play a vital role during cell differentiation). As cells become

differentiated, they lose some characteristics, including an abrupt drop of FSH receptors in their membrane.

The cell line utilized for this assay, not only was an established cell line, but also its elevated number of passages pointed out that the number of FSH receptors present in their membranes may be quite lower. This point states a problem with variability associated not only with the estradiol production, but also with that variability associated with sample analysis by ELISA. Cellular concentration in each well, death cell rates or half-life of hormones can provoke variability attributable to cell production of estradiol, while presence of certain interfering substances or concentration of secondary antibody are associated to analysis. In fact, technical specifications of ELISA kit informed about the lack of reliability of those samples comprised between 7 $pg \cdot mL^{-1}$ and 25 $pg \cdot mL^{-1}$.

With the aim to overcome this problem, an improved medium of culture was proposed in assay 3 (optimized mediums in subsection 2.3.2). McDonald *et al.,* 2006 described a remarkable decrease of aromatase activity (estradiol production) in sertoli cells when were cultured with concentrations of 10 $ng \cdot mL^{-1}$ of EGF[15], precisely that was the concentration used in our assays. Consequently, EGF was eliminated from medium in assay 3. Similarly, retinoic acid was also excluded. Galdieri and Nisticò, 1994 reported a reduction of estradiol production that fluctuates between 40% and 60% culturing sertoli cells at 0,25 μM[16], previous assays were performed at 1 μM, four times higher.

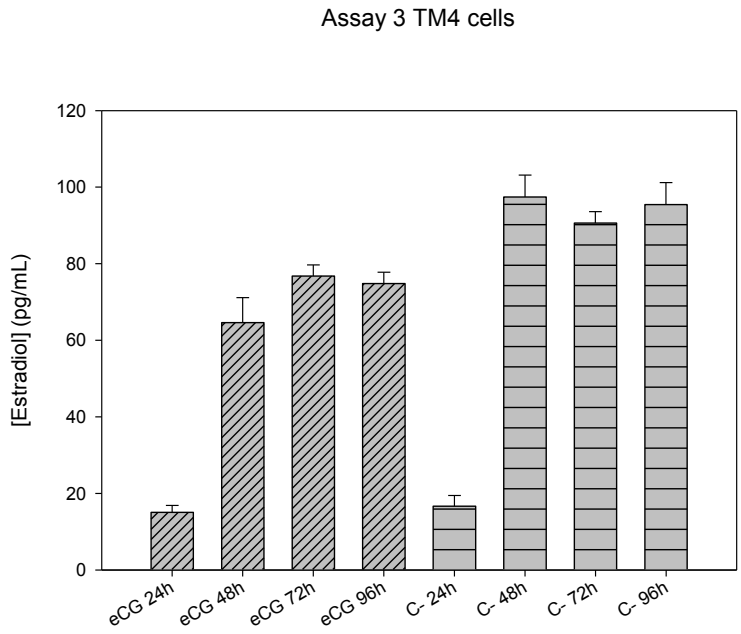

FIGURE 6. Graphical representation of estradiol production by sertoli cells (TM4) after elimination of EGF and retinoic acid from the culture medium. Commercial eCG in the figure corresponded to the native PMSG mentioned in text.

Despite of improvements applied in assay 3, the results obtained were incongruous (Figure 6). Remarkably, the global production of estradiol by native PMSG was increased a 60% when compared with assay 2. However, cultures without hormone (negative control) reached an unspecific production of estradiol eight times higher than those described in the previous assay.

Analyzing the biosynthesis of estradiol, it must be underlined that the only enzyme that acts on estradiol synthesis from 19-OH androstenedione is the estradiol-β-deshidrogenase (figure 7). Hence, any induction caused by the hormone must act in this point. By contrast, some articles use androstenedione, the precursor metabolite of 19-OH androstenedione[13]. Using androstenedione as substrate may expand the points where the

inductive hormone acts, mainly the enzyme P450 aromatase (NC-IUBMB: 1.14.14.1) which is the main enzyme involved in steroidogenesi (figure 7).

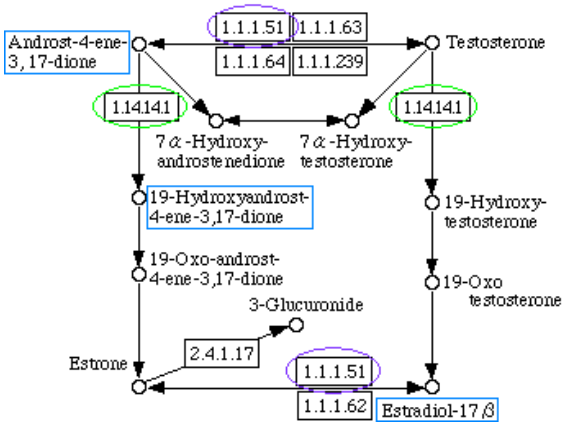

FIGURE 7. Biosynthetic pathway of estradiol. Green circles corresponds to the enzyme P450 aromatase, while purple circle corresponds to estradiol-β-deshidrogenase. Blue squares are the essential metabolites for estradiol production. Noticeably, estradiol can be produced through an alternative via where testosterone is the intermediate. More, the enzymes implicated in this alternative via are the same used for the estrone pathway.

Following this indication, substrate was changed to androstenedione in assay 4. Surprisingly, as hormone concentration was increased (expressed in International Units), the production of estradiol decreased gradually (Figure 8). This negative effect may be caused by a possible desensitization of FSH receptors in high hormone amounts present in the assay.

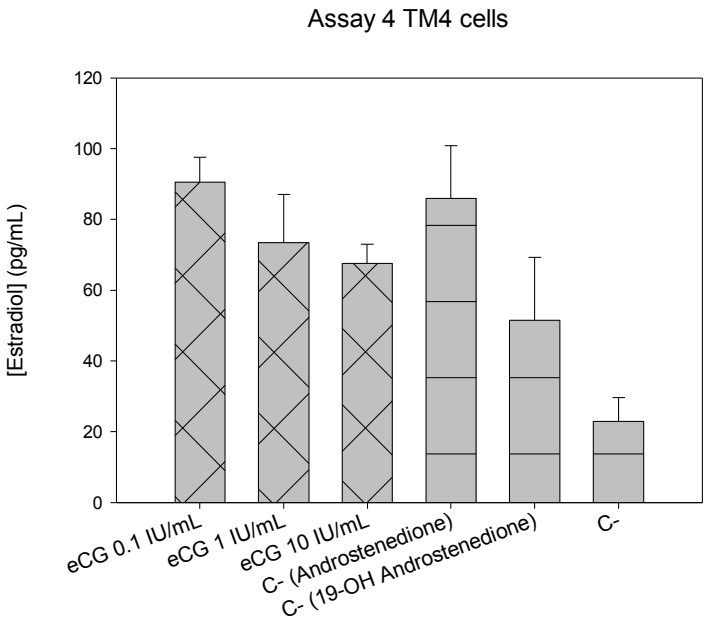

FIGURE 8. Estradiol production of cultures induced with native eCG at different concentrations expressed in International Units (IU). Samples are assayed with androstenedione instead of 19-OH androstenedione. Commercial eCG in the figure corresponded to the native PMSG mentioned in text.

Negative control with androstenedione as substrate, raised an inadmissible amount of estradiol (85 $pg \cdot mL^{-1}$). Similarly, negative control but using 19-OH androstenedione achieved 50 $pg \cdot mL^{-1}$, this value shows the huge variability present in this measurements using this cell line, since the same negative control reached values considerably higher in assays 2 and 3.

Finally, when lack of substrate was used, levels of estradiol produced were appreciably lower, demonstrating that both substrates, androstenedione and 19-OH androstenedione, conduced to an unspecific estradiol production

Therefore, sertoli cells (TM4) were not the appropriate cells for this analysis, since they cannot be induced by any hormone, FSH, native PMSG or recombinant

PMSG. The activity detected in assays 3 and 4 were probably generated by the basal expression of enzymes involved in steroid biosynthetic pathway.

The variability observed seems to be related with a low or null number of FSH receptors in the cytoplasm membrane compared with a primary culture, making impossible to detect significant differences between those cultures induced with hormone and those without hormone.

3.4.3 Determining a suitable protocol for granulose cells freezing

Three different cryovials of granulose cells were cultivated into three different 25 cm^2 flask and using three different protocols (described in section 2.3.3.3, granulose cells). During following seven days a visual checking was effectuated for establishing the best growing culture. The protocol used for Sertoli cells, called as standard in materials & methods and from now on, resulted in a reduced viability of granulose cells and consequently was excluded as a suitable protocol. By contrast, next two alternatives assayed demonstrate good viability rates (figure 9).

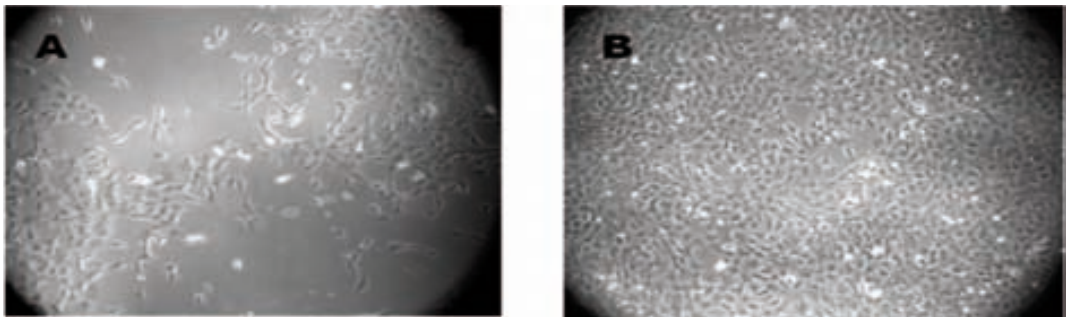

FIGURE 9. Visual comparison after seven days of cultivation. In **A** cells were suspended in growth medium, differently in **B** cells were suspended in serum. Image obtained by phase contrast optical microscope.

Just as shows the figure 9, the protocol in which the cell pellet was resuspended in mixture of serums and an additional centrifugation step was added (B), displayed the best cell viability rates and fast growing after a thawing process.

3.4.4 In vitro analysis of estradiol production by granulose cells stimulated with native PMSG

The levels of estradiol attained for granulose cells in assay 1 were particularly high in comparison with those reached for Sertoli cells (Figure 10). Similarly to Sertoli cells cultures, as hormone concentration increased the estradiol production drop drastically, indicating once again that high concentrations of hormone may provoke negative effects, basically by death cell or receptor desensitization.

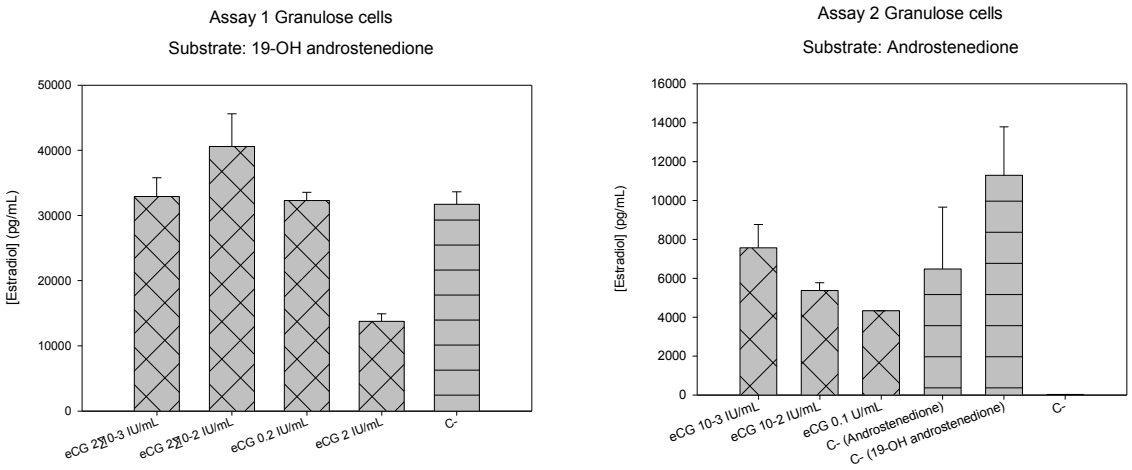

FIGURE 10. Levels of estradiol achieved by bovine granulose cells after induction with native PMSG (IU/mL). The substrate in assay 1 was 19-OH androstenedione and it was changed by androstenedione in assay 2, just as occurred for Sertoli cells. Commercial eCG in the figure corresponded to the native PMSG mentioned in text.

But once again, estradiol production in non-induced cultures was especially high which indicated an elevated basal expression. As a result of this excessive rank, a substrate change was analysed for identical reasons that Sertoli cells. 19-OH-androstenedione was changed in assay 2 by androstenedione. But the new substrate did not give good results since, once again, non-induced cultures produced the highest levels of estradiol. It was especially significant the control without hormone and without substrate in which any presence of estradiol was detected stipulating that both substrates were responsible of non-specific steroidogenic activity. Comparing levels of expression, must be underlined the differences between assay 1 and assay 2, where in the last one was lower, denoting the huge variability present among assays.

Consequently, granulose cells were not validated for a cell-based assay; because of big variability among assays and because of inadmissible levels reached for non-induced cultures.

3.4.5 In vitro analysis of progesterone production by Leydig cells (MLTC-1)

The MLTC-1 is an established transformed cell line and the response of which to chorionic gonadotropin has been widely described[12,17-21]. These cells have the ability to produce progesterone using cholesterol as a substrate after induction of CG or LH. Contrary to previous assays, in this case was expected to detect the LH activity of CG instead of the desired FSH. However, this method allowed discerning between those bioactive recombinant variants with those without apparent activity.

The objective of assay 1 was to assure the viability of MLTC-1 as proper cells for the desired *in vitro* assay. In bibliography was extensively reported how these cells produce progesterone after hCG induction. For this reason, in first assay hCG was used as control instead native PMSG. Forskolin was applied as positive control since its ability to

activate the adenylate cyclise (AC). Forskolin was a lipophilic molecule capable to go through cells membrane and increase the intracellular cAMP, one of the main secondary metabolites. This control informs about the metabolic capacity of the cell to trigger the expected response since when the hormone bounds to its receptor (a G protein-coupled receptor) this activates the AC.

Looking at Figure 11 became apparent the clear difference between cells induced with hCG or forskoline, with cells without hormone. Therefore, the analysis system returned reliable and coherent results (Figure 11).

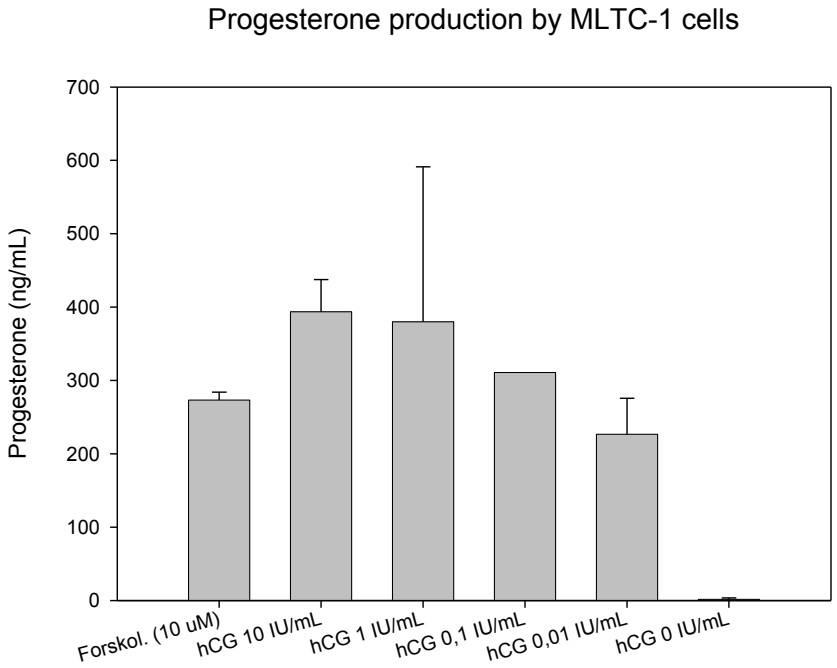

FIGURE 11. Levels of progesterone expressed by MLTC-1 cells after induction with hCG.

Despite the standard deviation were considerably high, especially the culture induced with 1 $IU \cdot mL^{-1}$, the main goal of this study was to determine if existed differences between the induced and no induced cultures. So the values evidenced the

capacity of these cells to response to the hormone stimulus through their receptors and therefore their susceptibility to be used for further experiments.

Once MLTC-1 cell line was validated, the purpose of the assay 2 was to prove the inductive aptitude of native PMSG.

Figure 12 displays the noticeable levels reached by a serial dilution of native hormone. Despite the dilutions applied, the levels of progesterone achieved by samples were remarkably similar, indicating that probably the values were out of range.

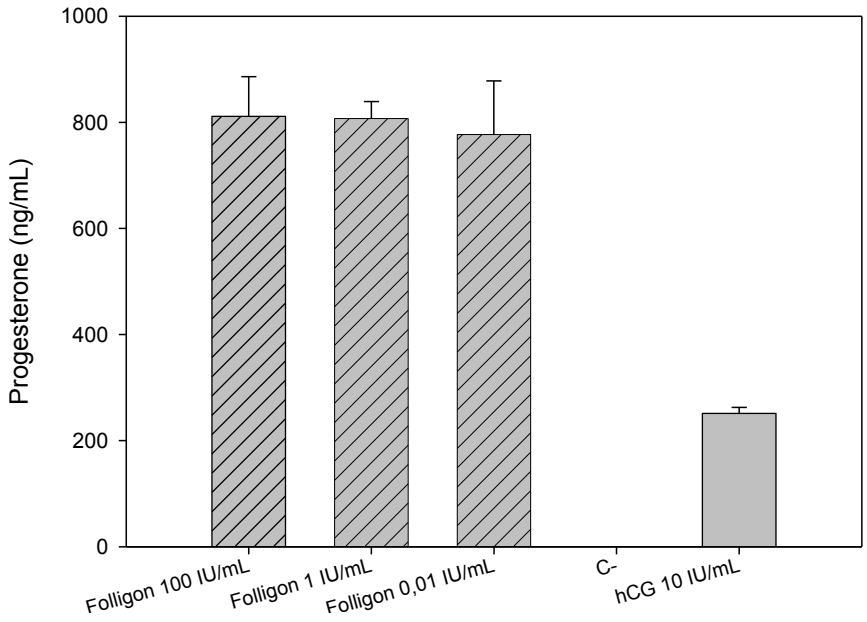

FIGURE 12. The graphic show the levels of progesterone produced by Folligon® induction. The Folligon corresponded to the commercial or native PMSG.

Summarising, native PMSG demonstrated its LH activity inducing progesterone production of MLTC-1 cells.

Therefore, qualitatively it can be exposed that Foligon® (commercial, native PMSG) was a good positive control, and that MLTC-1 cell line (Leydig cells) showed a

good response in front of this hormone and may be used to assay the recombinant variants produced in *Pichia pastoris*.

3.4.6 Advantages and disadvantages of cell lines

Following table 1 summarize main advantages and disadvantages of three cell lines used during this work.

TABLE 1. Main characteristics of three cell lines assayed. Further information can be found in ECACC for TM4 cells (#88111401) and in ATCC for MLTC-1 cells (#CRL-2065™). FSH receptor (FSHr), LH receptor (LHr) and CG receptor (CGr).

Cell lines	Metabolite measured	Receptors expressed	Advantages	Disavantages
TM4 (Sertoli cells)	Estradiol	FSHr	Expression FSHr	Primary culture. Loss of receptor
Granulose Cells	Estradiol	FSHr	Expression FSHr	Primary culture. Loss of receptor
MLTC-1 (Leydig cells)	Progesterone	LHr and CGr	Tumoral cells Easy detection by steroids	Expression LHr

3.4.7 Correlation between *in vitro* and *in vivo* LH actitvity

The objective of this experiment was to correlate the *in vitro* results (levels of progesterone) with LH activity *in vivo* determined through classic Steelman-Pohley bioassay, in order to develop a feasible and reproducible method to determine residual LH activity in purified FSH samples (from cows pituitary) avoiding costly and prolonged in vivo assays. The protocol followed for these assays were identical to those used for Leydig cells procedures, but in this case 96-well plates were established as suitable culture platform to analyse the samples and standards in quintuplicate.

With the purpose to correlate the progesterone measured and the LH activity measured *in vivo*, a calibrate with FSH samples was performed. The correlation is presented in figure 13.

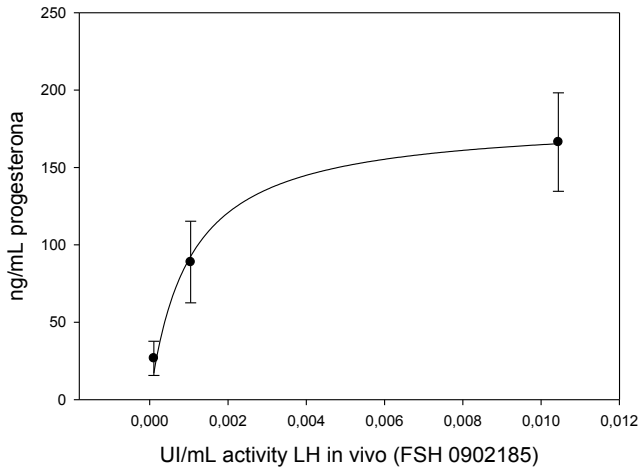

FIGURE 13. Calibration between in vivo LH activity with progesterone levels in cultures.

The non-linear regression presented a logarithmic growth with two different periods; first period corresponded to a rapid increase. In this zone, the measurements were quite sensitive making appropriated for the purpose of this experiment (values ranged between 0 $IU \cdot mL^{-1}$ and 0.003 $IU \cdot mL^{-1}$). By contrast, second period of the regression showed a slow but constant growth. In this period (values higher than 0.004 $IU \cdot mL^{-1}$) the sensitivity of the calibration decreased considerably and it cannot be used for correlating *in vivo* and *in vitro* assays.

The equation that expressed the regression curve is,

$$y = \frac{a \cdot x}{(b + x)} \qquad (2) \qquad \text{where a=181.220 and b=0.001}$$

Attempting to corroborate the calibration, one FSH sample and one LH sample with two dilutions each were assayed in quintuplicate and a theoretical activity *in vivo* was predicted with excellent results (table 2).

TABLE 2. Comparison between LH activities calculated *in vivo* and theoretical LH activities *in vivo* of two samples and their dilutions.

Samples	Protein concentration ($mg \cdot mL^{-1}$)	Theoretical activity *in vivo* ($UI \cdot mL^{-1}$)	Calculated activity *in vivo* ($UI \cdot mL^{-1}$)	SD between samples ($UI \cdot mL^{-1}$)
FSH dilution 1	1,00E-04	0,0010	0,00113	±0,0006
FSH dilution 2	1,00E-05	0,0001	0,00009	±0,000009
LH dilution 1	5,00E-05	0,0092	0,01069	±0,008
LH dilution 2	1,25E-04	0,0231	0,02229	±0,0024

In conclusion, a good correlation between in vitro and in vivo assays were find out within the range 0 and 0.004 $IU \cdot mL^{-1}$, being able to predict the residual LH activity in FSH samples, without the necessity to assay with animals.

3.5 CONCLUSIONS

In this chapter, the development of a cell-based assay as well as the cell culture techniques associated with the cell lines has been reported. Three different cell lines were tested as suitable platforms of response after induction by hormones. Finally, only MLTC-1, were selected as the adequate cell line despite the fact that activity measured corresponds to LH activity instead of the desired FSH. The main conclusions obtained from this work are:

- An applicable freezing protocol for granulose cells was optimized allowing recovering good rates of viable cells.

- The population doubling time, maximum cell concentration and lag phase of TM4 (sertoli cells) were elucidated providing valuable data to design subsequent analysis.
- TM4 cells were excluded as candidates for *in vitro* assay because of their poor steroidogenic activity detected. Probably the lack of FSH receptors in the cell membrane caused these low levels. Moreover, this deficient activity enable negative control reach similar or higher levels of estradiol produced.
- Similarly to TM4, granulose cells were rejected as well by analogous reasons. Despite the increase of estradiol concentration in the supernatants, the huge variability between assays and the inadmissible levels reached by non-induced cultures make no advisable these cells for the analysis.
- MLTC-1 (Leydig cells) was proposed as proper platform because of their capacity to produce big amounts of progesterone after induction by hCG and native PMSG. Therefore they may be used for further experiments with the recombinant variants expressed in *Pichia pastoris*.
- A feasible *in vitro* method was developed to predict *in vivo* LH actitivity of samples, avoiding prolonged and costly *in vivo* assays with animals.

