Genetic Engineering and Biotechnology of Growth Hormones

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1. Introduction

In its modern conception, biotechnology is the use of genetic engineering techniques to manipulate microorganisms, plants, and animals in order to produce commercial products and processes that benefit man. These techniques, which are the backbone of the biotechnological revolution that began in the mid 1970s, have permitted the isolation and manipulation of specific genes and the development of transgenic microorganisms that produce mainly eukaryotic proteins of therapeutic use, such as vaccines, enzymes, and hormones.

Biotechnology is present in diverse areas such as food production, degradation of industrial waste, mining, and medicine. Recent achievements include drug production in transgenic animals and plants, as well as the commercial exploitation of gene sequences generated by the human genome project and similar projects of plants and animals of commercial interest that are and will be in process.

Human growth hormone was, after insulin, the second product of this new technology. This product was developed and commercialized initially by Genentech, and was used clinically for treating growth problems and dwarfism (1). Furthermore, growth hormones from different animal species have also been produced in transgenic organisms and these have been used in different examples in the aquatic animal and livestock sectors.

2. The growth hormone (GH) family

GHs belong to a family of proteins with structural similarity and certain common functions that include prolactin (Prl), somatolactin (SL), chorionic somatomammotropin (CS), proliferin (PLF) and proteins related to Prl (PLP) (2). This family represents one of the most physiologically diverse protein groups that have evolved by gene duplication. The two most studied members of this family have been GH and Prl, which have been described from primitive fish to mammals; however, other members of the family are not so amply distributed or studied.

2.1 Structure of growth hormones

GHs (see Figure 1), in general, have a molecular weight of around 22,000 Daltons (22 kDa or simply 22k) and do not require post-translational modifications. They are synthesized in somatotrophs in the hypophysis, intervening as an important endocrine factor in postnatal somatic growth and lactation.
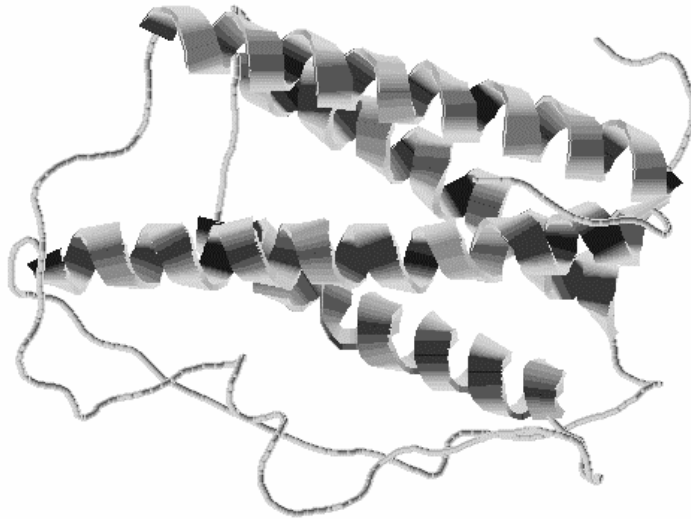

Fig. 1. Growth hormones' consensus tridimensional structure. The GHs have in general 190 aminoacidic residues, four alpha helixes, and two sulphide bonds

2.2 Hormones of the human growth hormone family

HGH22k

HGH22k (or HGHN) is the main product of the GH gene (hGH-N) active in the hypophysis and it is responsible for postnatal growth as well as being an important modulator of carbohydrate, lipid, nitrogen and mineral metabolism. It is the best known hormone and the only one of the HGH family that has been commercialized.

As mentioned, besides being the cure for hypophyseal dwarfism, HGH22k postulated benefits are as an anabolic in athletics and for the treatment of trauma because of its postulated regenerative properties (3).

HGH20k

In addition to the mRNA of HGH22k, an alternative processing pathway of the primary transcript of the hGH-N gene generates a second mRNA that is responsible for the production of the 20k isoform of HGH or HGH20k. Its smaller size is due to elimination of the first 45 nucleotides of the third exon of the mRNA and of the amino acids that correspond to positions 32-46 of the hormone, producing a protein with 176 amino acid residues (4).

This isoform comprises approximately 10% of all the GH produced in the hypophysis and although it has not been shown to be the etiological agent of any known disease, it is known that its levels are significantly higher in patients with active acromegaly and in those with anorexia nervosa (5).

The administration of exogenous HGH20k suppresses endogenous secretion of HGH22k in healthy subjects, which suggests that the regulation of secretion of both hormones is physiologically similar (6). In vitro findings suggest that both hormones can equally stimulate bone remodeling and allow anabolic effects on skeletal tissue when they are administered in vivo to laboratory animals (7).