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CHAPTER 4

Cloning and expression of Human Serum Albumin and Pregnant Mare Serum Gonadotropin fusion protein

4.1 INTRODUCTION

In this chapter, the cloning and expression of a recombinant PMSG hormone in *Pichia pastoris* is described. According to the previous works with PMSG, an insufficient half-life of the product in the bloodstream was the cause of its lack of efficacy. In many cases the efficacy of a pharmaceutical recombinant protein depends heavily on the intrinsic pharmacokinetics of the natural protein. Therapeutic recombinant proteins, especially those expressed in yeast may be rapidly cleared from the bloodstream after administration, requiring high dose rates or frequent administration to maintain effective therapeutic levels. There are multiples approaches to improve protein or peptide pharmacokinetics and pharmacodynamics, some of them are described hereafter; 1) amino acid sequence manipulation to decrease immunogenicity, proteolytic cleavage or increase bioavailability[1,2], such as Proleukin® a recombinant IL-2 (Prometheus Inc, CA, USA) or Betaseron® a recombinant interferon beta 1b (Bayer Healthcare, Leverkussen, Germany), whose free cysteine are mutated[3]. 2) Genetic fusions with albumin binding peptides[4,5], such as the antitissue factor D3H44 Fab, which its albumin binding peptide increases its half-life by approximately 40-fold[4]. 3) Conjugation of albumin binding fatty acids, such as Levemir® (NovoNordisk, Bagsværd, Denmark) a long-acting human insulin analogue for maintaining the basal level of insulin. This long half-life is caused by the conjugated myristic acid (Myristoylation) and its ability to bind to albumin into the bloodstream[6]. 4) Polyethylene glycol (PEG) conjugation, such as PEGasys® (Roche, Basel, Switzerland) an Interferon α conjugated to PEG, with a prolonged half-life[7] 5) Genetic fusions to Fc regions of immunoglobins G, such as Enbrel® (Pfizer, Gronton, Connecticut, USA) a Tumor Necrosis Factor fused to Fc region[8] or 6) Genetic fusion to Human Serum Albumin, such as Albutropin™ (Human Genome Sciences, Rockville, IN, USA) a long-acting recombinant growth hormone created by fusing the gene of human growth hormone to the human serum albumin gene[9].

Among all these different approaches, genetic fusions with albumin is an attractive option since it may increase half-life of the active molecule, resulting in a less frequent administration and increased bioavailability. In addition, it may reduce the dosing rates and avoid undesirable side effects as well as a possible reduction in immunogenicity. The main advantage over chemicals conjugations, such as PEGylation is that it does not require lengthy and costly post-production chemical processing.

Albumin, with a molecular mass of 67 kDa, is the most abundant protein in plasma, present at 50 $mg \cdot mL^{-1}$ (600 μM), and has a half-life of 19 days in humans. Albumin serves to maintain plasma pH, contributes to colloidal blood pressure, function as carrier of many metabolites and fatty acids, and serves as a major drug transport protein in plasma.

Human serum albumin (HSA) has been widely used as a stabilizing component in pharmaceutical and biologic products, such as vaccines, recombinant therapies and coatings for medical devices. Besides traditional extraction techniques involving the fractionation of plasma obtained from blood donors, last decade recombinant DNA technology has been used to examine the expression of rHSA in *Escherichia coli, Bacillus subtilis, Saccharomyces cerevisiae, Pichia pastoris, Kluyveromyces lactis*, plants and transgenic animals. However, pharmaceutical companies are currently producing rHSA through yeast species, basically, *S. cerevisiae*[10,11], *K. lactis*[12,13] and *P. pastoris*, being this last specie the most widely used as expression system. By this system, rHSA can be produced on a large scale and afterwards purified to such an extent, that the content of yeast derived contaminants amount are less than 1 ng/250 mg of rHSA[14-19].

Successful fusion proteins require not only the desired component proteins, but also suitable linkers to connect the protein domains. Linkers in fusion proteins generally consist of stable peptide sequence, including the glycine-serine linker $(GGGGS)_n$ and α-

helix-forming peptide linkers, such as $A(EAAAK)_nA$ (n=2-5), which can provide structure flexibility, improve protein stability, or increase biological activity.

However, stable peptide linkers do not allow for the separation of the two fusion protein domains *in vivo* and have several limitations including steric hindrance between two functional domains, altered biodistribution and metabolism of the protein moieties due to the interference with each other, and incorrect folding of the fusion protein [20-22].

In a previous work, A. Ubach reported in her thesis how the non-modified recombinant PMSG expressed in X-33 strain did not have activity in immature female rats following the Steelman-Pohley assay. This lack of *in vivo* activity of the recombinant hormone may be caused by its probable short half-life in blood circulation. To prevent the rapid clearance of PMSG we proposed to create a fusion protein containing the human serum albumin fused to the beta chain of PMSG. In this chapter the molecular design, cloning and expression of a HSA fused to PMSG is described. HSA has been genetically fused to the C-terminus or N-terminus of the β chain of PMSG with a linker peptide of 35 aminoacids length which substitutes the CTP present in the β subunit of the native hormone, this approximation was previously used successfully by Sugahara *et al*., 1996 and Fares *et al*., 1998 [23,24]. This chimera protein has been suitably expressed in *P. pastoris*, subsequent purification experiments and *in vitro* evaluations have been performed successfully.

4.2 MATERIALS AND METHODS

4.2.1 Design of the genetic fusion

Sequence of HSA fused to N-terminal and C-terminal of β subunit as well as sequence of α subunit alone of equine chorionic gonadotropin were entrusted to be synthesized with the codon usage optimized for *P. pastoris* to GeneArt (Regensburg, Germany). In both fusions, the C-terminal peptide (CTP), composed of 35 amino acids, was deleted in order to reduce the problems associated to 12 O-glycosylation points present in this part of the protein. It is though, that in both, hCG and PMSG, CTP is involved in the half-life of the protein into the bloodstream, but does not affect the bound with the receptor. Coherently, the fusion with HSA (more than 450 hours of half-life) replaces the ability of CTP region and may increase considerably the half-life of the fusion protein. The eliminated C-terminal peptide regions were replaced by a linker composed of 35 amino acids rich in Glycine (Gly, G) and Serine (Ser, S), accurately composed of seven repetitions $(GGGGS)_7$. In order to carry out the fusions, amino acidic sequence of beta subunit (P08751) was modified as shown figure 1. First, signal sequence (20 amino acids) located in N-terminal was removed and last 35 amino acids corresponding to CTP were also eliminated.

Entire amino acid sequence β subunit (169 aa)

```
        10         20         30         40         50         60
METLQGLLLW MLLSVGGVWA SRGPLRPLCR PINATLAAEK EACPICITFT TSICAGYCPS

        70         80         90        100        110        120
MVRVMPAALP AIPQPVCTYR ELRFASIRLP GCPPGVDPMV SFPVALSCHC GPCQIKTTDC

       130        140        150        160
GVFRDQPLAC APQASSSSKD PPSQPLTSTS TPTPGASRRS SHPLPIKTS
```

Mature sequence without CTP (114 aa)

```
        10         20         30         40         50         60
SRGPLRPLCR PINATLAAEK EACPICITFT TSICAGYCPS MVRVMPAALP AIPQPVCTYR
        70         80         90        100        110
ELRFASIRLP GCPPGVDPMV SFPVALSCHC GPCQIKTTDC GVFRDQPLAC APQA
```

FIGURE 1. Amino acid modifications on PMSG beta subunit sequence. The mature sequence without CTP corresponds to the sequence which is used for the HSA fusion. First 20 amino acids in Italic conform the signal sequence and last underlined 35 amino acids agree with C-terminal region (Serine and threonines involved in O-glycosylation are in bold). Marked amino acids form part of determinant loop Cys 10th and Cys 11th.

Similarly, Human serum albumin (P02768, 609 aa) amino acid sequence was modified and only the mature sequence was selected for the fusions. First 18 amino acids corresponding to the signal peptide and next 4 propeptide amino acids were eliminated. Figure 2 shows the final amino acid sequence with the linker of both fusions.

HSA-GS-βPMSG (740 aa)

```
        10         20         30         40         50         60
HHHHHHDAHK SEVAHRFKDL GEENFKALVL IAFAQYLQQC PFEDHVKLVN EVTEFAKTCV
        70         80         90        100        110        120
ADESAENCDK SLHTLFGDKL CTVATLRETY GEMADCCAKQ EPERNECFLQ HKDDNPNLPR
       130        140        150        160        170        180
LVRPEVDVMC TAFHDNEETF LKKYLYEIAR RHPYFYAPEL LFFAKRYKAA FTECCQAADK
       190        200        210        220        230        240
AACLLPKLDE LRDEGKASSA KQRLKCASLQ KFGERAFKAW AVARLSQRFP KAEFAEVSKL
       250        260        270        280        290        300
VTDLTKVHTE CCHGDLLECA DDRADLAKYI CENQDSISSK LKECCEKPLL EKSHCIAEVE
       310        320        330        340        350        360
NDEMPADLPS LAADFVESKD VCKNYAEAKD VFLGMFLYEY ARRHPDYSVV LLLRLAKTYE
       370        380        390        400        410        420
TTLEKCCAAA DPHECYAKVF DEFKPLVEEP QNLIKQNCEL FEQLGEYKFQ NALLVRYTKK
       430        440        450        460        470        480
VPQVSTPTLV EVSRNLGKVG SKCCKHPEAK RMPCAEDYLS VVLNQLCVLH EKTPVSDRVT
       490        500        510        520        530        540
KCCTESLVNR RPCFSALEVD ETYVPKEFNA ETFTFHADIC TLSEKERQIK KQTALVELVK
       550        560        570        580        590        600
HKPKATKEQL KAVMDDFAAF VEKCCKADDK ETCFAEEGKK LVAASQAALG LGGGGSGGGG
       610        620        630        640        650        660
SGGGGSGGGG SGGGGSGGGG SGGGGSSRGP LRPLCRPINA TLAAEKEACP ICITFTTSIC
       670        680        690        700        710        720
AGYCPSMVRV MPAALPAIPQ PVCTYRELRF ASIRLPGCPP GVDPMVSFPV ALSCHCGPCQ
       730        740
IKTTDCGVFR DQPLACAPQA stop
```

βPMSG-GS- HSA (740 aa)

```
        10         20         30         40         50         60
SRGPLRPLCR PINATLAAEK EACPICITFT TSICAGYCPS MVRVMPAALP AIPQPVCTYR
        70         80         90        100        110        120
ELRFASIRLP GCPPGVDPMV SFPVALSCHC GPCQIKTTDC GVFRDQPLAC APQAGGGGSG
```

```
       130        140        150        160        170        180
GGGSGGGGSG GGGSGGGGSG GGGSGGGGSD AHKSEVAHRF KDLGEENFKA LVLIAFAQYL
```

```
       190        200        210        220        230        240
QQCPFEDHVK LVNEVTEFAK TCVADESAEN CDKSLHTLFG DKLCTVATLR ETYGEMADCC
       250        260        270        280        290        300
AKQEPERNEC FLQHKDDNPN LPRLVRPEVD VMCTAFHDNE ETFLKKYLYE IARRHPYFYA
       310        320        330        340        350        360
PELLFFAKRY KAAFTECCQA ADKAACLLPK LDELRDEGKA SSAKQRLKCA SLQKFGERAF
       370        380        390        400        410        420
KAWAVARLSQ RFPKAEFAEV SKLVTDLTKV HTECCHGDLL ECADDRADLA KYICENQDSI
       430        440        450        460        470        480
SSKLKECCEK PLLEKSHCIA EVENDEMPAD LPSLAADFVE SKDVCKNYAE AKDVFLGMFL
       490        500        510        520        530        540
YEYARRHPDY SVVLLLRLAK TYETTLEKCC AAADPHECYA KVFDEFKPLV EEPQNLIKQN
       550        560        570        580        590        600
CELFEQLGEY KFQNALLVRY TKKVPQVSTP TLVEVSRNLG KVGSKCCKHP EAKRMPCAED
       610        620        630        640        650        660
YLSVVLNQLC VLHEKTPVSD RVTKCCTESL VNRRPCFSAL EVDETYVPKE FNAETFTFHA
       670        680        690        700        710        720
DICTLSEKER QIKKQTALVE LVKHKPKATK EQLKAVMDDF AAFVEKCCKA DDKETCFAEE
       730        740
GKKLVAASQA ALGLHHHHHH stop
```

FIGURE 2. HSA-GS-βPMSG and βPMSG-GS-HSA amino acid sequences. Underlined regions correspond to linker and marked H are the 6xHis tail.

On the other hand, native α subunit sequence (P01220) was formed by 120 amino acids. First 24 amino acids corresponding to signal sequence were removed (figure 3).

Entire amino acid sequence α subunit (96 aa)

```
        10         20         30         40         50         60
FPDGEFTTQD CPECKLRENK YFFKLGVPIY QCKGCCFSRA YPTPARSRKT MLVPKNITSE
        70         80         90
STCCVAKAFI RVTVMGNIKL ENHTQCYCST CYHHKIstop
```

FIGURE 3. The α subunit sequence without the signal sequence and with a stop codon at the end of the sequence.

4.2.2 Strains

Escherichia coli DH5α was used for plasmid construction and amplification. The wild-type phenotype *Pichia pastoris* X33 strain (Invitrogen Co.) was employed as a host strain for expressing α subunit and the fusions of β subunit and HSA. Both subunits were expressed under the transcriptional control of the AOX1 promoter.

4.2.3 Media composition

E. coli strains were cultured in Luria broth (LB) medium supplemented with 50 $\mu g \cdot mL^{-1}$ zeocin (InvivoGen) as required. *P. pastoris* strains were cultured in YPD medium (1 % yeast extract, 2 % peptone, 2 % glucose) supplemented with 100 $\mu g \cdot mL^{-1}$ of zeocin when required. Shake flask cultures were performed using a BMGY and BMMY medium (*Pichia Expression Kit*, Invitrogen Co.). BMGY is composed of 1 % w/v yeast extract (Panreac, 403687), 2 % w/v peptone (Panreac, 403695), 100 mM potassium phosphate, pH 6.0, 1,34 % w/v Yeast nitrogen Base (YNB) without ammonium sulphate and amino acids (Sigma, Y1251), 1 % w/v glycerol (Panreac, 201339). This medium is supplemented with $1 \cdot 10^{-4}$ % w/v biotin (Sigma, B-4501). BMMY was prepared identically with the exception of carbon source, instead of glycerol, a 0,5 % v/v of methanol (Panreac, 131091) was used. The YNB, biotin, and methanol components of both mediums were sterilised separately by microfiltration and then added to the shake flask.

For strain maintenance, *P. pastoris* strains were grown in YPD agar plates and maintained at 4ºC for up to one month. Long term stocks were prepared as recommended by Invitrogen and stored at -80ºC.

4.2.4 Shake flask cultivation conditions

Small scale cultures were carried out in a 1 L baffled shake flasks as it is stated into the manual *P. pastoris Expression Kit* (Invitrogen Co.). First, 25 mL of BMGY were inoculated with a single colony from a fresh plate or directly with 50 μL from a cryovial and incubated at 30ºC and 200 rpm in an orbital shaker for 16-18 h. Subsequently, cells were harvested using sterile centrifuge bottles at 3000 xg for 5 minutes at room temperature. To induce the expression, supernatant was decanted and the cell pellet resuspended to an OD_{600}=1 in BMMY medium to start induction. The cultivations were grown at 30ºC and 200 rpm adding methanol 100 % to 0,5 % (v/v) every 24 hours during 3 or 4 days. Finally, cells were harvested and supernatants were stored at -20ºC to be analysed.

4.2.5 Plasmid and strain construction

Recombinant DNA methods were performed, essentially, as described in Sambrook *et al.,* 2000. Oligonucleotides were synthesised by Sigma-Aldrich. DNA sequencing was performed at Macrogen Inc. (Seoul, Korea) using specific primers in order to verify the complete nucleotide sequence of both subunits cloned in frame.

Three different vectors with the selected sequences (pMA-α, pMK-HSABeta and pMK-BetaHSA) were electroporated to *E.coli* DH5α. Plasmids from resulting colonies were examined and digested with NotI (ER0595, Fermentas) and EcoRI (ER0271, Fermentas). Bands according to α, βHSA and HSAβ cassettes were purified (Illustra GFX purification Kit, 28-9034-70, GE Healthcare) and ligated with T4 DNA Ligase (EL0011, Fermentas) at room temperature for 1 hour with pPICZαA (Invitrogen)[25] vector digested likewise with *NotI* and *EcoRI*. Ligations were electroporated to DH5α again and plasmids pPICZα-α, pPICZα-HSAβ and pPICZα-βHSA from resulting colonies were confirmed by digestion analysis and sequencing (figure 4). In order to evaluate the expression of

subunit β fused to HSA without the presence of α subunit, pPICZα-HSAβ and pPICZα-βHSA were electroporated directly to X-33 *P. pastoris* without further subcloning steps.

Besides, α and β subunits were docked into the same expression vector. Vector pPICZα-α was opened with *BamHI* (ER0051, Fermentas) and dephosphorylated with shrimp alkaline phosphatase (EF0511, Fermentas). On the other hand, pPICZα-HSAβ and pPICZα-βHSA were digested with *BamHI* and *BglII* (ER0082, Fermentas) and the bands according to the expression cassette composed of AOX1 promoter, α -factor signal peptid, the β subunit and HSA fusions and AOX1 terminator (2230 pb) were purified and ligated with *BamHI*-digested pPICZα-α.

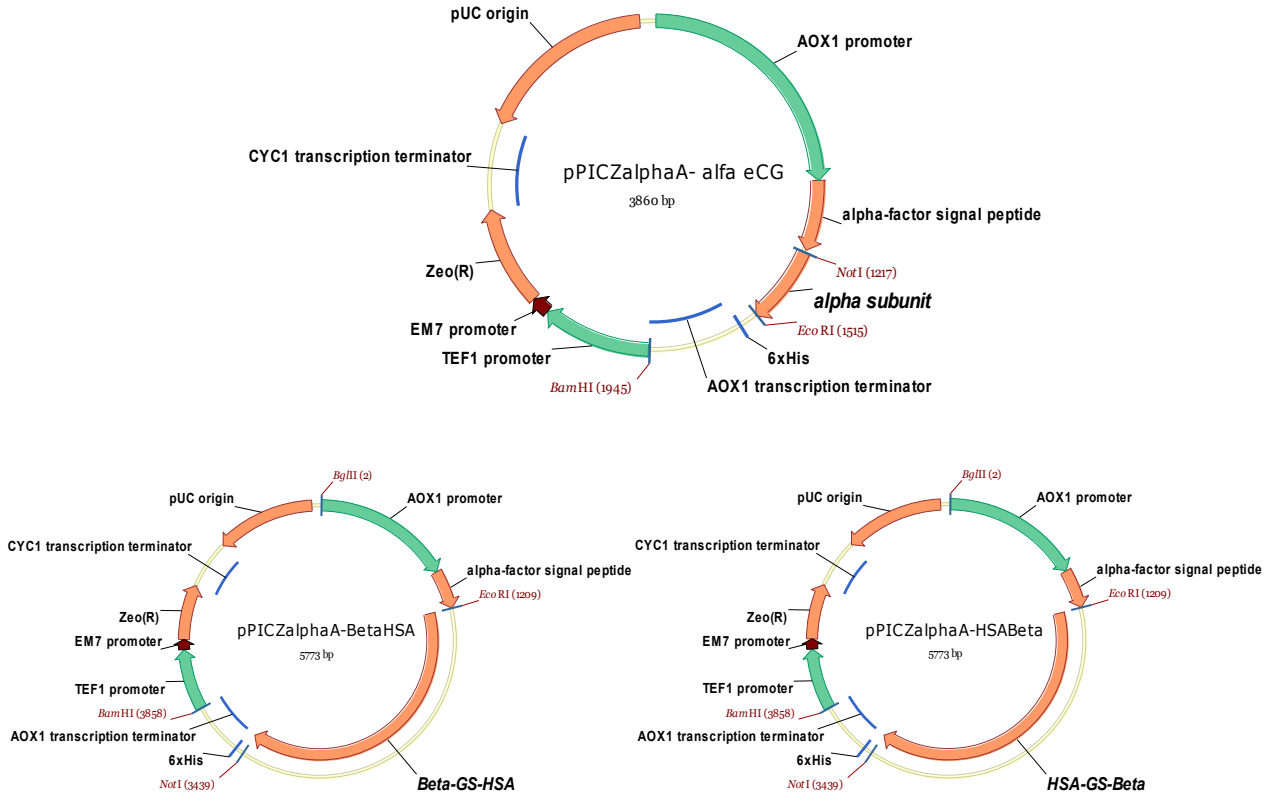

FIGURE 4. Vectors pPICZα-alfa, pPICZα-HSAβ and pPICZα-βHSA with the restriction sites used during subcloning

Therefore, after ligation two different vectors, pPICZαA-α+βHSA and pPICZαA-α+HSAβ (figure 5), with both subunits controlled by its own AOX1 promoter as well as its own AOX1 terminator were obtained and transformed into DH5α.

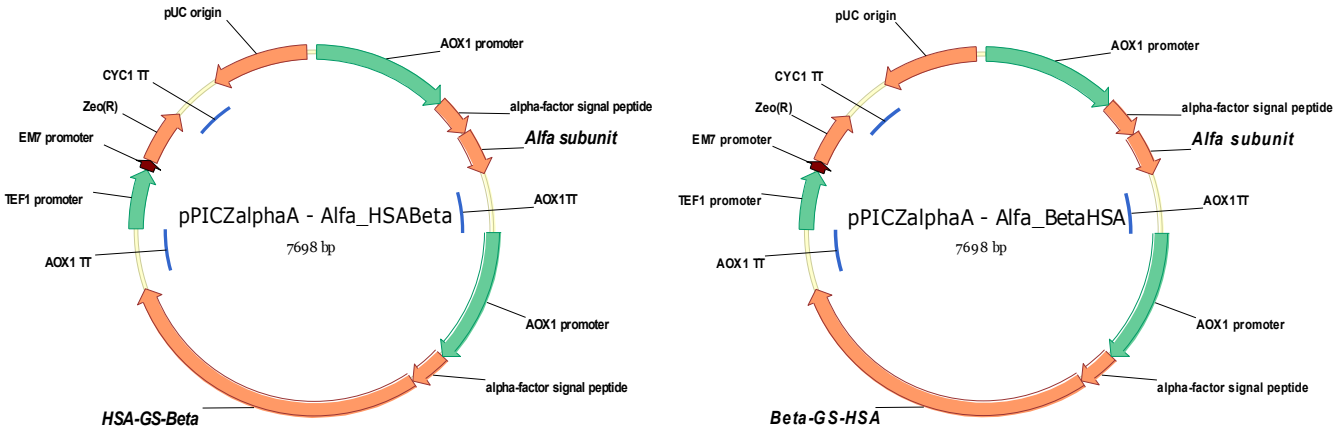

FIGURE 5. Vectors pPICZαA-α+βHSA and pPICZαA-α+HSAβ transformed to X-33.

Once, confirmed by sequencing, vectors were electroporated to *P. pastoris.*

4.2.6 Vector electroporation into *P. pastoris*

In *P. pastoris*, integration of expression vector into precise site of the genome is directed via specific digestion with a restriction enzyme creating highly recombinant DNA-free termini[26]. Using pPICZαA, *SacI* is used to linearize vectors in AOX1 promoter region and therefore plasmids are integrated in this region[27]. However, as is mentioned above, vectors pPICZαA-α+βHSA and pPICZαA-α+HSAβ contained two copies of AOX1 promoter region controlling α and β subunits independently and thus, it was not possible to linearize with *SacI*. Alternatively, an unique restriction site *BamHI* near to *SacI* was used for this purpose.

As the efficiency to insert *BamHI*-linearized vectors were presumibly low, an alternative protocol implemented by Wu *et al.,* 2004 to increase the transformation capacity of competents cells was carried out[28]. The protocol is detailed below:

1. Grow 10 mL overnight cultivation with YPD at 30ºC and 200 rpm
2. Use the overnight cultivation to inoculate 100 mL of YPD to a final OD_{600} of 0.1. Incubate at 30ºC and 200 rpm until OD_{600} reach 1.3-1.5.
3. Centrifugue cells at 4000 xg and 4ºC.
4. Resuspend pellet with 30 mL of LiAc 100 Mm, DTT 10 mM, Sorbitol 0,6 M and Tris-HCl 10 mM pH=7,5. Incubate the resuspension at room temperature for 30 minutes.
5. Centrifugue cells at 4000xg and 4ºC.
6. Wash the pellet with 30 mL of chilled 1 M Sorbitol.
7. Centrifugue cells at 4000xg and 4ºC.
8. Wash the pellet with 15 mL of chilled 1 M Sorbitol.
9. Centrifugue cells at 4000xg and 4ºC.
10. Wash the pellet with 15 mL of chilled 1 M Sorbitol.
11. Centrifugue cells at 4000xg and 4ºC.
12. Wash the pellet with 5 mL of chilled 1 M Sorbitol.
13. Centrifugue cells at 4000xg and 4ºC.
14. Resuspend pellet cells with 1 mL of chilled 1 M Sorbitol and prepare aliquots of 80 µL. Cells are now ready to be used inmediately or can be stored at -80ºC.

Note: Is important to emphasize that using fresh cells higher levels of transformants are obtained.

All reagents mentioned above were purchased to Sigma-Aldrich.

4.2.7 Confirmation of vector insertion through genomic amplification

Vector integration into the genome may be confirmed by PCR amplification using specific primers located either into the vector cassette or into the flanking genomic sequences. First, genomic DNA must be isolated. The protocol used as well as buffers necessaries are detailed below:

Buffers

2xTENS buffer - 0,12 g/L Tris, 0,15 g/L EDTA, 0,12 g/L NaCl pH 8.0

2xSTES buffer - 2xTENS buffer, 1 g/L SDS

TE buffer- 10 mM TrisHCl pH 8.0, 1 mM EDTA

1. An overnight cultivation of 5 mL YPD is inoculated with a single colony from a fresh plate.
2. Transfer the volume of cultivation necessary to adjust the OD_{600} to 1 into a microcentrifuge tube. Rectify the volume to a final 1 mL with milliQ® water.
3. Pellet cells, discard supernatant and resuspend them with 220 μL of milliQ® water.
4. Fill a half of microcentrifuge tube with acid-washed glassbeads (G8772, Sigma) and 250 μL of 2x STES buffer.
5. Pour suspended cells to the glass beads tube and add 30 μL of phenol:chloroform (77617, Sigma).
6. Vortex samples for 30 min at 4ºC.
7. Centrifuge sample at 13000 rpm during 5 minutes and transfer supernatant to a new tube.
8. Add 100 μL of 3M of sodium acetate pH 5.2 (141633.1210, Panreac) and 1000 μL of Isopropanol (211090, Panreac).
9. Mix the sample gently and centrifuge for 20 minutes at 13000 rpm at room temperature.

10. Discard the supernatant and add 50 μL of phenol:chloroform and 50 μL of 2x STES buffer.
11. Centrifuge 5 minutes at 13000 rpm. Recover the supernatant to a new tube and add 200 μL of 70% ethanol (161086, Panreac).
12. Centrifuge sample at 13000 rpm for 5 minutes, discard carefully the ethanol and let the pellet dry completely.
13. Pellet can be resuspended with TE buffer pH 7.6.

Note: Colony PCR is a fast alternative protocol which can also be used for checking positive transformants.

The quality of the genomic DNA was verified by agarose gel. Usually, with only 1 μL of this purified genomic DNA is enough for using it as template for next PCRs.

Reagent composition of PCR often must be optimized every experiment. For these genomic amplifications was used: 25 μL of RedTaq ReadyMix™ PCR reaction mix (R2523,Sigma), containing 3mM $MgCl_2$, 0.4 mM dNTP mix, 0.06 U/μL *Taq* DNA polymerase, 1 μL of 2μM forward and reverse primers and finally 1 μL of purified genomic DNA.

The program used for genomic amplification was substantially modified with the intention to improve the efficiency and reliability. A temperature gradient of 0.3 ºC/second was used to increase the temperature from 30ºC to 70ºC.

Step 1. 10 min 93ºC

Step 2. 1 min 94ºC

Step 3. 1 min 30 seconds 30ºC

Step 4. 0.3ºC/sec to 70ºC

Step 5. 3 min 72ºC

Repeat 4 times the cycle; step 2 to step 5

Step 6. 1 min 94ºC

Step 7. 1 min 51ºC

Step 8. 2 min 72ºC

Repeat 34 times the cycle; step 6 to step 8

Step 9. 10 min 72ºC

The primers used to confirm the specific integration into the genome were obtained from Invitrogen. Primer forward 5' AOX1: 5´-GACTGGTTCCAATTGACAAGC-3´, and 3' AOX1 primer reverse 5´-GCAAATGGCATTCTGACATCC-3´.

4.2.8 Purification process

Below are described the different steps used to purify the recombinant PMSG protein. Chromatographic separations were performed manually with a syringe.

After the cell culture the yeast cells were removed by a single step centrifugation at 5000 rpm for 10 minutes. The supernatant was then filtered through a 0,22 µm microfilters (6872-1302, Whatman). Previously to apply the concentrated to a HisTrap FF 1mL column (17-5319-01, GE Healthcare), the sample was adjusted to the composition and pH of the Binding buffer (20 mM NaH_2PO_4, 0.5 M NaCl, 50 mM Imidazole, pH 7.4). Once the sample was applied to the column was eluted with the Elution buffer (20 mM NaH_2PO_4, 0.5 M NaCl, 500 mM Imidazole, pH 7.4). Finally, the buffer of the eluate was changed to assay buffer (30 mM $Na_2HPO_4 \cdot 12\ H_2O$, 130 mM NaCl, pH 7.2) through a desalting column (17-1408-01, GE Healthcare).

4.3 RESULTS AND DISCUSSION

4.3.1 Construction of pPICZαA-α+βHSA and pPICZαA-α+HSAβ and clones confirmation by specific genomic PCR amplification.

To study the heterologous expression of PMSG with HSA fused to N- and C-terminal of β subunit, three different sequences were synthesized by GeneArt (Regensburg, Germany); α subunit, HSA fused to N-terminal of β subunit and HSA fused to C-terminal of β subunit. These three sequences were cloned individually into the vector pPICZαA and electroporated into the *E.coli* strain DH5α for plasmid replication. Vectors pPICZαA-βHSA and pPICZαA-HSAβ were linearized with their unique *SacI* restriction site present into the promoter AOX1 and electroporated to X-33 *P. pastoris* with the aim to evaluate their possible *in vitro* activity without α subunit.

On the other hand, and in contemplation of different expression patterns if α and β subunits were expressed in different vectors, both subunits were cloned in the same vector but each one individually controlled for its own AOX1 promoter. Afterwards, two different expression vectors containing α subunit and β subunit fused to HSA were constructed. Both subunits contained α -factor secretion signal fused in frame with a sequence of six histidines fused to HSA. The resulting vectors, pPICZαA-α+βHSA and pPICZαA-α+HSAβ were electroporated to *P. pastoris* and integrated into the AOX1 region of genome . Six transformants for each vector were adequately isolated and their integration was confirmed via genomic PCR amplification. Figure 6 shows the corresponding bands resulting from PCR with the specific primers AOX1 (Invitrogen).

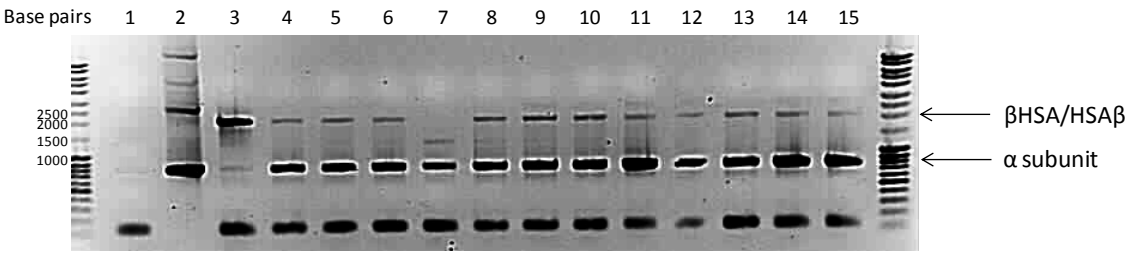

FIGURE 6. Genomic amplification in agarose gel. 1: Negative control; 2: Plasmidic positive control; 3: Genomic positive control; 4-9: α+HSAβ clones 1 to 6; 10-15: α+βHSA clones 1 to 6.

Figure 6 shows an intense band of ~ 800 base pairs (pb) which corresponds to a specific amplification of α subunit. On the other hand, only a slight amplification of ~2800 pb corresponding to βHSA and HSAβ cassettes were obtained. Subsequently, these bands were reamplified with specific inner primers and was demonstrated that all clones contained the adequate fusion cassette (data not shown).

Therefore, all transformants obtained for each construct were confirmed by genomic PCR amplification.

4.3.2 Expression analysis of pPICZαA-βHSA, pPICZαA-HSAβ, pPICZαA-α+βHSA and pPICZαA-α+HSAβ in shake flask

The expression of *P. pastoris* X-33 pPICZαA-βHSA, X-33 pPICZαA-HSAβ, X-33 pPICZαA-α+βHSA and X-33 pPICZαA-α+HSAβ were analysed by western blot and their growth was followed by means of OD_{600} measures. After 72 hours of cultivation, all clones reached similar optical density and their expression levels were visually compared by western blot without denoting differences between them (data not shown). So, one clone for each construction were selected for a deeper expression analysis in shake flask. Figure 7 compares growth curves during induction phase in BMMY of the expressing clones and a non-expressing wild type strain X-33. The arrows indicate the moment of methanol addition to final concentration of 5 $g \cdot L^{-1}$. The majority of expressing clones

reached OD_{600} higher than 25, except for the wild type. Suggesting that the expression of both rPMSG constructs may not affect the growth pattern.

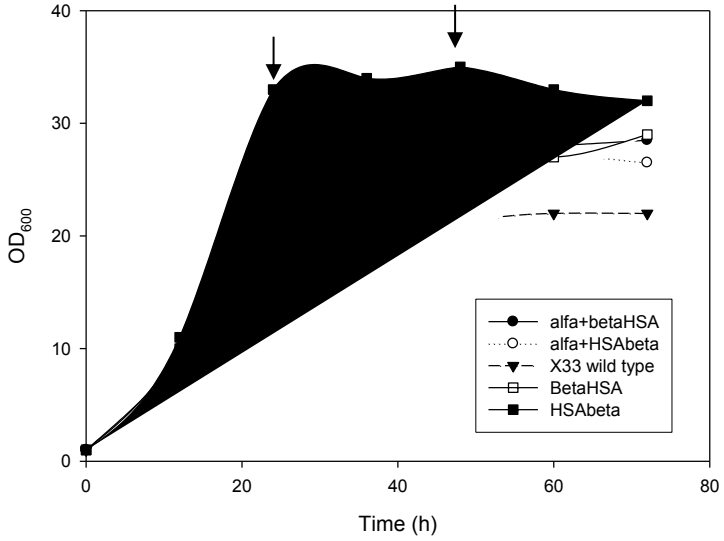

FIGURE 7. OD_{600} evolution of clones expressing the recombinant heterodimer rPMSG, the subunits βHSA and HSAβ and the wild type X-33 during induction phase growing in BMMY.

Figure 8 shows the signal corresponding to the samples extracted every 12 hours of α+βHSA and α+HSAβ after 15 minutes of exposition. Samples of βHSA and HSAβ are not shown in the figure.

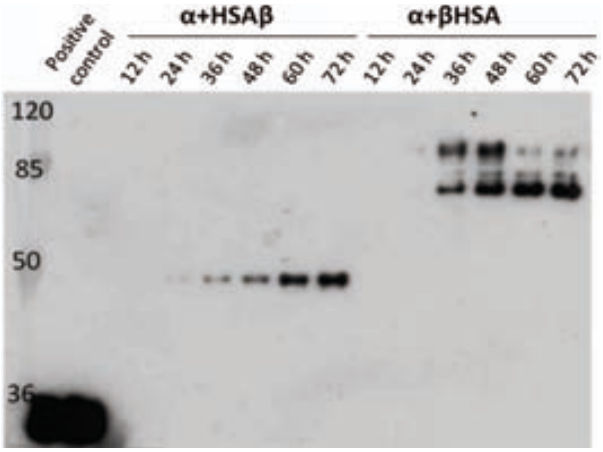

FIGURE 8. Western blot analysis of culture supernatants every 12 hours from shake flask cultivations of constructs α+βHSA and α+HSAβ using anti-6xHis antibody (11922416001, Roche). The positive control consists of a recombinant protein with a 6xHis tail.

As the 6xHis tail is located in both constructs at the free end of HSA, the signal corresponded to HSA fused to β subunit, while α does not contain any His tag and was not possible to detect by anti-6xHis antibodies.

Assuming that human serum albumin has a molecular weight of 67 kDa, and that β subunit without O-glycosylation sites has a weight of 17,87 kDa (Uniprot, P08751) and that only one N-glycosylation site at position Asn 33 should contribute with more or less 2,2 kDa[29,30], total molecular weight of β subunit fused to HSA should be ~ 87- 88 kDa. Considering this molecular weight approximation and the results showed in figure 8 two important observations can be ascertained.

First, construction α+HSAβ showed one unique and clear band around 50 kDa. Since in this construction the His tag is located at N-terminal of HSA and the molecular weight of this band is too small to be HSA, apparently occurred a unique cleavage in HSA region, near to C-terminal and linker sequence. As the other part containing linker and β subunit cannot be detected with anti-6xHis antibody was not possible to elucidate if actually is a specific proteolysis or may be an early termination of transcription.

Second, construct α+βHSA showed two majority bands; the upper band raised slightly above 85 kDa, which apparently coincided with the molecular weight of desired fusion protein, while a lower band was just below this weight. This result denoted, once again, degradation of the recombinant fusion, but in this case, only a part of the total entire product seemed to be affected for proteolysis. More, upper band reached its maximum signal at 48 hours, after this time it was vanished while the lower band increase its signal evidencing that possibly upper band was the substrate for proteases present in the cultivation media and the lower band was the product of their activity.

4.3.3 Purification of α+βHSA, α+HSAβ, HSAβ and βHSA constructs with HisTrap® FF columns (GE Healthcare)

Taking the advantage of the ability of the recombinant products to bind to affinity chromatography columns, in this case immobilized metal affinity columns, the centrifuged broth cultures of α+βHSA, α+HSAβ (purification of HSAβ and βHSA is not shown), were loaded to a prepacked Niquel Sepharose column using a syringe and subsequently eluted in different fractions of 500 μL. These prepacked columns are ideal for purifications of His-tagged recombinant proteins.

Figure 9 shows the protein eluted in each fraction. For α+βHSA construct, the major part of protein was eluted in fraction E2, by contrast α+HSAβ was purified in higher amounts and the protein was mainly eluted in fractions E1 and E2. Moreover, as a result of proteases activity in construct α+HSAβ an intense band around 50 kDa corresponding to a cleaved part of the protein was also purified. This band was previously detected in western blots of cultures showed in previous section, confirming that possibly a specific cleavage affect the fusion protein in which albumin was fused to N-terminal of β subunit.

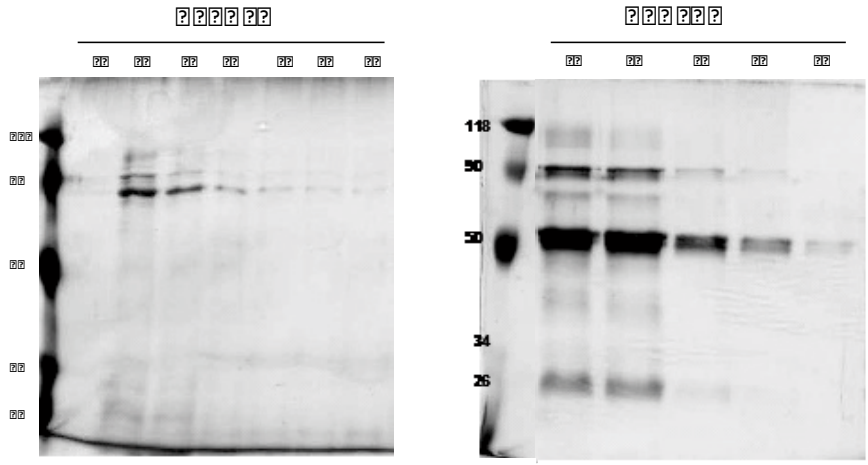

FIGURE 9. Silver stained SDS-PAGE. Supernatants of α+βHSA and α+HSAβ manually applied to the HisTrap FF columns and collected in different fractions

Thus, the purified band surrounding 90 kDa appeared to be the protein properly purified by Immobilized metal ion affinity chromatography (IMAC).

As recombinant proteins purified with IMAC system must be eluted using considerable amounts of imidazole, usually, after HisTag affinity purification a desalting step is recommended in order to eliminate all salts and imidazole present in elution buffer and therefore in samples.

Fractions E2 and E3 of α+βHSA were desalted in a HiTrap desalting columns of 5 mL (GE Healthcare) and 1 mL of samples were collected. Differently, fractions selected in α+HSAβ construct were E1 and E2. Next silver stained gel (Figure 10) shows how during the desalting step no noticeable loss of protein occurred.

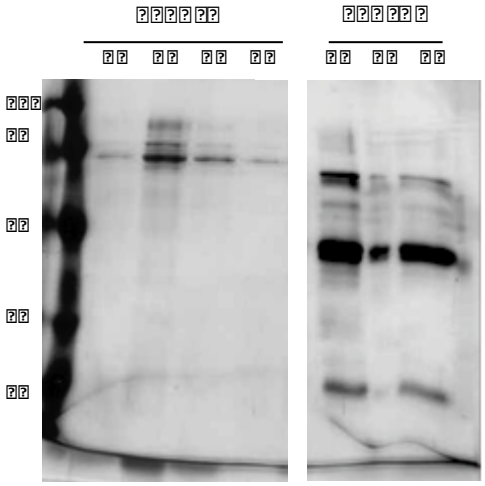

FIGURE 10. Silver stained SDS-PAGE. Fractions E2 and E3 from HisTrap columns of α+βHSA,and fractions E1 and E2 of α+HSAβ are manually applied to the HiTrap desalting columns.

After desalting step, main eluted samples of each construct were collected and their protein concentration was measured by Bradford assay. A total of 23,67 μg/mL of construct α+βHSA was finally recovered. By contrast, for α+HSAβ the total amount at the end of the purification was more than double (58,7 μg/mL). However, as it can be seen in figure 9 and figure 10 the largest part of purified protein is presented in a 50 kDa cleaved form. For its part, the subunits βHSA and HSAβ reached 30,65 μg/mL and 55,76 μg/mL respectively, confirming that the constructs with the HSA fused to the N-terminal of β subunit were expressed in slightly higher amounts than the others, but were affected by the degradation.

4.3.4 Activity determination of purified constructs through in vitro analysis with Leydig cells

Once all constructs were purified, and the protein concentration was determined by Bradford. In vitro activity of each construct was measured with Leydig cells cultures in a 12-wells culture plate. The positive control (commercial native PMSG, Foligon®) and

each construct were cultured in duplicate and assayed by ELISA in triplicate. Approximately, 10 μg·well^{-1} of each construct and the same amount of Foligon® was added to each culture in order to stimulate progesterone production by MLTC-1 cells. On the other hand, desalting buffer used in purification was used as negative control.

With the aim to minimize the variability in progesterone levels caused by the variable number of cells present in each well, the final progesterone concentration was expressed as ng progesterone·10^5 cells^{-1} balanced with cell concentration (Table 1).

TABLE 1. Comparison of progesterone produced by MLTC-1 cells stimulated with α+βHSA, α+HSAβ, βHSA and HSAβ.

	ng progesterone·mL^{-1}	Standard deviation	10^5 cells·mL^{-1}	ng progesterone· 10^5 cells^{-1}
Positive control (Foligon)	1020,296	89,647	1,575	647,807
Negative control	86,708	57,476	1,975	43,903
α+βHSA	591,942	228,147	1,900	311,548
α+HSAβ	120,737	75,825	1,980	60,980
βHSA	35,167	20,944	2,300	15,289
HSAβ	108,412	38,392	1,775	61,077

Next vertical bar chart (figure 11) compares levels of progesterone balanced with cell concentration. The highest values of progesterone were reached by the Foligon®-stimulated cultures. Despite an important standard deviation, levels of progesterone achieved by α+βHSA are remarkably considerable. By contrast, α+HSAβ attained similar levels to negative control and the constructs HSAβ and β HSA, denoting the importance of α subunit for the heterodimer activity.

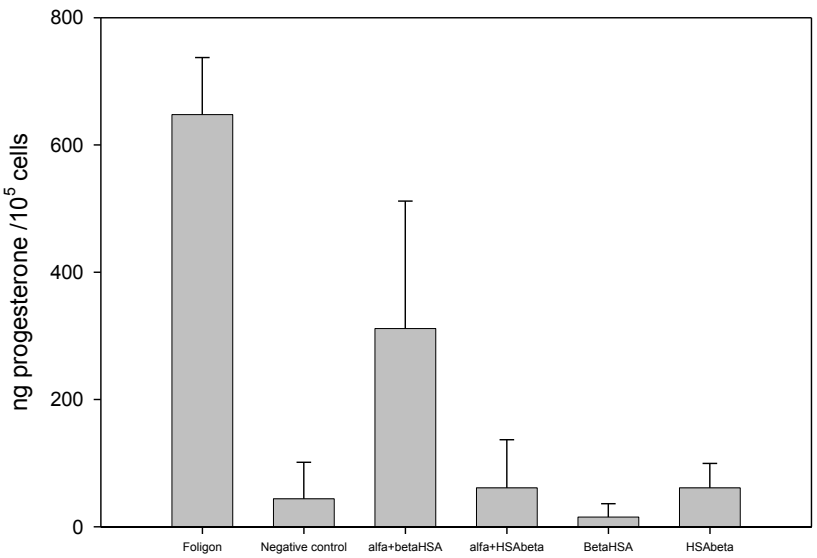

FIGURE 11. Different levels of progesterone production after stimulation with different recombinant fusions forms.

After this assay two major observations could be made:

First, the genetic fusion of Human serum Albumin to β subunit of PMSG may affect the *in vitro* capacity to stimulate progesterone production of MLTC-1 cells. Ignoring the standard deviation, levels of progesterone raised by α+βHSA were almost half of positive control denoting a probable negative effect of HSA fusion to β subunit. Similarly, Bai *et al*., 2006 described how a recombinant fusion protein containing granulocyte colony-stimulating factor (G-CSF) and transferrin (Tf) by inserting a small linker between the two protein domains suffered a loss of *in vitro* activity of 10% as compared to native Tf and G-CSF forms. Bai and co-workers designed larger spacers (linkers) between both proteins and obtained satisfactory results for G-CSF activity indicating an interference of the Tf domain with the G-CSF domain in the original fusion protein on the binding to its receptor[20]. In any case, is important to remark that α+βHSA still had a valuable capacity to stimulate progesterone production.

On the other hand, the construct α+HSAβ did not display *in vitro* activity. This lack of activity may be caused for the proteolytic cleavage into the aminoacidic sequence of HSAβ.

Second, the presence of α subunit, which until now had not been possible to detect, was confirmed. Since constructs βHSA and HSAβ without α subunit raised similar levels to negative control, might be concluded that not only α subunit was present, but it played an important role in LH receptor recognition. This result agrees with the assertion of Harold Papkoff in 1974 which reported the necessity of heterodimer formation for a full biological activity.

4.4 CONCLUSIONS

In this chapter, a set of vectors expressing different forms of recombinant hormone were constructed and their expression in X-33 *P. pastoris* was reported. Small scale affinity purification through HisTrap columns was set up and the *in vitro* activity of each construct was confirmed in MLTC-1 cells. The main conclusions obtained from this work are:

- A new fusion protein of PSMG fused with human serum albumin is able to elicit a strong follicle stimulating-like response on *in vitro* cultures of Leydig cells. These results confirm the correct formation of the heterodimer.
- A *P. pastoris* clone expressing the recombinant PMSG (α+βHSA) was selected for further scale up studies. By contrast, the clone expressing the construct α+HSAβ was rejected because its low activity *in vitro*, probably caused by its degradation.

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CHAPTER 5

Development of an upstream and downstream process for heterelogous production of rPMSG in P. pastoris

5.1 INTRODUCTION

This chapter describes the development of the cultivation of *P. pastoris* for production of recombinant PMSG. First, a series of batch fermentations were carried out in order to identify the best conditions. Subsequently, a fed-batch process was set up to produce enough quantities of the recombinant product used for *in vivo* efficacy tests.

5.1.1 BIOREACTOR BATCH CULTIVATIONS

In comparison with cultures in Erlenmeyer/shake flask, bioreactor cultivations allow to maintain controlled main environmental conditions such as the percentage of dissolved O2, temperature or pH. Additionally, cultivations in bioreactor not only permit to monitor these variables but also control them around optimal production conditions. Cultivations described in this section are carried out at low cellular concentration in order to determine the best conditions before proceed to high cell densities cultivations in which growth conditions are much more stressful for cells.

In chapter 4 is described how the rejected construct α+HSAβ is degraded from the beginning in shake cultivation. Similarly, the presence of proteases is suspected during cultivation of α+βHSA construct, but in this case the protein degradation does not affect the whole expressed protein.

With the objective to minimize the effect of the proteases during the cultivation of the α+βHSA construct, three different batch cultivations were designed with the objective to analyze independently two important parameters for protein stability, pH and temperature.

5.1.2 BIOREACTOR FED-BATCH CULTIVATIONS

In *P. pastoris* cultivations, fed-batch represents an important operational strategy since it allows achieving high cell concentrations and controlling the growth rate of the microorganism without notorious technical complications. An important requirement not achievable in shake flask cultures that must be considered in cultivations at high cell densities is that the growth on methanol requires high oxygen supply.

These high cell densities are accomplished under aerobic conditions because one of the substrates, usually carbon source, is administrated continuously at a rate acceptable for oxygen consumption. Differently, in batch cultures, where the substrate is added at the beginning, the oxygen concentration decrease exponentially because microbial can grow exponentially as well, becoming at the end a growth rate-limiting.

Typical features of a fed-batch culture are: quasy-steady state concentration of the limiting substrate and dissolved oxygen tension (DOT), slowly declining specific growth rate after an early and rapid augmentation, and an increasing of biomass concentration[1].

Several variants of fed-batch cultivation technique have been emerging lately because every protein usually needs an individualized improvement of its production due to its specific characteristics. For instance, Jahic *et al.,* 2003 developed the Temperature Limited fed-batch technique (TLFB) for control of proteolysis in *P. pastoris* bioreactor cultures[2]. On the other hand, Berdichevsky *et al.,* 2011 implemented an oxygen-limited cultivation that allowed an easy scale-up of process, but also reported higher concentrations of expressed recombinant monoclonal antibody, more homogeneous glycosylation and a reduction of antibody fragmentation[3].

Basically, fed-batch fermentation protocols usually include three different phases;

First phase consists of a batch growth on glycerol used to generate biomass while product formation is prevented due to repression of the AOX1, additionally growth rate

in glycerol is higher than in methanol. Growing first on glycerol signify that biomass can be generated without significant selection of those cells defective for heterologous gene, furthermore the cell death rate growing in glycerol is lower in comparison with methanol, avoiding the release of proteases to the medium.

Invitrogen Co. recommends an initial glycerol concentration of 40 $g \cdot L^{-1}$ which its total consumption will be detected by an abrupt increase of dissolved oxygen.

After the glycerol batch phase, a transition phase based on fed-batch of glycerol is initiated. Adjusting the feeding rate of glycerol, allows monitoring specifically the cell growth and therefore giving a suitable DOT for culture, avoiding limitations of the oxygen transfer capacity of the bioreactor. Additionally, when a Mut+ strain is cultivated under the growth-limited glycerol fed-batch, the AOX1 promotor is derepressed and the AOX activity, required for methanol oxidation, slightly increases. This procedure resulted in declining specific growth rate, due to declining residual glycerol concentration, but smooth transition to the subsequent methanol phase[4]. To avoid the declining of the specific growth rate, a glycerol exponential fed-batch phase is usually designed.

When the transition phase has been accomplished, begins the induction phase, where the methanol is added into the reactor using different strategies. The selected strategy is quite important since will vary in function of the phenotype, operational conditions and specific characteristics of the recombinant product. Since the residual methanol is the most important variable, it is important to monitorize it. If the on-line methanol concentration measurement was not possible, this should be monitored using the DOT electrode and feeding methanol at growth-limiting rate to keep a suitable constant DOT. However, at the beginning of induction phase, the AOX1 activity is still the growth-limiting factor, and the methanol concentration cannot be monitored by DOT

electrode, leading to a non-desired accumulation of methanol into the culture. This inconvenience can be avoided maintaining a low feeding rate, which often vary depending on the state of the culture.

In this chapter, the basis for a scalable process for the production of the rPMSG is explored. Different cultivation approaches are examined in order to prevent or reduce the proteolysis observed.

5.1.3 PROTEOLYSIS

Proteolysis has been described as one of the most important drawbacks in heterologous expression in yeast, especially in high cell densities cultures[5–8].

These proteolytic enzymes are also involved in essential roles inside the cell or in the extracellular environment. Proproteins processing, unwanted proteins degradation, inactivation of short-lived regulatory proteins or nutrients extraction from extracellular medium are some of these important roles.

Intracellular degradation of proteins is conducted mainly by proteasome and by vacuolar proteases; Proteasomes are very large protein complexes which main function of is to degrade unneeded or damaged proteins, such as missfolded proteins, by proteolysis. Proteasome system is conducted by 8-kDa peptide target, called ubiquitin, The tagging reaction is catalyzed by enzymes called ubiquitin ligases. Once a protein is tagged with a single ubiquitin molecule, this is a signal to other ligases to attach additional ubiquitin molecules. The result is a polyubiquitin chain that is bound by the proteasome, allowing it to degrade the tagged protein. Whereas in vacuolar system, proteins are transported to the vacuole and subsequently degraded by vacuolar proteases[9].

Vacuolar proteases can be classified in different manners. Endoproteinases are those which hydrolyse peptide bonds within polypeptide chain, and exopeptidases hydrolyse the carboxyl- or amino-terminal and are designed carboxypeptidases and aminopeptidases, respectively.

Furthermore, can be classified depending on its active site; serine, metallo-, thiol and aspartyl proteases. Serine proteases have a higher optimum pH and are called alkaline proteases, while aspartyl have lower pH and are called acid proteases.

5.1.3.1 The effect of proteases on secreted recombinant proteins

The secreted proteins can be proteolytically degraded in the culture by; extracellular proteases, cell-bound proteases and/or by intracellular proteases from lysed cells. However Mattanovich and co-workers determined that the wild type strain of *Pichia pastoris* DSMZ 70382 did not secrete any protease to the medium, at least growing on glucose[10].

Though several publications have described in *Pichia pastoris* the problem of secreted protein degradation, proteases in *P. pastoris* are not well characterized and no in-depth studies on the conditions that promotes the proteolysis are exactly known[11].

In *S. cerevisiae* the major source of proteases found in the medium are vacuolar proteases, followed by the proteasome and proteases of the secretory pathway[12]. Vacuolar proteases soluble within the vacuole are endoproteinase A and B, carboxypeptidase Y and S, aminopeptidase I and yscCo[9,13]. These proteases are non-specific and do not require ATP.

Degradation of the recombinant protein entails several problems such as the reduction of product yield, total or partial loss of biological activity when the protein is truncated and important complications during downstream processing because of similar

physic-chemical and/or affinity characteristics of the product and degradation intermediates.

5.1.3.2 Different approaches to control the proteolysis in *P. pastoris*

Several strategies to overcome the undesired proteolytic cleavage have been proposed in the literature. These strategies can be classified into three groups: Cultivation-level strategies, cell-level and protein-level. Some of these different approaches have been previously reviewed in Macauley-Patrick *et al*., 2005 and Jahic M. *et al*., 2003.[11,14]

1. *Cultivation approaches*. Different variations of physical parameters can be applied in order to control the proteolysis. For instance, setting the pH at 3,0 was optimal in the production of insulin-like growth factor and cytokine growth-blocking peptide[15,16]. In contrast, pH 6,0 was optimal in production of recombinant mouse epidermal factor and human serum albumin[17]. Notwithstanding, Invitrogen recommends to low the pH at 3,0 since the majority of *P. pastoris* proteases have its optimal pH around 5,5-8,0.

   Clare *et al*., 1991 found that product stability can be further enhanced by the addition of amino acid-rich supplements (e.g peptone, casaminoacids) to the culture medium[5]. Probably these amino sources repress the protease induction caused by nitrogen starvation or directly acting as a competing substrate for proteases.

   Reducing temperature has been another widely used alternative since the rate of proteolysis is lowered because of pure thermodynamic reason, increasing cell viability or enhancing protein folding[18]. In this way, Li and co-workers increased almost 4 times the amount of herring antifreeze proteins reducing the temperature from 30ºC to 23ºC, achieving in consequence an improvement of cell viability[19]. Jahic *et al*., 2003 applied a technique called temperature-limited fed-batch (TLFB). In these

cultures, classical methanol limitation is replaced by a temperature growth-limitation. Obtaining lower cell death, less proteases and increasing considerably the yield of a fusion protein[20].

Reducing the specific growth rate (μ) has also been reported as a successful tool. In this way production of hirudin was significantly improved maintaining a lower specific growth rate[21].

2. *Cell-level approaches*

Using vacuolar protease-deficient strains of *Pichia pastoris*, such as SMD1163 (his4 pep4 prb1), SMD1165 (his4 prb1) and SMD1168 (his4 pep4) has been proved to help reduce degradation[4]. Proteinase A (PEP4) and proteinase B (PRB1) are disrupted in these strains. Proteinase A is a vacuolar aspartyl protease required for the activation of carboxypeptidase Y and proteinase B. In turn, proteinase B has about half the activity of the processed enzyme before being activated by proteinase A. Thus, disrupting pep4, the activity of carboxypeptidase Y and proteinase A are eliminated, and a partial reduction of proteinase B activity. In a prb1 mutant only proteinase B is eliminated, and SMD1163 show substantial reduction of all three of these protease activities. Unfortunately, these protease-deficient strains seem that show lower viability, lower growth rate and are more difficult to transform.

A lot of other approaches modifying the genetic background of cells have been proved, some of them successfully. Disruption of kex1 gen in *Pichia pastoris*[22], mutant deficient yeast aspartic protease 3 (YAP3) in *S. cerevisiae*[23], disruption of YPS1 in *Pichia pastoris*[24] or simply combination of them usually report a total or partial reduction of proteolytic effect.

3. Protein level approaches

Specific single mutations in recognition sites of proteases can be an effective approach[25,26]. Eliminating these targets many proteases are no able to recognize these sites[27]. Similarly, Gustavsson and co-workers designed stable linker peptides for a cellulose-binding domain lipase fusion protein in order to decrease proteolysis in this point[28].

In this chapter, only cultivations approaches have been considered. In order to test if pH has a relevant effect on α+βHSA stability, an assay in 96-well plate has been designed and has reported clear results about the importance of this parameter. More, the repercussion of temperature and pH have been analysed independently in three different batch cultivations and the integrity of α+βHSA have been checked by western blots of the supernatants. Besides, the cytosolic fractions of the pelleted cells have been analyzed by western blot too, evidencing that temperature and pH have a relevant effect on the rPMSG. Subsequently, both parameters have been implemented in fed-batch cultivation and finally, a strategy with rich medium feeding has been implemented in fed-batch to reduce rPMSG degradation.

5.2 OBJECTIVES

The work described in this chapter has a major objective, to set up an adequate process for production of rPMSG with the appropriate quantity and quality to be used in the subsequent *in vivo* efficacy analysis.

5.3 MATERIALS AND METHODS

5.3.1 Strain and plasmid

A *P. pastoris* X-33-derived strain containing the expression vector pPICZαA-α+βHSA integrated in its genome AOX1 locus was used throughout this study.

5.3.2 Inoculum

Incoculum was produced in a 1 L shake flask with 100 mL buffered glycerol-complex medium (BMGY): Yeast nitrogen base without amino acids, 134 $g \cdot L^{-1}$; phosphate buffer (132 mL of 1 M K_2HPO_4 and 868 mL 1M KH_2PO_4), 100 $mL \cdot L^{-1}$; biotin, 400 $\mu g \cdot L^{-1}$; and glycerol, 10 $g \cdot L^{-1}$. Cells were grown for 16 hours at 30ºC on a shaker with 250 rpm.

5.3.3 Bioreactor cultivation conditions in batch

To analyze independently pH and temperature, the same protocol was applied to three cultivations; the initial OD_{600} was set up at 2, agitation was constant at 1000 rpm, the variables of O_2 loop control system was kept also immutable. More, once first batch were finalized with a 4 $g \cdot L^{-1}$ of methanol, it was initiated a second batch with 10 $g \cdot L^{-1}$ of methanol (figure 1). These two sequential batches were performed in order to obtain enough protein to be analyzed.

To reproduce the shake flask conditions the same culture medium BMMY was used scaling up the volume to 2 litres and controlling pH, temperature, dissolved O_2 and methanol concentration throughout methanol sensor.

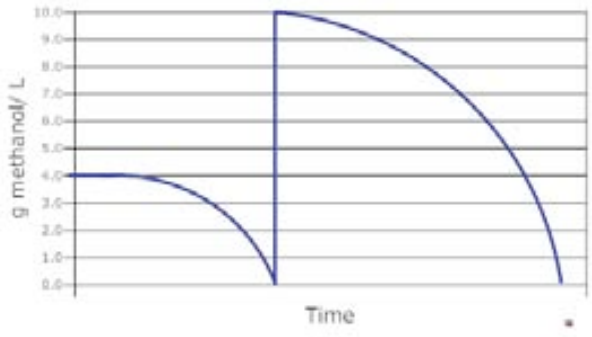


FIGURE 1. Simulation of different substrate addition phases in bioreactor cultivations in batch

Bioreactor batch cultivations were carried out at a working volume of 2 L in a 7 L bench-top bioreactor. Temperature was adjusted specifically to the conditions desired in any case. Similarly, pH was settled at desired condition and maintained by automatic addition of 30% NH_3 (addition of acid was not necessary). The duration of each batch was conditioned to the total consumption of methanol. The airflow was maintained at 2 $L \cdot min^{-1}$, assuring a minimum of 30% dissolved oxygen along the cultivation. The DO_2 control values were; P-gain 9.00, I-time 1500s, D-time 7s and the cycle time 5s.

5.3.4 Media composition in batch cultivations

Bioreactor batch cultivations were carried out using BMMY having the following composition: 1 % w/v yeast extract (Panreac, 403687), 2% w/v peptone (Panreac, 403695), 100 mM potassium phosphate, pH 6.0, 1,34% w/v Yeast nitrogen Base (YNB) without ammonium sulphate and amino acids (Sigma, Y1251), methanol was added as described in previous section (Panreac, 131091). This medium was supplemented with $1 \cdot 10^{-4}$ % w/v biotin (Sigma, B-4501). The YNB, biotin, and methanol components of both mediums were sterilised separately by microfiltration and then added to the bioreactor.

5.3.5 Equipment description

An autoclavable bioreactor of Applikon Biotechnology (Schiedam, The Netherlands) was used for the experiments (Figure 2A). The jacketed stirred tank of borosilicate glass and stainless steel had a capacity of 7 litres.

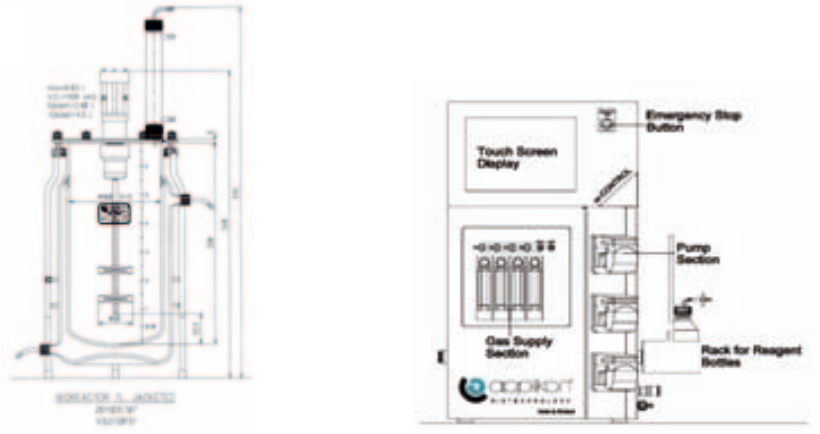


FIGURE 2. A. Scheme of jacketed stirred tank used during batch and fedbatch cultivations. B. Front view scheme of ez-Control Bio Controller

A B

The Applikon autoclavable Bio Reactor Systems basically consisted of the stirred tank described above and the ez-control which included the actuator console for aeration, liquid addition, agitation and temperature control (figure 2B).

The actuator console part of the ez-Control supported the following actuators:

- pH control: acid/base addition
- DO control: aeration with air, nitrogen or oxygen, stirrer speed control
- Temperature control: Thermo Circulator, cold water valve or heating blanket
- Level control: Level/Foam pump

For supervisory control and data acquisition was used BioXpert V2 program (Applikon Biotechnology) with expert system features developed for a real time on-line, and off-line data processing and fermentation process control.

A "low-drift" titanium membrane tip Applisens DO2 sensor (Z010007025) was used for measuring the partial pressure of dissolved oxygen in media. Additionally, an Applisens sterilizable pressurized gel electrode was used for pH control (Z001023551).

To measure methanol concentration an on-line methanol detector and sensor unit of Raven Biotech Inc.(Vancouver, Canada) was used. This sensor was designed to monitor methanol and other organic volatile compounds in the concentration range of 0.1-1.0 % v/v in aqueous solutions during the fermentation of yeast *P. pastoris*.

5.3.6 Intracellular protein analysis by glass beads disruption.

In order to analyze the recombinant hormone integrity in intracellular fractions the following protocol was applied: First the volume of culture necessary for having the same cell number (~$2 \cdot 10^9$ cells, considering that 1 OD_{600} for *P. pastoris* is equivalent to $2 \cdot 10^7$ cells/mL)[29] in each sample was calculated and subsequently centrifuged, the supernatant was discarded and cells were resuspended with 500 μL of PBS. Afterwards, cell suspension was centrifuged at 6000 rpm for 10 minuts at 4ºC and the harvested cells resuspended once again with 500 μL of PBS. This step was repeated three times in order to wash cells thoroughly. Last wash with PBS included 10 μL of protease inhibitor (Sigma, P8215) and 500 μL of glass beads were added to the tube. The tube was vortexed for 10 min at 4ºC and finally centrifuged, after the centrifugation the supernatant containing the intracellular fraction was ready to be analyzed.

5.3.7 Detection of α+βHSA degradation in the culture supernatant

In order to observe the proteolytic degradation of the recombinant product an experiment was carried out. Previously produced α+βHSA in batch at pH 5,5 and 30ºC, was purified by affinity chromatography using the HisTrap FF columns. Later, the purified construct α+βHSA was diluted to 2 μg·mL^{-1} with 0,04 M Britton-Robinson's buffer adjusted to various pH values (ranging from pH 3,5 to 6,5) and it was used to detect degradation. The flowthrough sample from the affinity purification was used as the protease solution. The flowthrough (5 μL) was mixed with the α+βHSA solution (5 μl) (at a ratio of 1 part flowthrough: 1 part α+βHSA v/v) and the final volume was adjusted to 50 μL with buffer. The reaction mixtures were incubated at 30ºC for 120 hours in 96-well microplate. Samples were taken from the reaction mixture every 20 hours and stored at -20ºC for SDS-PAGE analysis. The intensity of the band was quantified by the free-software ImageJ (National Institute of Health, NIH).

5.3.8 Medium and cultivation conditions in fed-batch cultures

Shake flask cultures were used to inoculate a volume of 3 litres of fermentation medium: H_3PO_4 85%, 26.7 mL·L^{-1}; $CaSO_4 \cdot 2H_2O$, 0.93 g·L^{-1}; K_2SO_4, 18.2 g·L^{-1}; $MgSO_4 \cdot 7H_2O$, 14.9 g·L^{-1}; KOH, 4.13 g·L^{-1}; Glycerol, 40 g·L^{-1} and trace salts solution, 4.3 mL·L^{-1} of the fermentor medium. The trace salts stock solution contained: $CuSO_4 \cdot 5H_2O$, 6 g·L^{-1}; KI, 0.8 g·L^{-1}; $MnSO_4 \cdot H_2O$, 3 g·L^{-1}; $NaMoO_4 \cdot 2H_20$, 0.2 g·L^{-1}; H_3BO_3, 0.2 g·L^{-1}; $CaSO_4 \cdot 2H_2O$, 0.5 g·L^{-1}; $ZnCl_2$, 20 g·L^{-1}; $FeSO_4 \cdot H_2O$, 65 g·L^{-1}; biotin, 0.2 g·L^{-1}; H_2SO_4, 5mL·L^{-1}.

Fed-batch cultures were performed in a 7 L bench-top bioreactor (Applikon Biotechnology, Schiedam, The Netherlands), with an initial working volume of 3 L. The agitation, pH, temperature, dissolved oxygen tension (DOT), pressure, air-flow, oxygen flow rate, micro burettes feeding rates, and antifoam addition were automatically

controlled. The specific set-points for pH and temperature were described individually for each of the fermentations. The pH was controlled by addition of 30% NH_3. DOT was controlled in cascade at the level of 20-30% air saturation by agitation up to 1200 rpm with aeration rate 2 $L \cdot min^{-1}$ and by feeding with pure oxygen when the rpm reached the maximum 1200. Foaming was automatically controlled by means of a level electrode and antifoam A (A5758, Sigma-Aldrich). The initial OD_{600} for cultivations was 0,5 and the inoculums were previously growing with BMGY in a shake-flask overnight.

As it was described in the introduction, the cultivation process comprised three phases:

Firstly, the batch growth phase was performed using glycerol (40 $g \cdot L^{-1}$) as a sole carbon. A cellular yield of 25 to 42 $g \cdot L^{-1}$ dry cell weight was expected for this stage (about OD_{600}=80). When the glycerol was consumed after approximately 25 hours, a feed containing 550 $g \cdot L^{-1}$ glycerol and 12 $mL \cdot L^{-1}$ trace salts solution was started. Except for those cultures with exponential feeding rate, a constant feed rate of 908 $\mu L \cdot min^{-1}$ was added to the reactor by an automatic micro burette MicroBU-2031 from Crison Instruments (Alella, Barcelona, Catalunya). After 4 hours of glycerol fedbatch, when cell concentration was approximately 50 to 62 $g \cdot L^{-1}$ wet cells (about OD_{600} =160), the glycerol feed was replaced with a feed containing 100% methanol and 12 $mL \cdot L^{-1}$ of trace salts. The initial feed rate was 0,180 $mL \cdot min^{-1}$ and during the first 2 hours, methanol will accumulate in the fermentor. After two hours of methanol feeding the cells have completely synthetized the enzymes for methanol degradation. It would take approximately two more hours to consume the accumulated methanol in the medium. After these two hours, the methanol concentration in the culture is limiting, and the feeding rate was maintained for at least 1 hour before doubling the feed to 0,360 $mL \cdot min^{-}$

^{1}. After 2 hours at 0,360 mL·min^{-1}, the feed rate was incremented again to 0,545 mL·min^{-1}. This feed rate was then maintained throughout the rest of the fermentation.

5.3.9 Pre-programmed exponential feeding rate in glycerol fed-batch phase.

For a smooth transition to subsequent methanol phase a slight increase of AOX activity in this phase was tested for the fed-batch in rich medium. M. Jahic simulated a *P. pastoris* glycerol-limited chemostat culture using data from Veenhuis *et al*., 1983 and concluded that residual glycerol concentration was very low at dilution rates below 0,2. On the other hand, Veenhuis *et al*., showed that when the methylotrophic yeast *Pichia angusta* was grown in a glucose-limited chemostat culture, the AOX activity in the cell increased with lower dilution rate due to the repression/derepression regulation mechanism of AOX. With the dilution rate lower than 0,2, the AOX activity did not increase[11,30]. Using these data a exponential glycerol fed-batch phase with a constant specific growth rate of 0,18 h^{-1} was designated.

If a quasi-steady state was assumed for the residual substrate concentration, the specific growth rate of the culture at time (t) can be obtained from fed-batch substrate balance (eq.1).

$$(t) = \frac{Y_{x/s} F(t)\, S_0}{V(t) X(t)} \qquad (1)$$

By integrating the fed-batch cell mass balance (eq.2) and combining it with equation 1, the feeding rate F(t) for a fixed specific growth rate under growth-limiting conditions can be expressed by equation 3.

$$X(t)\, V(t) = X(t_0)\, V(t_0) \exp(\quad (t \quad t_0)) \qquad (2)$$

$$F(t) = \frac{\left[X(t_0)\,V(t_0)\right]}{Y_{X/S}\,S_0}\exp\left[\quad(t \quad t_0)\right] \qquad (3)$$

This feeding rate equation can be applied if V, X and $Y_{X/S}$ are known at t_0 and the yield can be assumed as a constant along the fermentation. At start of fed-batch phase, t_0, the biomass concentration is around 25 to 42 $g{\cdot}L^{-1}$ wet cells (OD_{600} around 80) the culture volume is 3 L, and the glycerol feed concentration wass fixed at 550 $g{\cdot}L^{-1}$. The biomass/glycerol yield was considered to be constant at 0,5 g X·g $glycerol^{-1}$ under these equation.

For programming reasons, the feeding rate (eq.3) was expressed as a function of the previously added feed rate in equation 4. In this way, the feed rate (F) did not depend on the absolute time because the new addition rate can be expressed as the product of the previous feed rate and the exponential factor. The equation only requires the initial value of $F(t_0)$ when the induction phase starts and the time between two additions (Δt: 1 min).

$$F(t + \quad t) = F(t)\exp\left(\quad t\right) \qquad (4)$$

5.3.10 Analyses

5.3.10.1 Cell and protein concentration

The cell concentration was monitored by measuring the optical density (OD_{600}) at 600 nm. The total amount of protein present in the medium was analyzed according to Bradford (1976), after removal of the cells by centrifugation.

5.3.10.2 Methanol concentration in medium

To monitor on-line methanol concentration a methanol detector and sensor unit of Raven Biotech Inc. (Vancouver, Canada) was used. A calibration curve was obtained by adding increasing amounts of methanol to the bioreactor containing sterile culture medium. The methanol analysis was used to adjust the methanol feed rate to ensure there was no over-feeding during the AOX induction phase. This feeding was carried out by the micro burette MicroBU-2031 from Crison Instruments that dispense the proper volume in order to keep the set point of methanol concentration.

5.3.10.3 Western blot

Western blots were carried out according to the protocol detailed in material and methods chapter. Briefly, for the recombinant pregnant mare serum gonadotropin (rPMSG) detection, a mouse anti-His_6 antibody was used (11922416001, Roche) with a dilution 1:1000. As a secondary antibody an anti-mouse horseradish peroxidise labelled antibody was used with a dilution 1:20000 (ImmunoPure Antibody, Thermo Scientific, 31430).

5.3.10.4 Purification

Below is described the methodology for purification of rPMSG protein. Chromatographic separations were performed on an ÄKTA purifier 10 (GE Healthcare).

After fermentation the yeast cell were removed by a single step centrifugation at 5000 rpm for 30 minutes. The supernatant was then filtered through a glass microfiber filters (1820-055, Whatman). Later, the filtered supernatant was concentrated about 20 times by ultra-filtration (P3B010A01, Pellicon 2 mini-cassettes 10 kDa membrane, Millipore) and buffer changed with 2 L of 10 mM Tris-HCl pH 7.0. Previously to apply the concentrated to a HisTrap FF 1 mL column (17-5319-01, GE Healthcare), the sample

was adjusted to the composition and pH of the binding buffer (20 mM NaH_2PO_4, 0,5 M NaCl, 50 mM Imidazole, pH 7,4). Once the sample was applied to the column, it was eluted with a linear gradient of elution buffer (20 mM NaH_2PO_4, 0,5 M NaCl, 500 mM Imidazole, pH 7,4). Finally, the buffer of the eluate was changed to assay buffer (30 mM $Na_2HPO_4 \cdot 12\ H_2O$, 130 mM NaCl, pH 7,2) through a desalting column (HiTrap, 17-1408-01, GE Healthcare).

5.3.10.5 *In vitro* activity

The ability of rPMSG to elicit a biological response was demonstrated by determining its capacity to stimulate steroidogenesis by MLTC-1 Leydig cells (Chapter 3). Approximately $1 \cdot 10^5$ cells·mL^{-1} were cultured in growth medium (Gentamicin 50 μg·mL^{-1}, Horse serum 9%, Fetal Bovine Serum 4.5% and RPMI-1640) at 37 ºC in a CO_2 incubator for 2 days and then stimulated with the corresponding doses of commercial eCG (Foligon®) and rPMSG in 1 mL of assay medium (Horse serum 9%, Fetal Bovine Serum 4.5%, Bovine serum albumin (BSA) 0.1%, Metil-isobutil-xantina (MIX) 0.2 mM and RPMI-1640 for 8 hours under identical conditions. Progesterone secreted into the medium was estimated by commercial ELISA kit (Neogen, ref.402310). Samples and controls were tested in quintuplicate.

5.3.10.6 Flow cytometry. Cell viability staining

For viability staining a culture sample of 2 μL was diluted with PBS to 300 μL before adding 2 μL of propidium iodide (PI) 1 mg·mL^{-1} (Sigma, P4864) and samples were analyzed within 10 minutes. PI fluorescence was measured in triplicate through a 670 nm LP filter (FL3) in a XL z44284 cytometer (Cytometry facilities, Scientific Park of Barcelona).

In order to perform viability control, fresh samples from fed-batch were heated at 75ºC during 20 minutes. Different increasing concentrations of amphotericin to 80 $\mu g \cdot mL^{-1}$ were also used as a negative control. Controls were also analyzed in triplicates and were used to define a drawn rectangle in the plot which defined viable cells and non viable to quantify the percentage of viability.

5.4 RESULTS AND DISCUSSION

5.4.1 Methanol-limited fed-batch culture at pH 5,5 and 30ºC in defined medium.

In order to obtain the necessary amount of protein to perform *in vivo* bioactivity assays, a high cell density fed-batch process was carried out. The first approach was to use the standard growth conditions for *P. pastoris* at pH 5,5 and 30ºC. As discussed in the introduction, these conditions are more frequently used for protein production in *P. pastoris*[31]. Figure 4 shows the evolution of OD_{600}, methanol concentration, pH, DOT and temperature. After 25 hours of glycerol batch (B), began the transition phase (T) with fed-batch of glycerol and after 4 hours started the fed-batch of methanol as can be seen for the increase of methanol concentration.

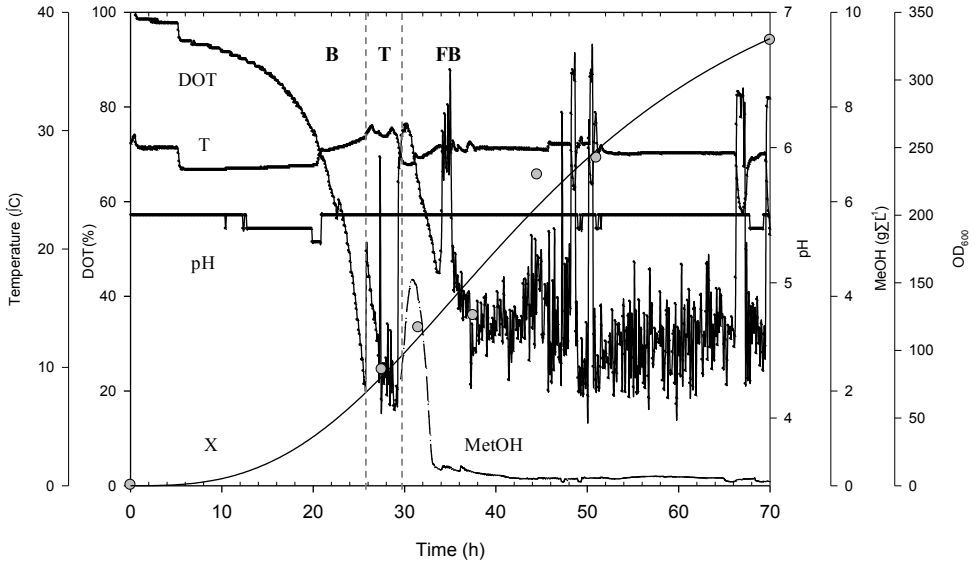


FIGURE 4. Temperature (T), OD600 (X), pH, Dissolved oxygen tension (DOT) and Methanol (MeOH) in a fed-batch cultivation with *P. pastoris* producing rPMSG at pH 5,5 and 30ºC.

The induction phase lasted around 40 hours, reaching a final OD_{600} of 329. The methanol accumulates during first 2 hours until 4,5 g methanol· L^{-1}, after this point the concentration drop drastically to negligible values, in this point the concentration of methanol was limiting.

During cultivation, different samples were taken at different times. Figure 5 shows a silver stained SDS-PAGE showing the time course of the cultivation. After 45 hours of cultivation a thin band appeared and its intensity slowly increased as the culture progresses. On the right part of figure 5, a western blot shows the degradation present in samples took at 50 and 70 hours of cultivation. The band above 86 kDa coincides with the intact rPMSG, by contrast the rest of bands tagged with the antibody anti-6xHis apparently were the result of specific degradation in several points of the protein.

A quantifiable assay for protease detection was performed with the objective to detect the presence of proteases into the culture medium. Different dilutions of the

supernatant were assayed but in all samples the levels of proteases detected were insignificant (data not shown).

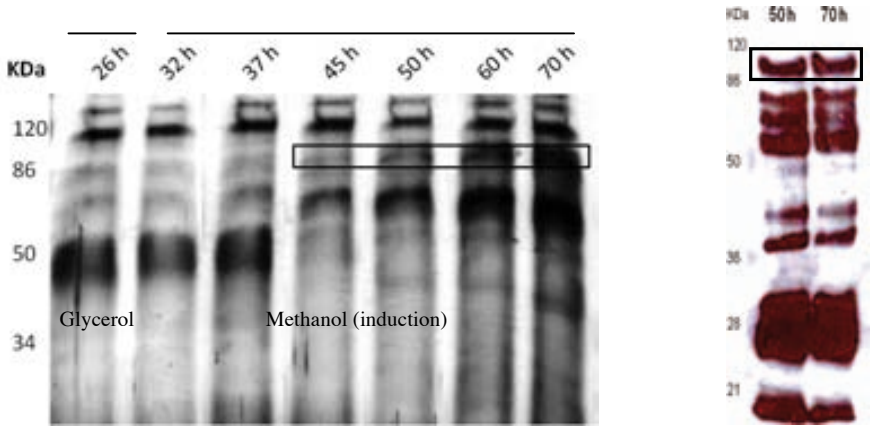


FIGURE 5. On the left a silver staining analysis of culture supernatants extracted at different times from the fedbatch cultivation. The black rectangle shows the appearance of rPMSG. In the right part, a western blot analysis (antibody anti-6xHis) of samples taken at 50 and 70 hours of cultivation. The black rectangle indicates the entire rPMSG while the rest of bands corresponded to fragments of the protein containing the C-terminal of HSA.

In conclusion, the rPMSG was produced in fed-batch fermentations, but unfortunately the degradation observed in shake-flask experiments was still happening in spite of having the grow conditions under control.

5.4.1.1 Purification of fed-batch at pH 5,5 and 30ºC

After 40 hours of induction the medium was centrifuged at 4ºC and a final volume of 2250 mL was recovered and the supernatant subsequently filtered to eliminate all cells and cellular debris still present.

The filtered supernatant was concentrated through tangential ultrafiltration (10 kDa) with Pellicon 2 system to 150 mL. Subsequently, the concentrated supernatant was

diafiltered with 2 litters of Tris-HCl pH 7.0 in order to leave the sample in a suitable buffer for the chromatography.

Before the affinity column step, the sample was adjusted to the proper concentration of Imidazole and the same pH of the binding buffer. Subsequently, the concentrated was applied to HisTrap FF 1 ml affinity column and the retained protein in the column was eluted with approximately 15% of elution buffer and 85% of binding buffer (Figure 6).

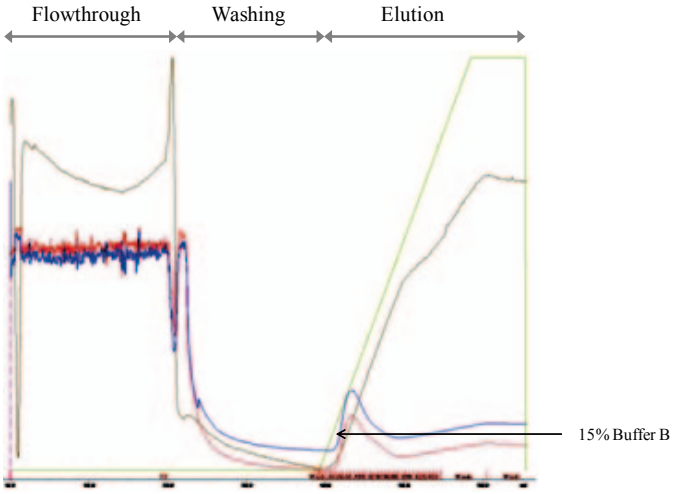


FIGURE 6. Chromatogram of affinity chromatography using a 1 mL HisTrap® FF column (GE Healthcare). The absorbance at 280 nm is shown in blue and at 254 nm in red. The conductivity is shown in brown while in light green is displayed the increase of elution buffer during the elution.

The flowthrough and fractions were checked in a coomasie-stained SDS-PAGE (figure 7). The major part of the rPMSG was retained into the affinity column and was properly eluted within fraction from A5 to B5. Similarly, the degradation fragments of the protein were also purified since they contained the 6xHis tail as well. It is important to note the enormous amount of degraded protein present at the bottom of the gel. These

small fragments, smaller than 24 kDa, were appropriately detected in the western blot of figure 5.

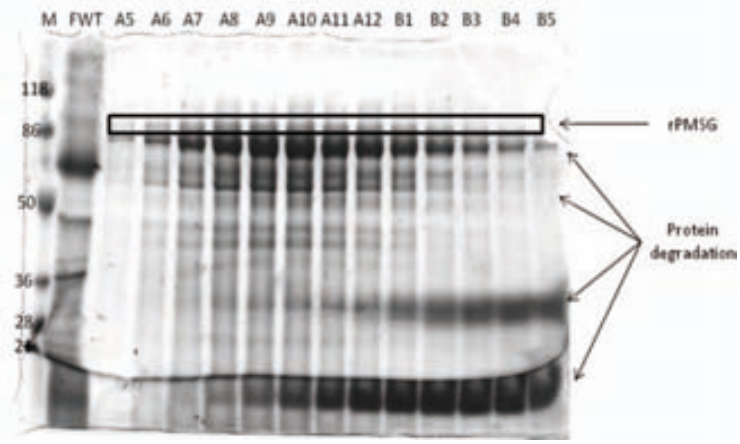


FIGURE 7. Coomasie-stained SDS-PAGE of flowthrough (FWT) and fractions purified by IMAC affinity column. The black rectangle indicates the rPMSG, while the rest of bands eluted at the same time correspond to degraded fragments purified due to the presence of 6xHis-tail.

Fractions from A6 to B1 were collected and poured together with a final volume of 4 mL. With the objective of eliminating the large amounts of imidazole present in the purified sample, which would be detrimental for in vitro and in vivo assays, a desalting step was carried out. For this purpose, 3 desalting columns HiTrap (GE Healthcare) of 5 mL were connected sequentially, since each column had a capacity for 1,5 mL. Buffer used to perform the desalting was the buffer described by the pharmacopeia (30 mM $Na_2HPO_4 \cdot 12\ H_2O$, 130 mM NaCl, pH 7,2) for rPMSG. The flow rate was 2 $mL \cdot min^{-1}$ and fractions of 0,5 mL were collected. In figure 8 the early elution of rPMSG can be followed through the absorbance at 280nm (blue), later the conductivity increased similarly indicating the change of buffer.

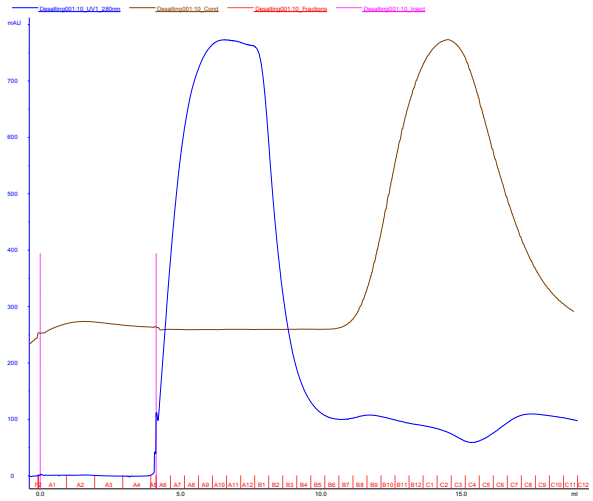


FIGURE 8. Chromatogram of the desalting step using three columns of 5 mL HiTrap® (GE Healthcare) connected in series. The absorbance at 280 nm is shown in blue (first peak). The conductivity is shown in brown (second peak).

Fractions from A5 to B5 were analyzed in a coomasie-stained SDS-PAGE (Figure 9). Finally all samples were gathered with a final volume of 6 mL and they concentration estimated in 420, 8 $\mu g \cdot mL^{-1}$ by Bradford assay.

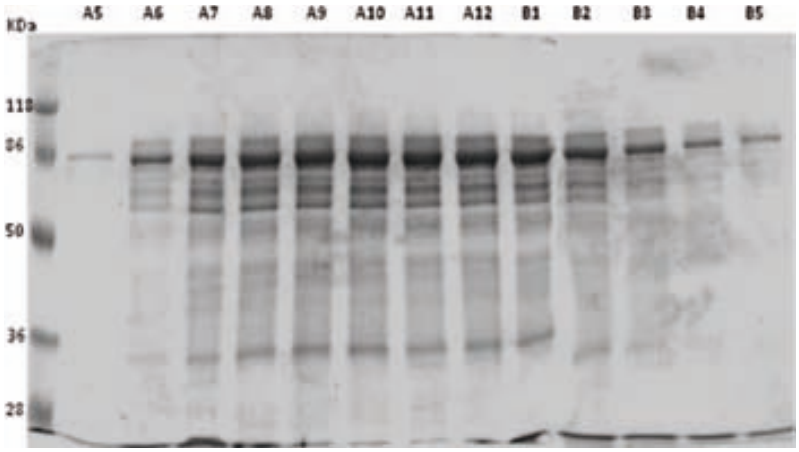


FIGURE 9. Coomasie-stained SDS-PAGE of desalted rPMSG with HiTrap columns.

Finally, it was demonstrated that only in two-step purification, the rPMSG can be purified from other proteins of the supernatant, but unfortunately it was not possible to purify from the rest of degradation fragments from itself, which in the end, make up the majority of the purified.

5.4.1.2 *In vitro* analysis of rPMSG produced in fed-batch pH 5,5 and 30ºC

Collected samples from desalting column were assayed in MLTC-1 cells as is described in materials and methods. Two different concentrations of purified rPMSG were assayed; 10 μg·mL^{-1} and 1 μg·mL^{-1} of rPMSG. The activity detected for 10 μg·mL^{-1} rPMSG represented a 77.4% of the positive control (10 mIU·mL^{-1}, Folligon). By contrast, when 1 μg·mL^{-1} of rPMSG was tested no appreciable activity was found, achieving similar levels of progesterone to the negative control, which was only assayed media without hormone (Figure 10).

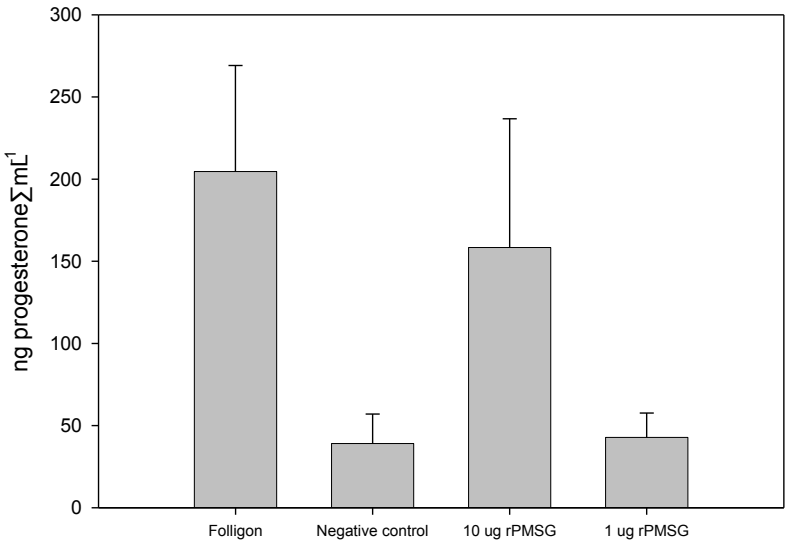


FIGURE 10. Levels of progesterone produced after induction with Folligon (commercial PMSG) and rPMSG at two different concentrations.

Therefore, despite the degradation of the protein, it was proved that the rPMSG produced in fed-batch at pH 5,5 and 30ºC still conserved an important part of its *in vitro* activity activating the production of progesterone by MLTC-1 cells to similar levels of commercial PMSG.

5.4.2 Determination of proteolytic degradation conditions

Based on the experiment reported on Kobayashi *et al*., 1999, a 96-well plate was used to perform the individual incubations at different pH and different times to reproduce the conditions in the culture, and study the effect of such conditions on the proteolytic degradation of recombinant product. The purified rPMSG was previously produced in a fed-batch cultivation at pH 5,5, 30ºC, and subsequently purified through affinity chromatography as described in materials & methods. Later, the recombinant hormone was incubated with the flowthrough from purification which may contain proteases released to the medium during the cultivation[32].

A sample was taken every 20 hours and analyzed in a coomasie-blue stained SDS-PAGE. Figure 11 shows how the pH affected enormously to protein degradation.

As observed in figure 11, at the time zero sample (marker as C in the figure) it appear two bands previously reported in chapter 4, the higher band just below 90 kDa matched with the recombinant hormone weight (~88 kDa). On the other hand, the lower band was described as a degradation product of fusion hormone since it can be detected in western blot.

At pH 3,5, the higher band corresponding to recombinant hormone was completely vanished after 20 hours and its proteolysis product was also almost consumed after 60 hours of incubation. Similarly, when the pH was increased to 4,5 the recombinant protein seemed to be degraded just after 20 hours but in this case the lower

band kept intact after 120 hours. At pH 5,5 not only the lower band kept intact but also after 20 hours of incubation the band concordant with fusion hormone was still quite visible. However, the best results were obtained when the purified protein was incubated at pH 6,5. At this pH the protein maintained its integrity utterly for the first 100 hours of preparation. Therefore, figure 11 clearly shows how low pHs had a big influence over product, potentially affecting the activity of proteases present in supernatant. On the other hand, not only protease-derived degradation might be the cause of the observed phenomenon but also protein instability under the tested conditions.

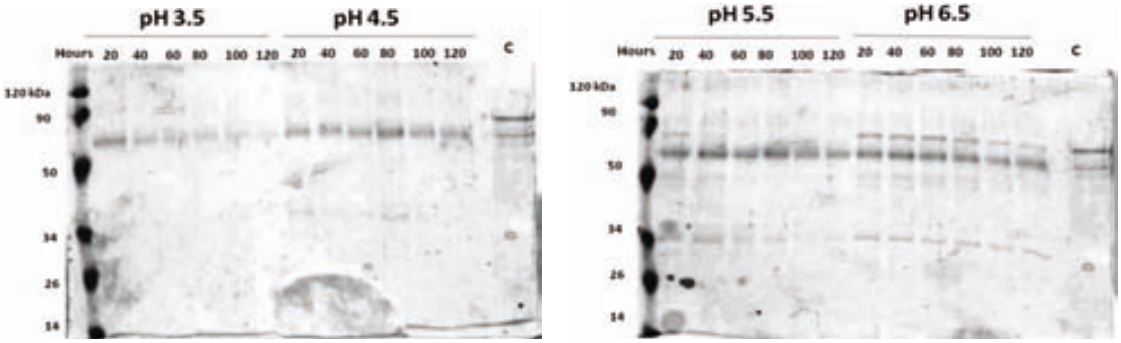


FIGURE 11. Coomasie-blue stained SDS-PAGE of samples taken every 20 hours for different pH. In the right part of gels (last well, C) is time 0.

In order to obtain quantitative data from this qualitative assay, the software Image J was used to analyze each the hormone degradation along the time[33]. Briefly, the entire lane corresponding to a specific time in gel is selected and the software displays an intensity profile as shows figure 12. Later, the area of the main peak was selected and subsequently integrated, giving a relative values which can be graph and contribute with

valuable information concerning with the relationship between the entire product and their degradation products.

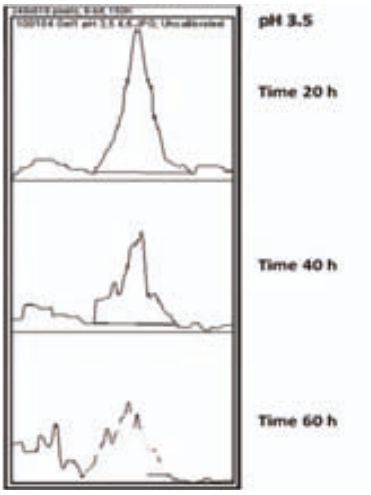


FIGURE 12. Example of a result obtained after the analysis of Image J program. At pH 3,5 can be observed how peak area decrease along the time.

As example in figure 13 the areas evolution of both bands at pH 5,5 and pH 6,5 along the time are displayed. At pH 5,5 the recombinant hormone (higher band) is degraded faster than pH 6,5 as it can be seen in the graph (bold symbols). By contrast, lower band which agree with the proteolysis product surprisingly increase at the beginning confirming that this band is feed by the degradation of the higher band. After 45 hours peak of this degradation product began to decrease progressively denoting that probably a mixture of proteases were found in the supernatant. In table 1 is shown the areas calculated of recombinant heterodimer at pH 5,5 and pH 6,5 and the percentage of residual protein along the time.

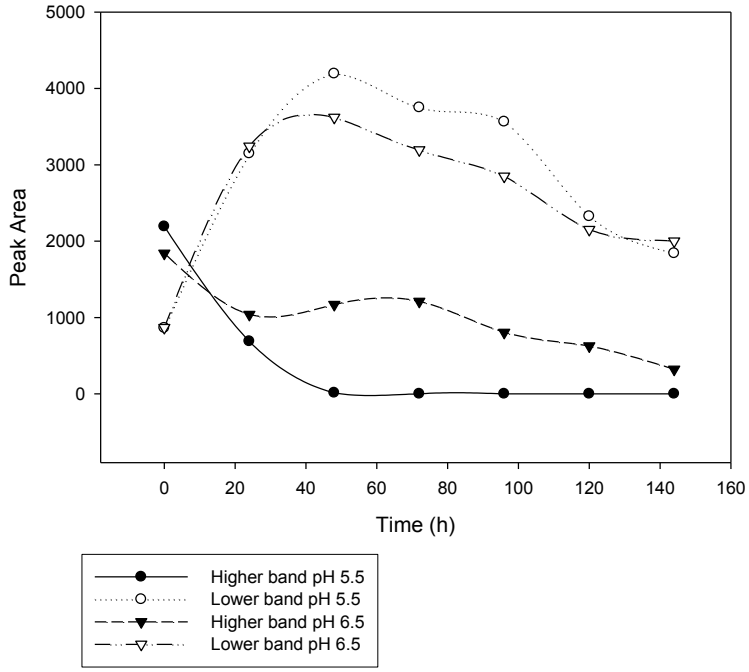


FIGURE 13. Area variation of peaks corresponding to α+βHSA (Higher band, bold symbols) and proteolysis product (Lower band, white symbols) at pH 5,5 and pH 6,5.

TABLE1. Percentage calculation of residual protein at pH 5.5 and pH 6.5. Percentage of areas of pH 3,5 and 4,5 are not presented because their values are significantly low.

	Area pH 5,5	Area pH 6,5
Time (h)	**% residual protein**	**% residual protein**
0	100	100
24	31,36	56,52
48	0,64	63,53
72	0	65,76
96	0	43,75
120	0	34,02
144	0	17,50

Looking at table 1 significant difference on protein stability can be seen, only pH 5,5 and pH 6,5 were compared because at lower pH (3,5 and 4,5) after 24 hours almost hundred percent of the heterodimer was totally degraded. Although at pH 5,5 the hormone can be quantified, after 48 hours almost the total amount of protein was vanished. Contrarily, at pH 6,5 the recombinant hormone still can be clearly quantified after 144 hours of incubation.

In conclusion, the pH was presented as an important parameter to control during cultivations in order to avoid the early degradation of rPMSG. Hence, working at pH 6,5 will be considered for further cultivations at higher cell densities.

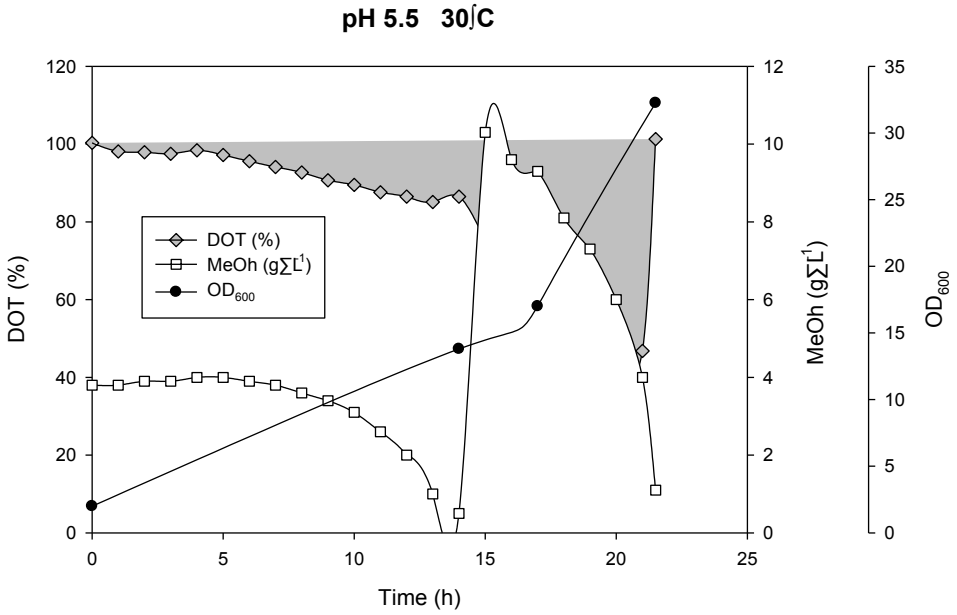
5.4.3 Comparison of batch cultivations at 30ºC. pH 6,5 and 5,5

Previous experiment based on rPMSG degration throughout incubation with supernatant, elucidated a clear effect of pH on recombinant hormone stability. At pH 6,5 the 65,76 % of the initial protein remained intact after 72 hours of incubation and after 144 hours a 17,5% was still quantifiable. The information provided by this experiment agrees with several publications which describe the augmentation of stability of human serum albumin expressed in yeast, basically *S. cerevisiae* and *P. pastoris* when the pH of cultivation is incremented to values higher than pH 6,0.[17,32,34]

Probably, this major stability of the recombinant protein at pH 6,5 is caused by the sensitivity of human serum albumin to acid proteases released to the medium from disrupted cells. Once the albumin is cleaved by any protease, then seems reasonably that the fusion protein become unstable and can be recognised by other proteases presents in the culture medium. Similar observation was pointed out in Jahic *et al.,* 2003, where the CBM-CALB fusion protein was affected not only for dissolved proteases, but also for cell-associated proteases[20].

Several batch cultivations were performed in bioreactor to confirm the effect of pH and temperature on product stability during cultivation. Figure 14 compares batch cultivations at pH 5,5 and pH 6,5. Basically the only difference was the final OD_{600} reached by the culture; at pH 5,5 OD was slightly higher than OD achieved by the culture at pH 6,5. Similarly, the oxygen and methanol consumption were analogous in both cultivations. Which suggest that tested differences on pH did not caused important variations on yeast growth. Two sequential batch of 4 $g \cdot L^{-1}$ and 10 $g \cdot L^{-1}$ of methanol can be followed by DOT.

The calculated μmax of second batch at pH 5,5 was 0,131 h^{-1} while the μmax of second batch at pH 6,5 was 0,154 h^{-1} which were similar with values described by Brierley *et al*., 1990 for the wild type strain[35], between 0,13 and 0,14 h^{-1}.



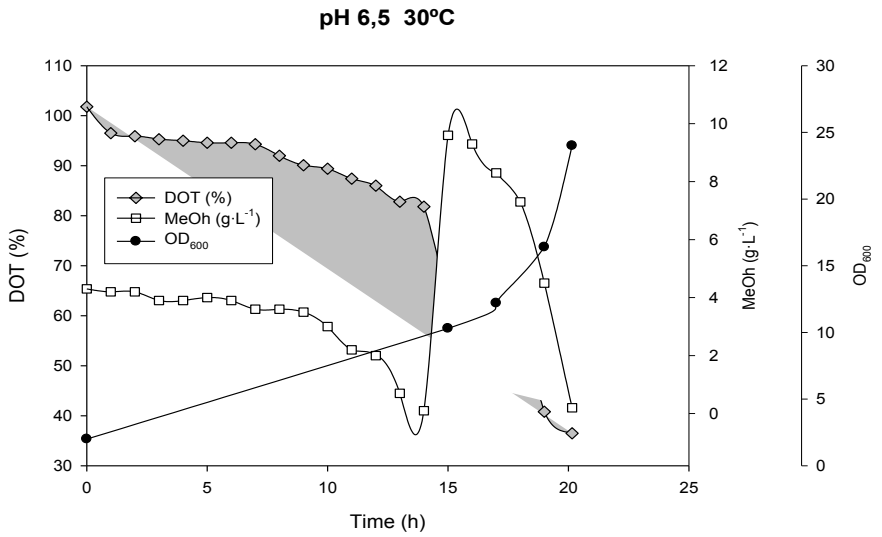


FIGURE 14. Comparison of bioreactor batch cultivations at different pH of *P. pastoris* expressing rPMSG in BMMY.

Several samples were extracted during the cultivations at different times and 10-fold concentrated, subsequently they were analyzed by western blot. Figure 15 compares the secreted and intracellular rPMSG expressed during cultivation at pH 5,5 and pH 6,5. The left side of the figure shows the secreted protein (supernatants) while in the right side; the western blot shows the cytosolic fraction. In spite of the supernatants did not exhibit any differences in the protein expression between both pH, it is worth noting a significant improvement in the quality of the protein coming from the cytosolic fraction at pH 6,5.

Despite this improvement, still degradation bands appeared in the intracellular fraction denoting that proteases may act on the rPMSG during the secretion pathway.

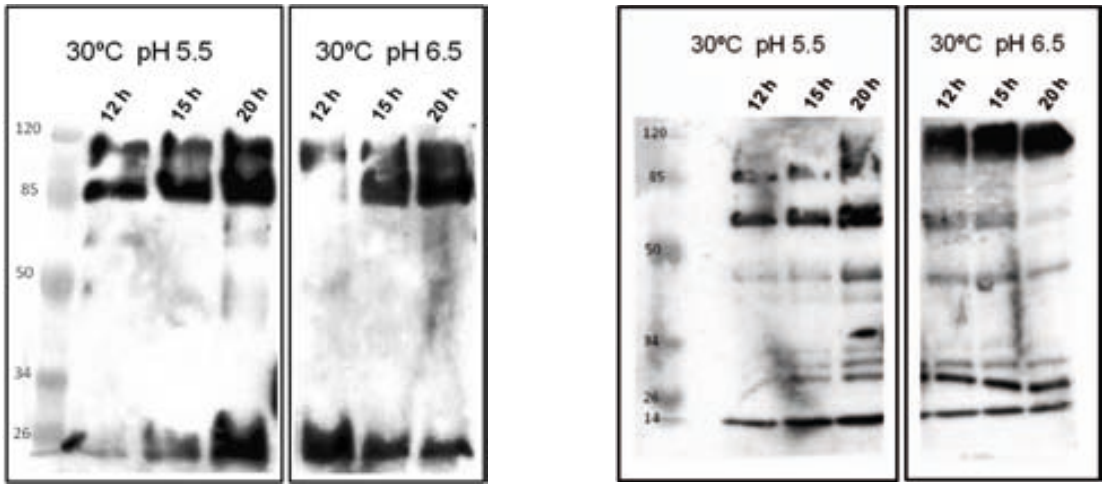


FIGURE 15. In the left side supernatants extracted at 12, 15 and 20 hours of cultivation and 10-fold concentrated. In the right side, the intracellular or cytosolic fractions of the same samples. Both analyzed by western blot.

The higher molecular weight of the rPMSG at intracellular fraction at 30ºC and pH 6,5 probably is caused by the presence of the α-factor secretion signal, which still was not removed.

Considering these results together with those obtained in the subsection 5.4.2, the use of pH 6,5 could result in an important improvement in the stability of the rPMSG during cultivations.

5.4.4 Comparison of batch cultivation at pH 5,5. 22ºC and 30ºC

Several publications reported that lowering temperature during *P. pastoris* cultivations may enhance the protein stability and therefore increase the final yield.[2,18–20,36,37]

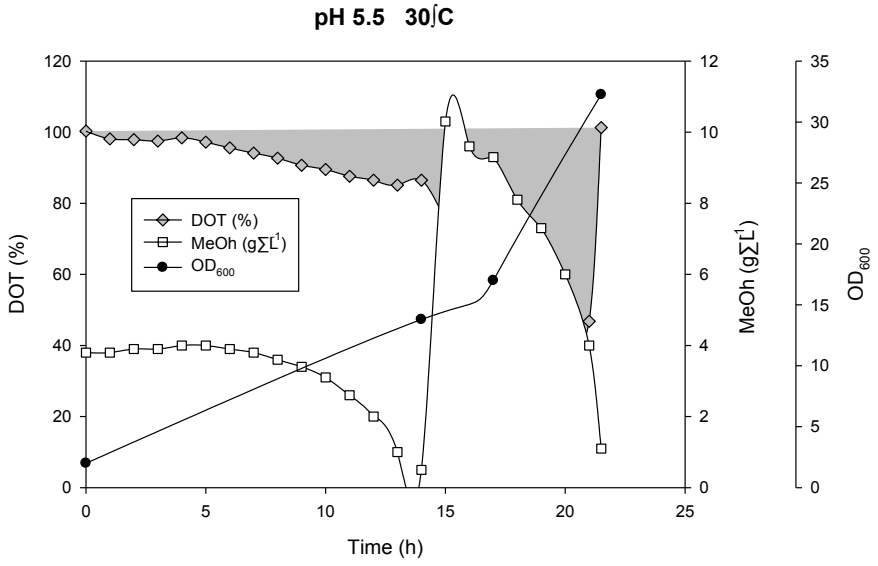
M. Jahic exposed that better yields can be attained reducing the temperature of cultivations with proteolysis problems, mainly for three reasons. First, pure thermodynamic reasons, at lower temperature the activity of proteases drop significantly. Second, at lower temperatures cell viability increment considerably and third, cells

growing at low temperature fairly reduce their protein synthesis machinery and the quality of these proteins is positively increased.

Since *P. pastoris* has the ability to grow at different acceptable specific growth rates (μ) depending on the temperature of the medium (from 15ºC to 30ºC), lowering the temperature, cell viability may be incremented and therefore the final protein yield may be augmented.

Figure 16 compares the effect of temperature in batch cultivations. As might be expected, lowering the temperature to 22ºC clearly affects the specific growth rate (μ). The μ_{max} at 30ºC was 0,131 while the μ_{max} at 22ºC drop to 0,095, which is similar to the value attained by Jahic *et al*., 2003 with a maximum specific growth rate of 0,105 h^{-1} at 22ºC, but in this case, the cultivation was on minimal medium[2].

Hence, the temperature has a decisive role on the yeast growth in BMMY medium, much more than pH which suggests lower importance.



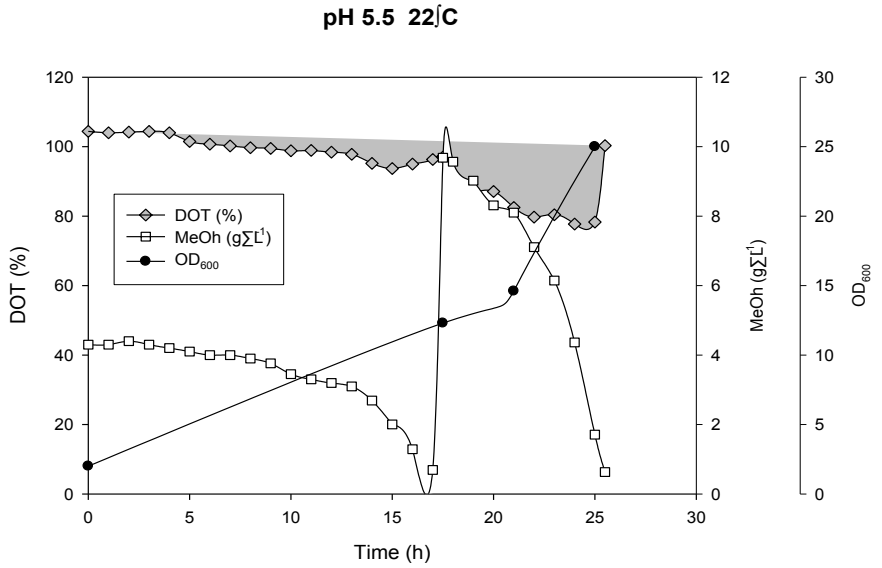


FIGURE 16. Comparison of bioreactor batch cultivations at different temperatures of *P. pastoris* expressing rPMSG in BMMY

Figure 17 compares the rPMSG secreted and the accumulated intracellularly during cultivation at 30ºC and 22ºC. The left side of the figure shows the secreted protein (supernatants) while in the right side; the western blot shows the cytosolic fraction.

Surprisingly, the supernatants did not show any noticeable difference between temperatures, and the quality of the protein secreted was very similar in both cases. But, similarly to the comparison between pH, when the cytosolic fraction was analyzed by western blot, significant variations were exposed. A unique and clear band of rPMSG appeared with no evidences of degradation, indicating that probably at 22ºC the intracellular activity of proteases was considerably reduced.

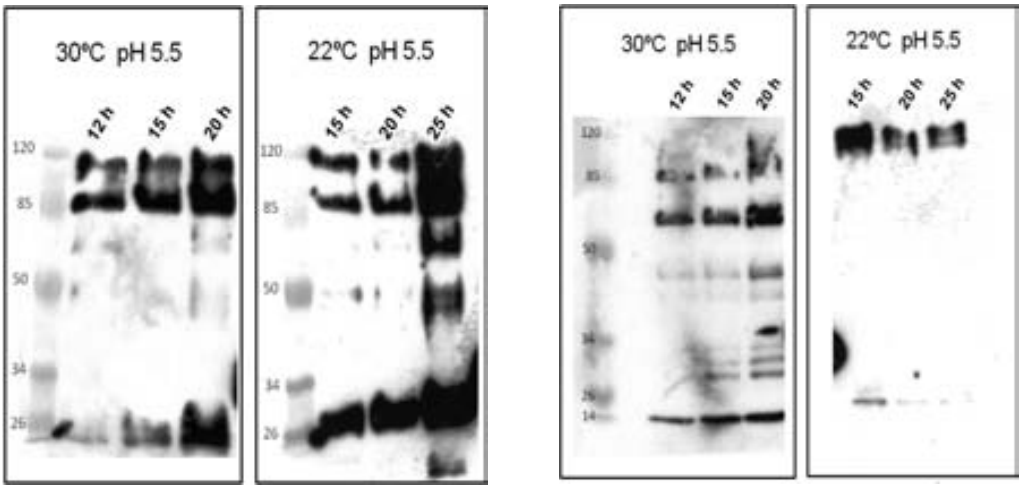


FIGURE 17. . In the left side supernatants extracted at 12, 15 and 20 hours of cultivation and 10-fold concentrated. In the right side, the cytosolic fractions of the same samples. Both analyzed by western blot.

Thus, lowering the cultivation temperature to 22ºC appeared as a good strategy to decrease the intracellular degradation of the protein.

The intracellular analysis of rPMSG pointed out that the hormone begins to be degraded during its secretion to the supernatant making it, at the end, vulnerable to the attack of no-specific proteases in the supernatant.

In conclusion, pH and temperature were defined as two important parameters for the expression of rPMSG. Despite the extracellular degradation was still evident, the stability improvement of intracellular protein may lead to higher yields of no-degraded rPMSG in the supernatant.

The data obtained from the batch experiments suggest that the best conditions were pH 6,5 and a temperature of 22ºC, the combination of such conditions was tested directly in a fed-batch culture.

5.4.5 Methanol-limited fed-batch cultivation at pH 6,5 and 22ºC in defined medium

Looking at the results obtained in batch, cultivations at pH 6,5 and 22ºC presented an improvement in the integrity of the intracellular rPMSG. For this reason, a fed-batch cultivation using both conditions was performed (figure 18). After 28 hours of glycerol batch (B), began the transition phase (T) with fed-batch of glycerol and after 4 hours started the fed-batch of methanol as can be seen for the increase of methanol concentration. During the transition phase, a progressively change of pH from 5,5 to 6,5 was accomplished in order to avoid a sharp increase of pH. On the other hand, the temperature was maintained at 30ºC during the fed-batch of glycerol with the aim to prevent a glycerol accumulation, but at its end and the beginning of methanol fed-batch was decreased to 22ºC. In figure 18 the temperature is not displayed because the ez-control unit could not maintain it and a cryostat unit was connected in order to keep the desired parameter.

With the aim to extent the cultivation time, at 96 hours, 3300 mL of broth culture were removed and stored at -20ºC for further purification (M1). On the other hand, at the end of fed-batch at 127 hours, the rest of broth culture (M2,1330 mL) was appropriately processed to be purified.

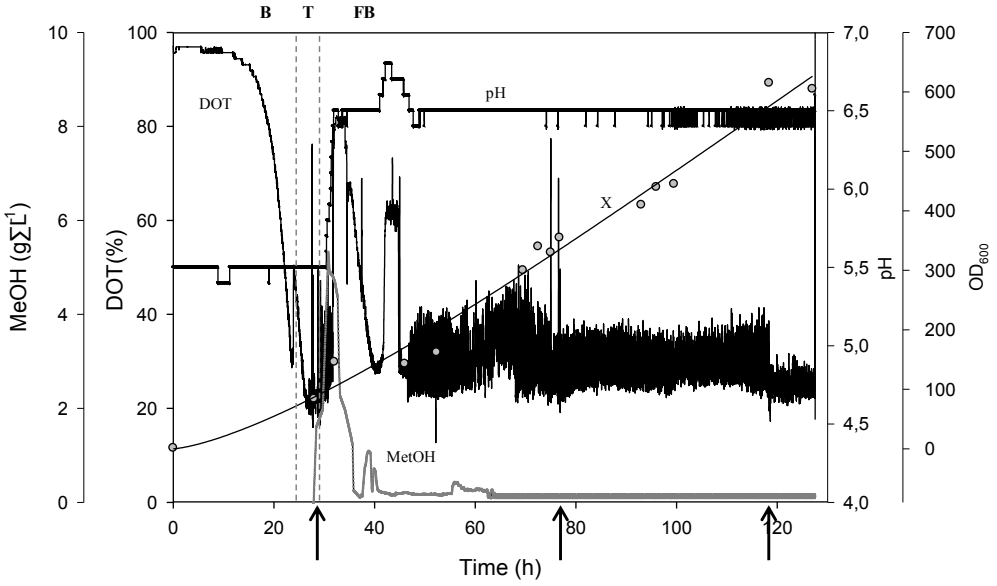


FIGURE 18. OD_{600} (X), pH, Dissolved oxygen tension (DOT) and Methanol (MeOH) in a fed-batch cultivation with *P. pastoris* producing rPMSG at pH 6,5 and 22ºC. Black arrows point out the addition of NH_4Cl.

In this culture, the induction phase was increased and lasted 96 hours, reaching a final OD_{600} of 605. In comparison with the fed-batch at pH 5,5 and 30ºC, levels of biomass were very similar at 70 hours achieving OD_{600} values around 300. The methanol accumulated considerably at the beginning until almost 5,5 $g \cdot L^{-1}$, but decrease after 8 hours to negligible values, in this point the methanol was limiting.

Kang *et al*., 2000 investigated the effect of nitrogen-source supplements on HSA degradation and clearly observed that cultivations with several nitrogen source or punctual additions of them into the culture noticeably minimized the effect of proteases on HSA[34]. Following these precepts, punctual additions, indicated in the figure 18 by black arrows, of two times 125 mL of NH_4Cl at 220 $g \cdot L^{-1}$ were added to avoid lack of nitrogen in the culture. The defined medium used for this fermentation, initially only contains enough nitrogen sources for 40 g $DCW \cdot L^{-1}$; for this reason, approximately every increment of 40 g of biomass were supplemented with 55 g of NH_4Cl.

The expression of rPMSG under these conditions was examined by western blot (figure 19). The time course of the culture shows how the proteolysis seriously affects the rPMSG. Until 100 hours of cultivation the band corresponding to rPMSG did not appear, while bands of degradation were evident from 70 hours of cultivation. One of the major bands, just below 50 kDa agree with a similar band of 45 kDa identified by Kerry-Williams and co-workers in recombinant HSA expressed by *S. Cerevisiae*. Kerry-Williams described how specific mutations in arginine 410, lysisne 413 and lysine 414 in the HSA sequence positively affect the integrity of the protein[26].

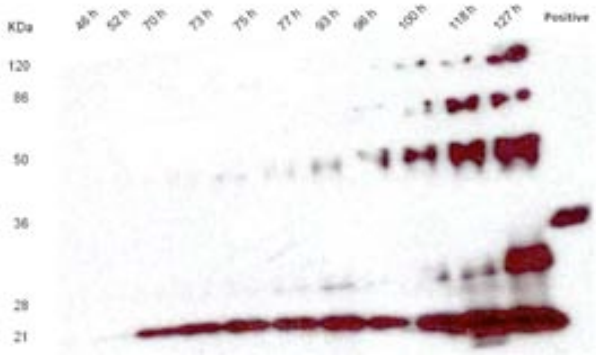


FIGURE 19. Western blot analysis (antibody anti-6xHis) of samples extracted at different times from the fedbatch cultivation at pH 6,5 and 22ºC.

Hence, the implementation of the determined pH and temperature in a fed-batch culture did not result in any important improvement related with rPMSG integrity. In order to evaluate if high-cell densities culture conditions could be stressful for cells as Hohenblum described in 2003, cell viability from fermentation samples were analyzed by flow cytometry[38].

5.4.5.1 Evaluation of cell viability by flow cytometry

Several studies proved that *P. pastoris* high cell density cultivations are susceptible to stress which may lead to decreased viability and increased cell lysis and therefore product proteolysis. Despite no significant extracellular protease activity was measured with the detection kit, the assay performed in 96-well microplate disclosed a progressive degradation of rPMSG at pH values below 6,5. For this reason, different samples from the induction phase of fed-batch were analysed by flow cytometry and results compared bibliographically.

In figure 20 the percentage of viable cells in cultivation maintained constant value of around 90% of viability; suggesting that rPMSG overexpression under the cited conditions did not have any deleterious effects on cell viability. These results agrees with those described by Jahic in 2003 in which cell death was low and constant and reached 4% dead cells after 150 hours of induction phase in methanol[2]. By contrast, Hohenblum reported in 2003 that viability decreased significantly below 70% during methanol fed-batch phase at 30ºC, indicating a stress situation triggered by the fermentation conditions.

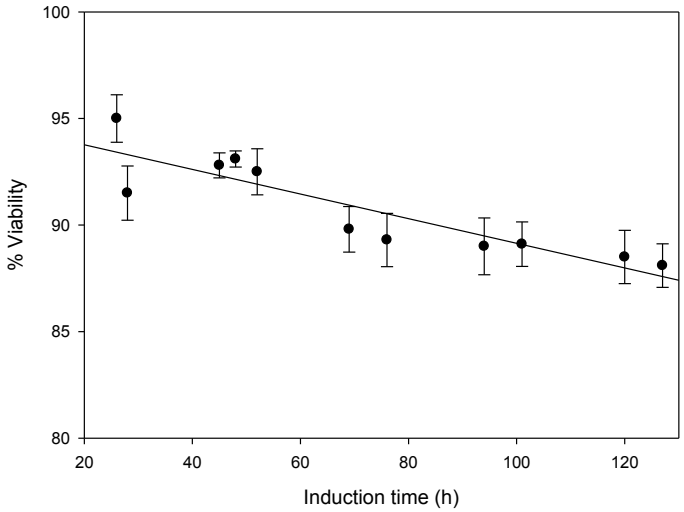


FIGURE 20. Percentage of viable cells in fed-batch culture at pH 6,5 and 22ºC.

In conclusion, these results suggested low levels of vacuolar proteases released to the culture medium by cell lysis when fed-batch cultures are performed under cited conditions. Hence, in agree with results obtained from intracellular western blots, it might be suggested that the product degradation takes place throughout the secretion pathway.

5.4.5.2 Purification of fed-batch pH 6,5 and 22ºC

As discussed above, at 96 hours of cultivation 3300 mL of culture broth were extracted (M1), centrifuged and the supernatant was filtered and subsequently concentrated to 130 mL and ultrafiltrated (10 kDa) with 2 liters of 10 mM Tris-HCl pH 7,0. Finally, it was stored at 4ºC with Complete EDTA-free (Roche, 04693132001) and 1 $mg \cdot mL^{-1}$ of pepstatin (Sigma, P5318-5mg) for 72 hours. The supernatant was not frozen in order to avoid freeze-thaw cycles which might affect rPMSG for further *in vitro* and *in vivo* experiments.

The rest of broth culture remaining into reactor (1330 mL, M2) were extracted at the end of cultivation (127 hours) and was processed independently of M1. After being centrifuged, the M2 supernatant was similarly filtered and subsequently concentrated to 150 mL and ultrafiltrated (10 kDa) with 2 litters of 10 mM Tris-HCl pH 7,0.

Thereafter, both batches M1 and M2 were processed independently, but exactly the same procedure was applied to each one.

Before the affinity column step, the samples were adjusted to the proper concentration of Imidazole and the same pH of the binding buffer. Subsequently, the concentrates were applied to HisTrap FF 1 ml affinity column and the retained protein in column was eluted with approximately 15% of elution buffer and 85% of binding buffer (Figure 21).

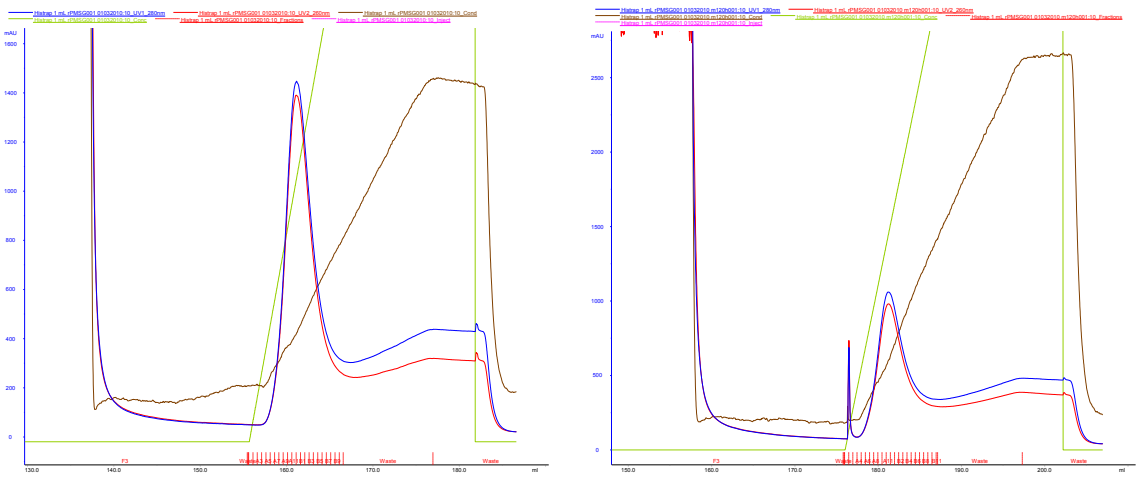


FIGURE 21. Chromatogram of affinity chromatography using a 1 mL HisTrap® FF column (GE Healthcare). The absorbance at 280 nm is shown in blue and at 254 nm in red. The conductivity is shown in brown while in light green is displayed the increase of elution buffer during the elution. Picture A and B correspond to M1 and M2 respectively.

Both purifications accomplished a unique and clear peak above the 1000 mAU eluted at 15% of elution buffer. The same fractions were selected for both purifications, having a final volume of 5 mL for each one. In both cases, the flowthrough and fractions of both purifications were checked in a coomasie-stained SDS-PAGE (figure 22). The eluted rPMSG appeared as an intense band arround 86 kDa in both SDS-PAGE. The diffuse bands which appeared above 86 kDa might be assumed as different degrees of glycosylation, since often glycoproteins migrate on SDS-PAGE as diffuse bands (figure 22).

The flowthrough in M1 and M2 samples was very similar, with an intense band around 50 kDa and another band with a molecular weight slightly lower than rPMSG present in the purified fractions. In order to elucidate if that band could be rPMSG, the flowthroughs were assayed *in vitro* together with the purified samples (next subsection).

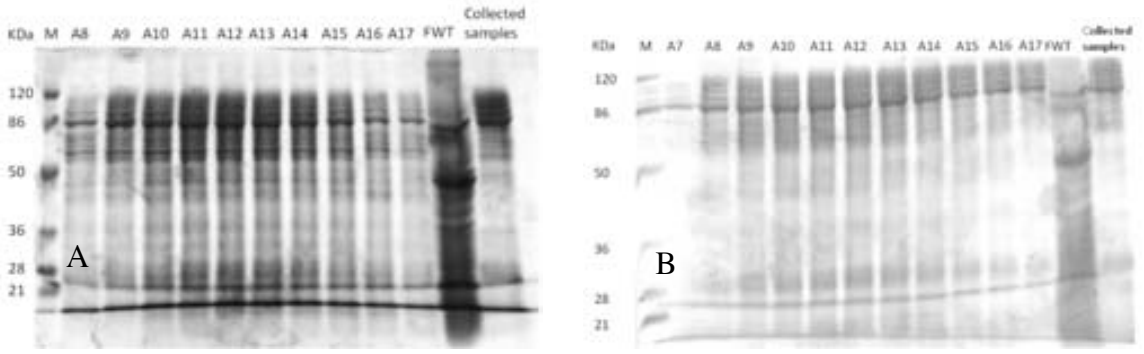


FIGURE 22. Coomasie-stained SDS-PAGE of flowthrough (FWT) and fractions purified by IMAC affinity column. A. M1 HisTrap purification. B. M2 HisTrap purification.

Large amounts of imidazole present into the collected samples were removed by connecting three desalting columns HiTrap of 5 mL. The buffer used to perform the desalting was 30 mM $Na_2HPO_4 \cdot 12\ H_2O$, 130 mM NaCl, pH 7.2 described by the pharmacopoeia. The flow rate was 2 mL·min^{-1} and fractions of 0,5 mL were collected. In figure 23 the early elution of rPMSG can be followed through the absorbance at 280nm (blue), later the conductivity increased similarly indicating the change of buffer.

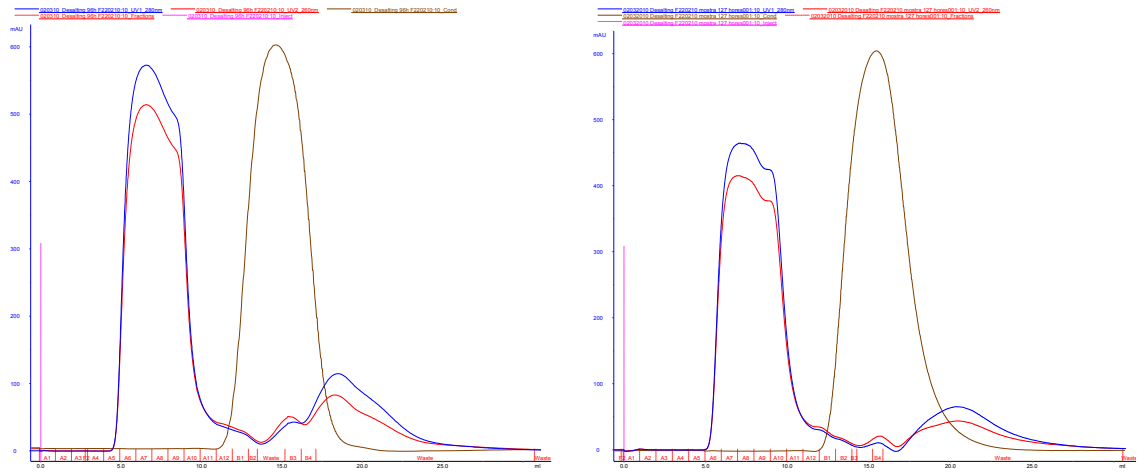
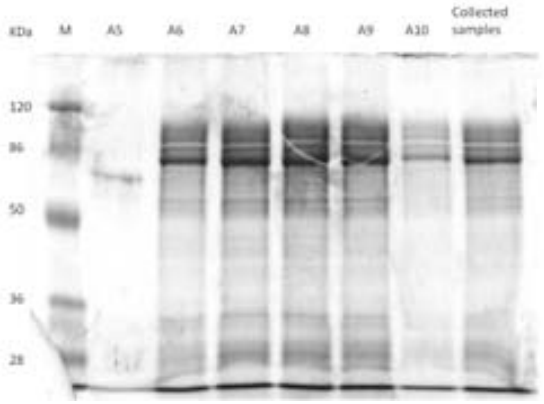


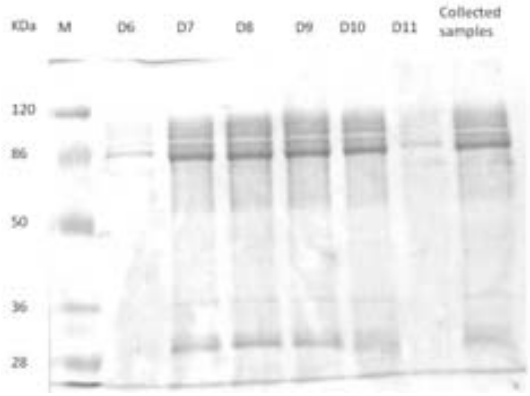
FIGURE 23. Chromatogram of the desalting step using three columns of 5 mL HiTrap® (GE Healthcare) connected in serie. M1 HisTrap purification. B. M2 HisTrap purification.

The six fractions, that appear in figure 24A and 24B for each SDS-PAGE, were gathered having a final volume of 6 mL for M1 and M2 (figure 24C). The rPMSG concentration was estimated by Bradford assay in 408,36 $\mu g \cdot mL^{-1}$ and 422,78 $\mu g \cdot mL^{-1}$ for M1 and M2 respectively. These concentrations of recovered protein were very similar to those achieved for the fed-batch at pH 5,5 and 30ºC (420,8 $\mu g \cdot mL^{-1}$ of rPMSG), but in that case, the induction phase only lasted 40 hours instead of 96 hours. Therefore, it may be assumed that new conditions could affect somehow the expression of rPMSG. However, for the fed-batch at pH 5,5 and 30ºC only 2,5 mg of total rPMSG could be recovered, otherwise for the fed-batch at pH 6,5 and 22ºC around 5 mg of rPMSG were successfully recovered.

A



B



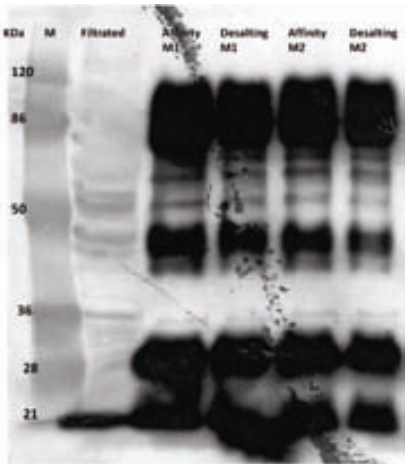


FIGURE 24. A.Coomasie-stained SDS-PAGE of desalted M1 sample with HiTrap columns. B. Coomasie-stained SDS-PAGE of desalted M2 sample with HiTrap columns. C. Western Blot anti-6xHis of affinity and desalting pools of M1 and M2 samples

5.4.5.3 *In vitro* analysis of rPMSG produced in fed-batch pH 6,5, 22ºC and minimal medium

In order to check if the rPMSG was active under these new conditions and they may contribute to improve the quality of the hormone, an *in vitro* assay was performed using the MLTC-1 cells. Collected fractions of sample M2 was assayed at unique concentration of 10 $\mu g \cdot mL^{-1}$. Furthermore, the flowthrough (FWT) from the affinity purification of M2 was assayed at the same protein concentration 10 $\mu g \cdot mL^{-1}$, in order to discover if traces of rPMSG were present.

As show figure 25, considerable levels of progesterone were surprisingly induced not only for the purified sample, but also by the flowthrough, indicating that probably traces of rPMSG did not bound to the column, or maybe some degradation fragment of rPMSG was able to induce the stereogenesis in MLTC-1 cells. Levels of progesterone achieved by both samples were similar to those obtained by the positive control (Folligon, 10 $mIU \cdot mL^{-1}$).

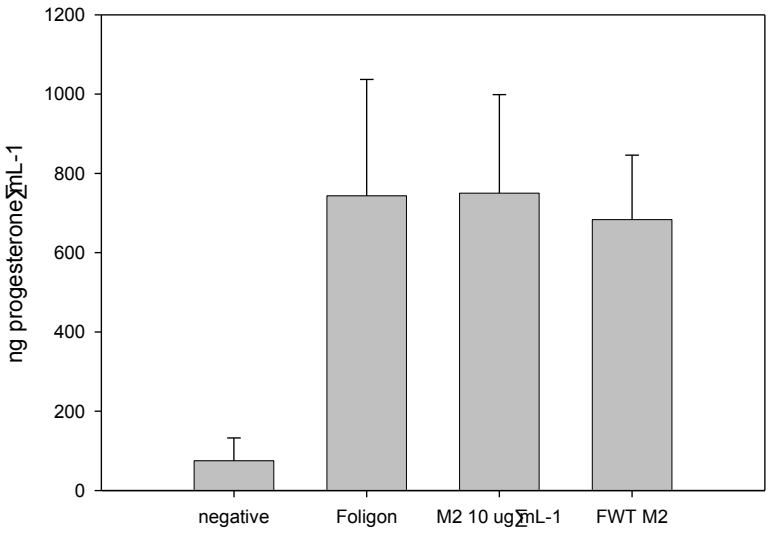


FIGURE 25. Levels of progesterone produced after induction with Folligon (commercial eCG) and rPMSG at two different concentrations for M1 and M2 samples. FWT correspons to the flow through from affinity purification.

These results were different to those obtained in the fed-batch at pH 5,5 and 30ºC, in which the rPMSG reached a 74% of the activity achieved by the native hormone. The levels of progesterone achieved during this assay for samples and controls were considerably higher for all samples, these differences pointed out the necessity to include the positive control in every assay in order to evaluate the extent of induction. Levels of progesterone induced for the rPMSG expressed at pH 6,5 and 22ºC were equal that those induced by Folligon, indicating the positive effect of new conditions. The improvement of biological activity in comparison with the results obtained for the fed-batch at pH 5,5 and 30ºC may be probably caused by the new process conditions that yielded in a larger fraction of non-degraded rPMSG.

5.4.6 Methanol Limited fed-batch fermentation at pH 6,5 and 22ºC in rich medium

The positive effect of using complex medium has been described in several articles. Complex media often contain yeast extract, peptone, casaminoacids or a defined cocktails of amino acids to help stabilize secreted proteins and prevent or decrease their proteolysis.[5,34,36]

Since all the attempted approaches to reduce the degradation of rPMSG did not succeed, another process approach was carried out. In this case, a continuous feeding of yeast extract and bacterial peptone in parallel to normal methanol feeding was implemented.

The batch phase for this fermentation was the same as previous cultivations, but in this case a 10 $g \cdot L^{-1}$ yeast extract and 20 $g \cdot L^{-1}$ peptone were added to the fermentation basalt salts medium (Invitrogen). The temperature was set at 30ºC in order to shorten this phase, but the pH was fixed at 6.5 from the beginning to avoid sharp changes.

First noticeable differences were found in glycerol fed-batch. Instead of feed with a constant rate as in previous cultures, a pre-programmed exponential feeding rate was applied. A feed containing 555 $g \cdot L^{-1}$ glycerol, 27 $g \cdot L^{-1}$ yeast extract, 54 $g \cdot L^{-1}$ peptone and 12 $mL \cdot L^{-1}$ trace elements solution was started. The initial feed rate was 432 $\mu L \cdot min^{-1}$ and the specific growth rate for the exponential was set up to 0.18 h^{-1}. Temperature and pH were the same of batch phase. After 4 hours, when the OD_{600} was approximately 80, the enriched glycerol feed was replaced with a step-wise transition to methanol phase. The feeding rates adapted were; first 128 $\mu L \cdot min^{-1}$ for 4 hours, followed by 2 hours at 256,8 $\mu L \cdot min^{-1}$, and finally a constant rate of 400 $\mu L \cdot min^{-1}$ was maintained until the end of the culture. This feeding solution contained 780,.6 $g \cdot L^{-1}$ methanol and 12 $mL \cdot L^{-1}$ trace elements solution. In parallel to the methanol addition, a constant feeding rate at 66 $\mu L \cdot min^{-1}$ of 108.6 $g \cdot L^{-1}$ yeast extract and 217.4 $g \cdot L^{-1}$ peptone was added during this phase.

In this stage, the temperature was decreased to 22ºC. The scheme of figure 26 show how were distributed the feedings. Antifoam and 30% ammonium were added when it was required by controller pumps; however methanol fedbatch and its enriched solution of yeast extract and peptone, and the glycerol feeding were all added through the microburetes. The operational scheme is represented in figure 26.

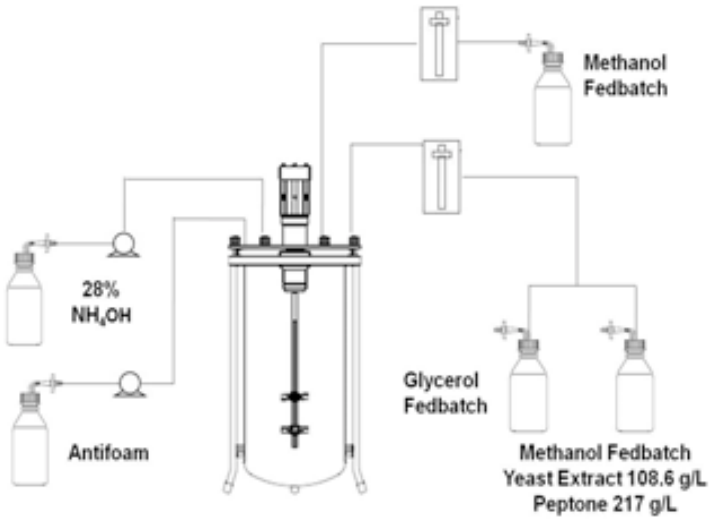


FIGURE 26. Scheme of the strategy followed to feed appropriately all solutions to the reactor.

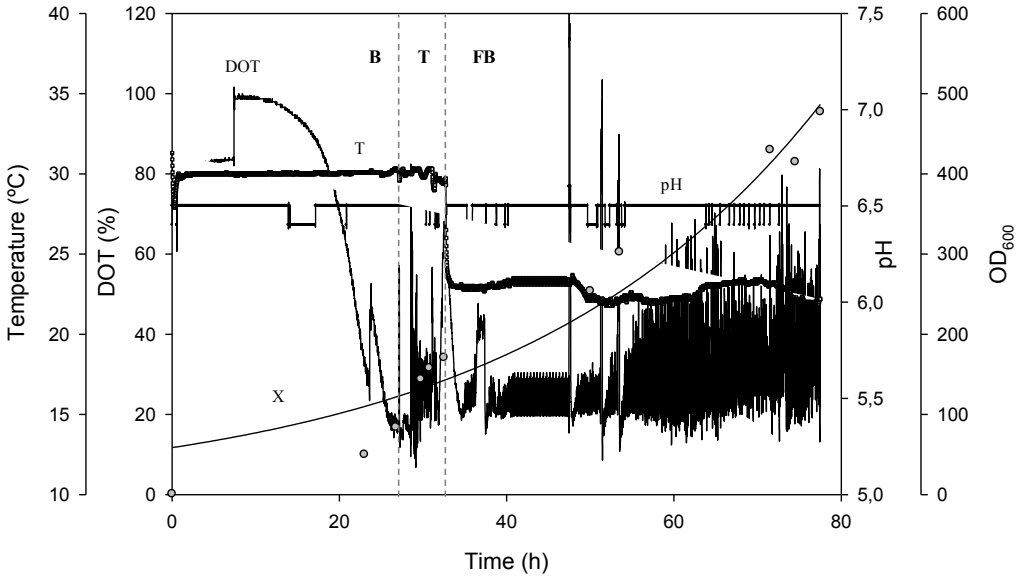


FIGURE 27. Temperature, OD_{600} (X), pH, Dissolved oxygen tension (DOT) and in a fed-batch cultivation with *P. pastoris* producing rPMSG at pH 6,5 and 22ºC in rich medium.

The presence of yeast extract and peptone into the initial medium in the batch phase did not affect the evolution of this stage, moreover despite the pH was set to 6,5 from the beginning, the length of this phase did not change significantly in comparison with previous cultivations. After 26,5 hours of the enriched glycerol phase (B), began 4 hours more of the transition phase (T) with glycerol, yeast extract and peptone. Finally, when fed-batch of methanol (FB) began, the temperature was decreased to 22ºC (Figure 27).

In this culture, the induction phase was very short in comparison with the cultivation in minimal medium, lasted 48 hours because of the difficulty to feed the concentrated rich solutions through microburetes. Thus, after 46 hours of induction the culture was stopped and the results were checked in SDS-PAGE and western blot.

The cell growth was affected by the presence of yeast extract and peptone into the medium reaching an OD_{600} of 477 after 48 h of induction in rich medium compared with a value of 360 obtained in the minimal medium cultures. This higher biomass may contribute to higher titers of rPMSG to the culture medium, but at the same time may enhance the levels of proteases released from dead cells (Figure 28). Moreover, some articles describes how the presence of rich amino acid sources into the minimal medium may brought out secretion of proteases from the host.

The expression of rPMSG with rich medium was examined by western blot (figure 28). Once again first signals in blot corresponded to degradation band instead of entire rPMSG. But in this case, they appeared sooner probably caused by the higher biomass achieved at that moment.

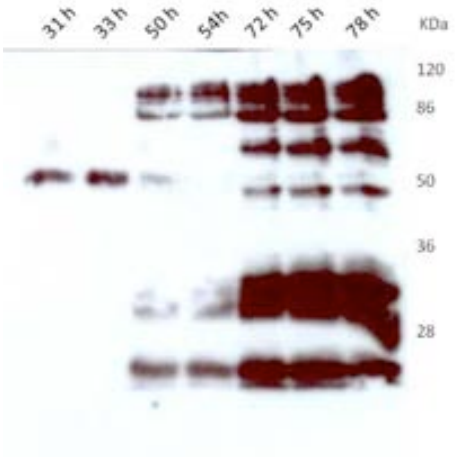


FIGURE 28. Western blot analysis (antibody anti-6xHis) of samples extracted at different times from the fedbatch cultivation at pH 6,5 and 22ºC and rich medium.

From the SDS-PAGE analysis it may be stated that the addition of rich medium for protease substrate competition had no effect on the proteolysis of rPMSG, such results

also strengthen the theory that degradation began inside the cell probably during the secretion pathway.

5.4.6.1 Purification of fed-batch at pH 6,5, 22ºC and rich medium.

After 48 hours of induction the culture was centrifuged in order to discard the cells and approximately 2250 mL of supernatant was recovered and subsequently clarified. The filtered supernatant was concentrated through tangential ultrafiltration (10 kDa) with Pellicon 2 system to 150 mL. Subsequently, the concentrated supernatant was diafiltered with 2 liters of Tris-HCl pH 7.0 in order to leave the sample in a suitable buffer for chromatography.

The concentration of Imidazole and the pH of the sample were adjusted before to apply to HisTrap FF 1 ml affinity column. Figure 29 shows elution chromatogram. As can be seen in the coomasie-stained SDS-PAGE (figure 29B), the elution fraction obtained from the column resulted very contaminated for other proteins. Probably, all this contaminant proteins are caused by the addition of peptone and yeast extract concentrates applied continuously to the culture.

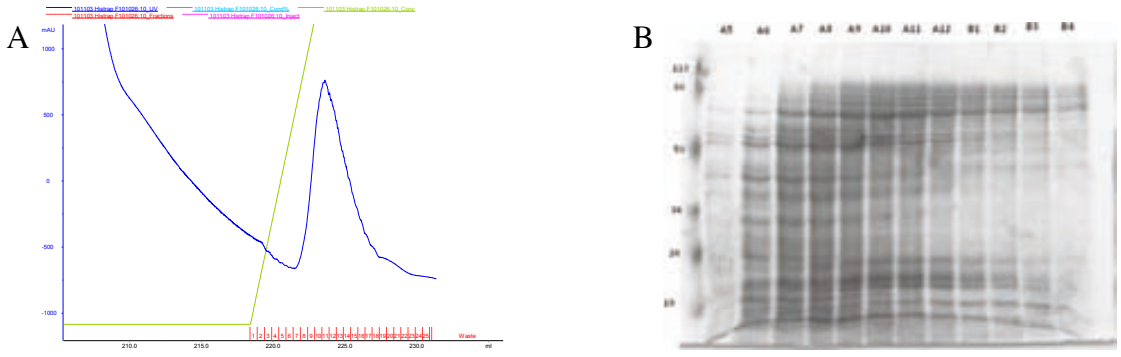


FIGURE 29. A.Chromatogram of rPMSG expressed in rich medium purified by affinity chromatography using a 1 mL HisTrap® FF column (GE Healthcare). B. Coomasie-stained SDS-PAGE of different fractions eluted in the affinity chromatography.

Fractions eluted from the affinity column were gathered together and were applied into three desalting columns HiTrap of 5 mL connected sequentially and eluted in buffer 30 mM $Na_2HPO_4 \cdot 12\ H_2O$, 130 mM NaCl, pH 7,2. The flow rate was 2 $mL \cdot min^{-1}$ and fractions of 0,5 mL were collected. In Figure 30A the low amount recovered non-degraded rPMSG can be observed. By contrast all the contaminants and proteolytic products appeared in the bottom of the gel. Despite this really low yield of rPMSG at the end of purification, western blot of figure 30B show a clear band around 86 kDa corresponding to rPMSG.

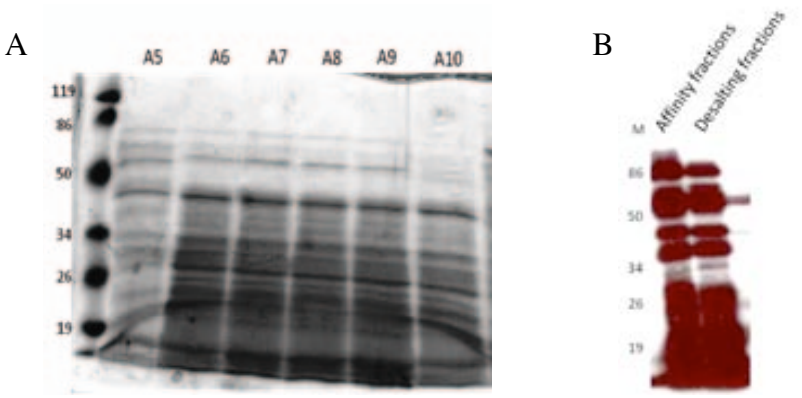


FIGURE 30. A. Coomasie-stained SDS-PAGE of fractions recovered in the desalting step (6 mL). B. Western blot analysis (antibody anti-6xHis) of pool samples obtained from affinity and desalting step.

The specificity of the primary antibody (Anti-6xHis) used in western blot analysis was tested in order to discard nonspecific bounds. In figure 31 a final sample of a fed-batch culture of *P. pastoris* expressing a recombinant enzyme was used as a negative control. The positive control is a recombinant protein with a 6xHis tail in its C-terminal, whereas the rPMSG sample was extracted 78 hours of fed-batch culture.

No unspecific bands appeared in the negative control, proving the specificity of the primary antibody.

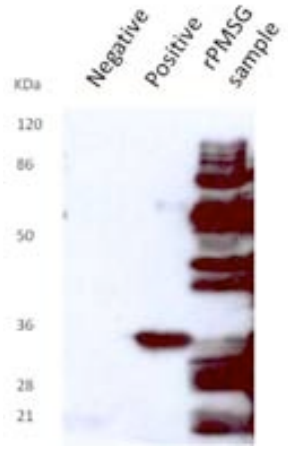


FIGURE 31. Specificity test of the primery antibody. The western blot proved its specificity.

The *in vitro* analysis of rPMSG produced during this fed-batch was not carried out because the objective of this operational approach was to eliminate or reduce substantially the proteolysis and that was not achieved.

5.5 CONCLUSIONS

In this chapter, high-cell density cultivation at pH 5,5 and 30ºC was performed with the aim to get enough amount of protein for *in vitro* and *in vivo* assays. After checking the noticeable degradation of rPMSG secreted under these conditions, the effect of pH was studied determining an optimal pH to avoid extracellular degradation of rPMSG. Moreover, another important parameter such as the temperature was also studied alone and in combination with the pH in batch cultivations, determining the best conditions to reduce intracellular proteolysis of rPMSG. Subsequently, once determined these favourable variables were implemented in fed-batch cultures with no apparent

improvements. Finally, another process approach using rich medium was implemented to reduce degradation by protease substrate competition with no positive effect in final yields of the protein. Main conclusions obtained from this work are:

- High-cell density cultivation in fed-batch was properly implemented at pH 5,5 and 30ºC, but a low yield of the entire rPMSG secreted could be detected, since almost all the protein expressed was present in different degradation bands. Despite this inconvenience, the rPMSG was purified and positively assayed *in vitro* with MLTC-1 cells.
- The best working pH to reduce the extracellular degradation of the rPMSG was found at pH 6.5, using the purified rPMSG as a substrate and the flow through of the affinity column as a proteases source. The assay, performed in a 96-well plate, demonstrated how after 72 hours of incubation, the rPMSG was completely banned at pH 5,5, while at pH 6,5 almost 66% of the protein remained integral. Therefore, evidencing that probably proteases released to the medium that affect the rPMSG were acid proteases.
- The pH 5,5 and pH 6,5 were compared in batch cultivations at 30ºC, and concluding that intracellularly, at pH 6,5 the state of rPMSG improved significantly, but unable to prevent the proteolysis at all.
- Temperatures at 30ºC and 22ºC were also compared in batch cultivations, establishing 22ºC as the best temperature to reduce the intracellular degradation of the rPMSG.
- These both parameters were implemented in fed-batch with unsuccessful results, but with a significant increase of the recombinant hormone bioactivity *in vitro* in comparison with the rPMSG produced at pH 5,5 and 30ºC.

- Cell viability analysis by flow cytometry suggested that rPMSG degradation mainly occurred during its secretion pathway inside the cell.
- Finally, a fed-batch with continuous addition of rich medium was implemented to reduce the proteolysis of the rPMSG. However, the yields of non-degraded protein recovered were as poor as previous approaches.

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CHAPTER 6

Evaluation of rPMSG efficacy in vivo

6.1 INTRODUCTION

Nowadays all hormones with veterinary applications are obtained from animal source; this often leads to commercial products with complex and indefinable mixtures of hormone isoforms. Normally this isoforms show different bioactivity in animals, and that is the main reason why in this moment *in vivo* assays with animals are still the only method recognized by the World Health Organization (WHO) and European Pharmacopoeia to measure biological activity.

Early studies used to detect FSH bioactivity were confusing and contradictory. A number of different *in vivo* bioassays, which were essentially based upon the effects of aqueous extracts of pituitary, placenta, or urine on the reproductive tracts of mice and prepubertal rats, were used. Finally, the assay developed in 1953 by Steelman and Pohley based on the stimulation of ovarian weight in gonadotropin treated immature rats, proved to be a robust specific *in vivo* bioassay for FSH activity[1,2].

Similarly, Parlow developed a method in 1961 to determine the bioactivity of LH through the depletion of ascorbic acid in the ovary measured after 4 hours. On the other hand, the pharmacopoeia describes that LH activity of PMSG is determined by the increase in the mass of seminal vesicles of immature rats.

However, Steelman-Pohley and Parlow bioassays have a number of important limitations; the assay's limited precision, its requirement for the sacrifice of large numbers of animals and its cumbersome data generation and interpretation procedures, which require highly controlled conditions and standardized procedures[3].

Therefore, ideally the existence of a source of homogenous recombinant hormones for veterinary purposes, such as the rPMSG, may be crucial for the substitution of this assay for other with fewer drawbacks.

In this chapter, the efficacy of rPMSG *in vivo* has been tested throughout the LH and FSH assays described in the European Pharmacopoeia, this pharmacodynamics assays reported an absence of activity of the recombinant hormone. For this reason, a pharmacokinetic study of rPMSG was conducted in rabbits and pointed out the rapid clearance of rPMSG from the blood-stream as possible cause of no activity.

6.2 OBJECTIVES

The objective in this part of the work is to characterize the behaviour of rPMSG *in vivo*, which is the potential effect of the product on model animals, pharmacodynamics; and also the response of the animals to eliminate the drug from its body, what is known as pharmacokinetic.

6.3 MATERIALS AND METHODS

6.3.1 Animals

1. ***FSH activity***

   Immature female Wistar rats, 21 to 28 days old, having no more than 10 g of difference between their weights were obtained from Harlan Laboratories. All the procedures used in this paper were in compliance with the European Pharmacopeia 5.0 01/2008:0719.

2. ***LH activity***

   Immature male Wistar rats, 19 to 28 days old, having no more than 10 g of difference between their weights were obtained from Harlan Laboratories. All the procedures used in this paper were in compliance with the European Pharmacopeia 5.0 01/2008:0498.

3. Half life of rPMSG

Six White New Zealand rabbit females of 2 kg of weight obtained from Granja San Bernardo S.L. were used for this experiment.

6.3.2 Efficacy bioassays for rPMSG

These assays were carried out in the department of medicine and animal surgery, Veterinary faculty, Universitat Autònoma de Barcelona, and were conducted by the Dra. Teresa Rigau.

Supernatants containing rPMSG from fed-batch cultivations were centrifuged, ultrafiltrated and purified throughout IMAC affinity columns. Finally, the buffer of fractions containing the rPMSG was changed to phosphate-albumin buffered saline pH 7.2 (10.75g·L^{-1} disodium hydrogen phosphate, 7.6g·L^{-1} NaCl, 10 g·L^{-1}).

6.3.2.1 Assay for FSH activity

The *in vivo* FSH bioactivity of rPMSG expressed in *P. pastoris* was determined in the FSH test of the Pharmacopoeia (Steelman & Pohley 1953). Five animals were injected with 200 µL s.c at three different doses of rPMSG; high, medium and low; 51 µg, 8,4 µg and 1,4 µg respectively, at 18, 21, 24, 42 and 48 hours after first injection. In parallel, the medium dose was assayed in combination with yeast mannan (SIGMA, M-3640). Yeast mannan is used to saturate the reticuloendothelial system of rats and thus, avoid the recognition of the mannose residues present in the product. With that strategy the objective is to elucidate if reticuloendothelial system would be main clearance system for rPMSG. One minute before each regular injection of rPMSG, approximately 10 mg of yeast mannan was injected. Also, Folligon 1000 (Intervet) was used as a standard at three

concentrations; high (18 units), medium (12 units) and low (8 units). After 48 hours since last injection, the animals were euthanized and their ovaries were weighted.

6.3.2.2 Assay for LH activity

The *in vivo* LH activity of rPMSG was determined in the hCG test of the Pharmacopoeia. Five animals were injected s.c. with a single dose of rPMSG (600 $\mu g \cdot mL^{-1}$) with a dairy injection during four days. As a standard, a LH sample courtesy of Calier Laboratories was injected at 4, 8 and 16 Units per injection. In parallel, a single concentration of 4 U per injection of hCG was used as positive control. The phosphate-albumin buffer was used as a negative control. After 24 hours since last injection, the animals were euthanized and their seminal vesicles were weighted.

6.3.3 Experimental determination of rPMSG pharmacokinetics

These assays were carried out in the Servei d'Experimentació Animal, Facultat de Farmàcia, Universitat de Barcelona, and were conducted by the Dr. Teresa Rodrigo Calduch and Dr. Rosa Ferraz Colomina.

A quantity of 0.5 mg of purified rPMSG/kg was administered to three rabbits. Two rabbits were administered with 25 μg of Folligon, and saline buffer was used for negative control. Blood samples of 3 mL were extracted to each rabbit at different times 0, 15 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, 8 hours, 24 hours and 48 hours. The serum was separated from blood samples through centrifugation and subsequently stored at -20ºC for further analysis by an specific PMSG ELISA (DRG Instruments, EIA-1298)

6.4 RESULTS AND DISCUSSION

6.4.1 FSH activity

The potency of PMSG is estimated by measuring the increase in the mass of the ovaries of immature rats in comparison with the effect of the International Standard of PMSG or of a reference preparation calibrated in International Units (I.U.). For these experiments, Folligon was used as a reference preparation at 8, 12 and 18 I.U. Similarly, the rPMSG was assayed at three increasing doses; 51 µg, 8,4 µg and 1,4 µg.

More, despite in the rPMSG most glycosylation sites no-determinant for protein conformation were eliminated to avoid the the Mannan-binding lectin pathway of complement, another medium dose of rPMSG was assayed with a solution of yeast mannan, an inhibitor of mannose receptor, in order to overload this lectin pathway and avoid a rapid clearance of rPMSG injected subcutaneously. Kogelberg et al., 2007 described how the concomitant intraperitoneal injection of mannan together with the recombinant antibody expressed in *P. pastoris* increased the presence of the protein 206-fold after 5 hours[4]. The mannose receptor is a member of the C-type lectin family[5], and is an endocytic and phagocytic pattern recognition receptor that binds in a calcium-dependent manner to mannose, fucose, and N-acetylglucosamine terminating glycans[6]. In liver, it is found on sinusoidal endothelium and Kupffer cells, where it is act as a molecular scavenger by clearing pathogenic microorganism and endogenous glycoproteins with high mannose structures[7]. Mannose receptor also plays an important role in cell activation and antigen presentation[7,8] and therefore, could be involved in the immune response to glycosylated protein therapeutics.

Logically, the phosphate-albumin buffer alone, and containing mannam were used as a negative controls (Table 1).

TABLE 1. Folligon (Intervet) at 18, 12 and 8 I.U. were used as reference preparation for high, medium and low doses respectively. Similarly, rPMSG were administered at three different doses and a dose was administered in combination with mannan.

	Standards			rPMSG			rPMSG + Mannan	Negative	
Doses	**Low (8 IU)**	**Medium (12 IU)**	**High (18 IU)**	**Low (1,4 µg)**	**Medium (8,4 µg)**	**High (51 µg)**	**Medium**	**Buffer**	**Mannan**
	170	226,1	244,8	20	21,6	35,2	21,2	36,9	19,9
	241,3	247,9	254,1	32,8	24,2	25,4	25,4	20	17,6
	150,3	212	180,6	24,3	19,9	19,3	25,1	21,4	15,5
	187,7	261,7	261,5	20,3	19	28,4	35,4	34,9	18,8
	220,2	247,1	233,5	19,3	24,2	34,9	37,1	23,7	17,3
Mean (mg)	**193,9**	**238,96**	**234,9**	**23,34**	**21,78**	**28,64**	**28,84**	**27,38**	**17,82**

Figure 1 shows clearly the positive effect of standard preparation on the ovarian weight in comparison with the rPMSG which only arise values ten-fold lower.

Similarly, the concomitant administration of mannan with rPMSG achieved similar levels than negative controls.

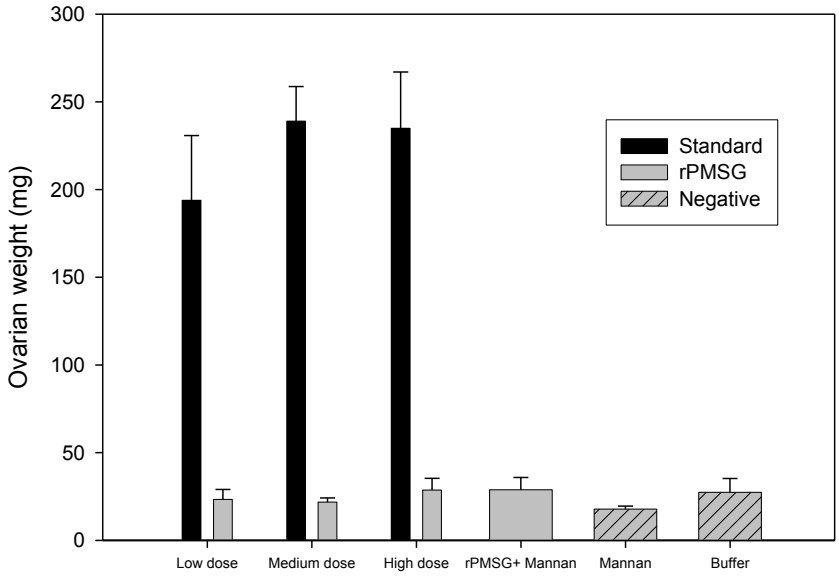

FIGURE 1. Effect of rPMSG and reference preparation on ovarian weight of immature rats after their administration stated for European Pharmacopoeia.

Therefore, these negative results certainly exposed that rPMSG had any effect on the ovarian weight of immature rats. Even the presence of mannan did not help to detect *in vivo* activity. Kogelberg et al., 2007 described that the concomitant injection of mannan with the recombinant protein increase the stability of it 206-fold after 5 hours, but after 24 hours, enzyme levels in blood fell below detection levels, also when mannan was added[4]. Several causes can be responsible for this lack of activity, not only the rapid clearance of rPMSG from blood-stream by the lectin pathway, but also the amounts of intact rPMSG in the samples administered were too low to trigger a positive response.

6.4.2 LH activity

The LH potency of PMSG (or eCG) is estimated by comparing under given conditions its effect of increasing the mass of the seminal vesicles (or the prostate gland) of immature rats with the same effect of the International Standard of chorionic

gonadotropin or of a reference preparation calibrated in International Units. For this experiment, human Chorionic Gonadotropin (hCG) was used as a reference preparation or positive control, but in this case injecting only 4 U per injection, instead of three different concentrations. Figure 2 presents how, at least, at this concentration the activity of the hCG was not detected since it achieved the same levels of negative control which was only phosphate-albumin saline buffer. Similarly, the injections of rPMSG at 600 $\mu g \cdot mL^{-1}$ did not trigger a significant growth of vesicles, revealing that the recombinant hormone did not have LH activity. On the other hand, LH sample used at three different concentrations clearly showed the positive effect on seminal vesicles growth. The increasing concentrations of LH, displays a step-wise increase of vesicles weight.

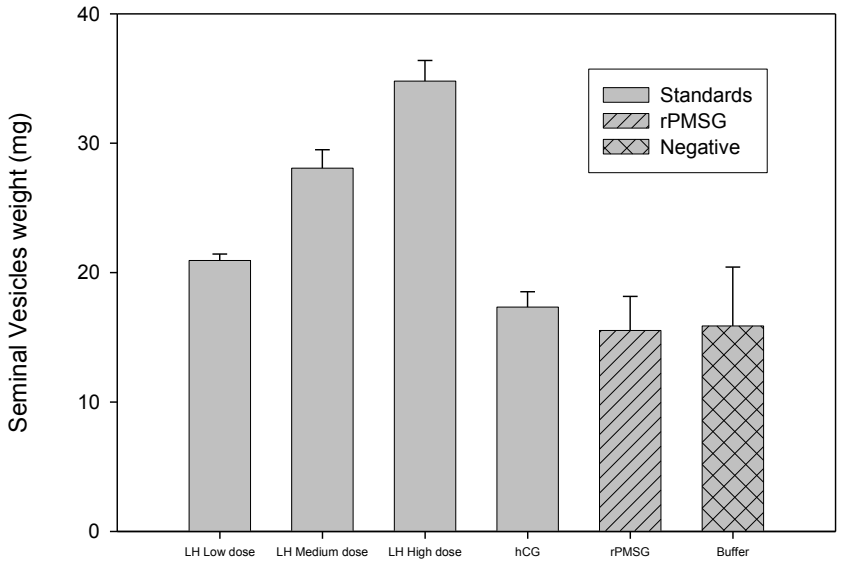

FIGURE 2. Effect of rPMSG and reference preparation on seminal vesicles of immature rats after their administration

These results coincide with those obtained in FSH activity, suggesting that probably the lack of LH and FSH activity *in vivo* may be caused by low concentrations of functional protein or by rapid clearance of rPMSG from the bloodstream.

6.4.3 Calculation of rPMSG and Folligon half-life by ELISA

With the aim to determine if lack of activity was provoked by fast elimination of rPMSG from the animal, it was attempt to determine the half-life of rPMSG and commercial eCG (Folligon) was ELISA. Figure 3 compares the evolution of 25μg of Folligon injected in two rabbits using the standards included into the kit and 1 mg of rPMSG injected in one rabbit.

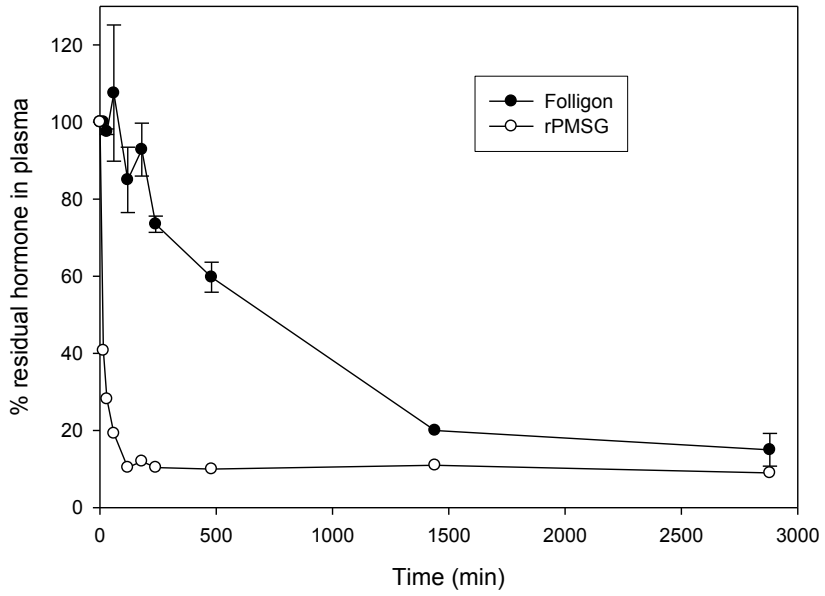

FIGURE 3. Percentage of residual Folligon and rPMSG remaining in the blood-stream.

The half-life of Folligon was estimated around 24 hours which coincide with the bibliography (BIBLIO). Besides, the rPMSG was analysed similarly, but in this case the

standards included into the kit were not used since the amounts of protein estimated were completely different from those injected to the rabbits. This difference may be explained since the amino acid chain, structure of the protein and glycosylation of rPMSG differed from native hormone. Alternatively, the rPMSG purified and properly quantified was used as calibration curve in the same plate. Figure 3 shows how rPMSG was unstable in the blood-stream unlike Folligon which after 2500 minutes still 20% of hormone was under circulation. The half-life of rPMSG could not be adequately calculated because after less than 2 hours the commercial ELISA was not able to detect such low amounts, the residual protein in circulation after 90 minutes was around 10% which represents approximately 1 $\mu g \cdot mL^{-1}$ of rPMSG.

Therefore, a plausible explication why the rPMSG did not have in vivo activity would be because of its really fast elimination from the blood-stream. This extremely rapid clearance can be explained by the Mannan-binding lectin pathway, which after administration subcutaneously of a mannosylated protein is quicly activated.

6.5 CONCLUSIONS

In this chapter, the *in vivo* FSH and LH activity of rPMSG was tested with negative results. Both assays reported any activity from the recombinant hormone expressed in *P. pastoris*. This lack of activity was investigated throughout a study of the half-life by ELISA, indicating that probably a rapid elimination of the protein may be the cause of it.

- Both FSH and LH assays were performed as described European Pharmacopoeia demonstrating an absence of bioactivity of rPMSG in both cases.

- A half-life study of rPMSG and Folligon throughout ELISA elucidated that a rapid clearance of the recombinant hormone expressed in *P. pastoris* from the blood-stream could explain the deficiencies in the bioassays.

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CONCLUSIONS

The global conclusions extracted from this study are here highlighted:

The expression in *Pichia pastoris* of an active form of a fusion protein between PMSG and HSA has been accomplished. The adequate folding of HSA fused at N-terminal of PMSG β subunit (rPMSG) has been demonstrated through the capacity of the recombinant hormone to induce a steroidogenic response in Leydig cells (MLTC-1). Nevertheless, the expression analysis elucidated the effect of proteases on rPMSG, yielding to a poor non-degraded protein recover.

With the aim of providing sufficient quantities of rPMSG for *in vivo* studies, the development of fed-batch cultivation strategy and purification procedure has been performed. Different cultivation approaches has been examined to reduce the proteolysis with no significant improvements, however setting up the temperature at 22ºC and the pH at 6,5 derived in an eloquent improvement of *in vitro* activity probably caused by larger amount of non-degraded protein assayed.

The analysis of cell viability by flow cytometry, the assays to determine the effect of extracellular proteases and the analysis of cytoplasmic fractions, pointed out an early degradation of rPMSG during the secretion pathway, making it unstable and vulnerable to extracellular proteases.

The *in vivo* studies performed in rats as mentioned the European Pharmacopoiea evidenced a lack of FSH and LH activity of the rPMSG expressed in *Pichia pastoris*. More, an evaluation of rPMSG half-life in rabbits indicated the rapid clearance of the recombinant hormone from the blood-stream as a possible cause for this lack of activity.

Specific conclusions

- First, a cell-based assay has been developed with the aim to evaluate, batch-to-batch the adequate conformation of rPMSG. Either Sertoli cells (TM4) and granulose cells were rejected because of their poor steroidogenic activity detected. Instead, Leydig cells (MLTC-1) have been presented as an acceptable cell line for this purpose.
- Second, two different constructs have been cloned and expressed in X-33 *P. pastoris*. The expression analysis revealed that construct α+HSAβ suffered a specific cleavage which invalidated this construct for further scaling up experiments. On the other hand, the α+βHSA construct was selected for high cell concentration cultures. Despite the expression analysis also exposed degradation of this construct, a small portion of the secreted protein was still non-degraded. More, the adequate conformation of this construct has been verified with the *in vitro* assay.
- Third, fed-batch cultivation and a purification procedure have been developed successfully, obtaining enough non-degraded rPMSG to induce MLTC-1 cells.
- Fourth, better conditions of pH and temperature to reduce the degradation have been found. Batch cultivations comparing pH 5,5 and 6,5 have been performed discerning that pH 6,5 notably reduced intracellular degradation. Similarly, batch cultivations comparing 30ºC and 22ºC revealed that at 22ºC the non-degraded fraction of rPMSG intracellularly was majority. Moreover, the stability of the secreted rPMSG in a cultivation supernatant have been found to be higher at pH 6,5.
- Fifth, these better cultivation conditions have been successfully implemented in a fed-batch strategy. Improving significantly the *in vitro* activity of rPMSG in

comparison with the rPMSG expressed under *standard* conditions (pH 5,5 and 30ºC).

- Sixth, the intracellular analysis and the flow cytometry results evidencing that the degradation of rPMSG started during its secretion pathway, making impossible to eliminate the degradation with only cultivation approaches.
- The *in vivo* activity has been studied as mentioned the European Pharmacopoeia. The rPMSG did not present FSH or LH activity in rats. More, pharmacokinetic studies of rPMSG have been performed in rabbits pointing out the rapid clearance of the recombinant hormone from the blood stream as one of the possible causes for the lack of activity.