HGHV

Several isoforms also derive from the GH gene expressed in the placenta (hGH-V)(Table 1). The most abundant mRNA from this gene in the placenta at terminus also codifies for a 22 kDa isoform. A less abundant isoform (HGHV2) originate from a species of mRNA that retains the fourth intron and due to this, it codifies for a 26 kDa protein that anchors to the membrane and which could have a local action (8). A 25 kDa protein is also derived by glycosylation of residue 140 of asparagine from the 22 kDa isoform (9, 10). Finally, two new transcripts of this gene have recently been identified: one already known that as in the case of the HGH20k also produces a 20 kDa protein, and another novel splicing variation that results in a mRNA known as hGHV3, that traduces into a 24 kDa isoform (11).

Isoform	Size	Length	Characteristic
• HGH-V22k	22kDa	191aa	Main isoform.
• HGH-V25k	25kDa	191aa	Glycosylated version of HGH-V22.
• HGH-V2	26kDa	230aa	Retains the fourth intron.
• HGH-V20K	20kDa*	176aa	Deletion of aa residues 32 to 46.
•HGH-V3	24kDa*	219aa	Alternate processing at level of exon 4.

*Only the mRNAs that codify each have been identified.

Table 1. HGH-V isoforms generated by alternative splicing and processing

During pregnancy, while hypophyseal HGHN progressively disappears from the maternal circulation until undetectable values are reached at weeks 24 to 25, HGHV progressively increases until birth, suggesting that it has a key role during human gestation (12). It has also been found that in cases of intrauterine growth restriction, circulating levels of HGHV measured between week 31 and birth are lower than those reported in normal pregnancy (13, 14, 15).

Finally, although low levels of this hormone have been associated with intrauterine growth retardation, cases of hGH-V gene deletion have also been reported, but without an apparent pathology (16).

2.3 Human chorionic somatomammotropin (HCS)

HCS is detected in maternal serum from the fourth week of gestation, increasing throughout the pregnancy in a linear fashion, and reaching high production levels of a couple of grams

per day at the end of gestation. These actions result in both elevation of glucose and amino acids in the maternal circulation. These are in turn used by the fetus for his/her development. It also generates free fatty acids (by lipolytic effect), which are used as an energy source by the fetus (17, 18). Little is known about the HCS physiological role, and still is not known its action mechanism. Producing rHCS by biotechnology will help to advance these investigations.

2.4 In vitro bioassays for GHs and CSHs

As stated above except for HGH22k, the functions of the rest of hormones of the human GH family have been not completely defined. Their biological activities are being studied, classifying them into at least two general categories:

a. Somatogenic activities. These involve linear bone growth and alterations in carbohydrate metabolism; effects that are in part mediated by local and hepatic generation of insulin-like growth factor-I (IGF-I). The somatogenic activity of HGHV has been studied by stimulating body weight increase in hypophysectomized rats, reporting a linear increase comparable to that produced by HGH22k (19).

b. Lactogenic activities. These include stimulation of lactation and reproductive functions (20). The lactogenic activities of this hormone have been studied using a cell model (by mitogenic response to Nb2 cells) and a response that is parallel to HGH22k has been reported, although it is significantly less (19).

2.5 The human GH locus

Besides the two hGH genes (normal and variant), three HCSs complement the multigenic HGH family from the human genome and these are arranged in the following order: HGH-N, HCS-1, HCS-2, HGH-V y HCS-3 (21, 22) (Figure 2). While HCS-1 appears to be a pseudogene, HCS-2 and HCS-3 are very active in the placenta and interestingly; mature versions of the hormones that they codify are identical (23).

In the last few years, in our laboratory, all the hGH and HCS genes have been cloned and expressed in cell culture, and the factors that affect their levels of expression have been particularly studied (24).

In the same way, and using polymerase chain reaction (PCR) with consensus primers, several new genes and complementary DNAs (cDNAs) to the mRNA of numerous GHs have been isolated in our laboratory, mainly from mammals (unpublished results).

3. Growth hormone of animal origin

3.1 Bovine growth hormone (BGH)

Bovine growth hormone (BGH) or bovine somatotropin improves the efficiency of milk production (per unit of food consumed) (25), and the production (body weight) and composition (muscle: fat ratio) of meat (26). In the case of milk cows, this permits a reduction in the number of animals needed for milk production and a subsequent savings in maintenance, feeding, water, drugs, etc. It also reduces the production of manure, and nitrogen from urine and methane (27).
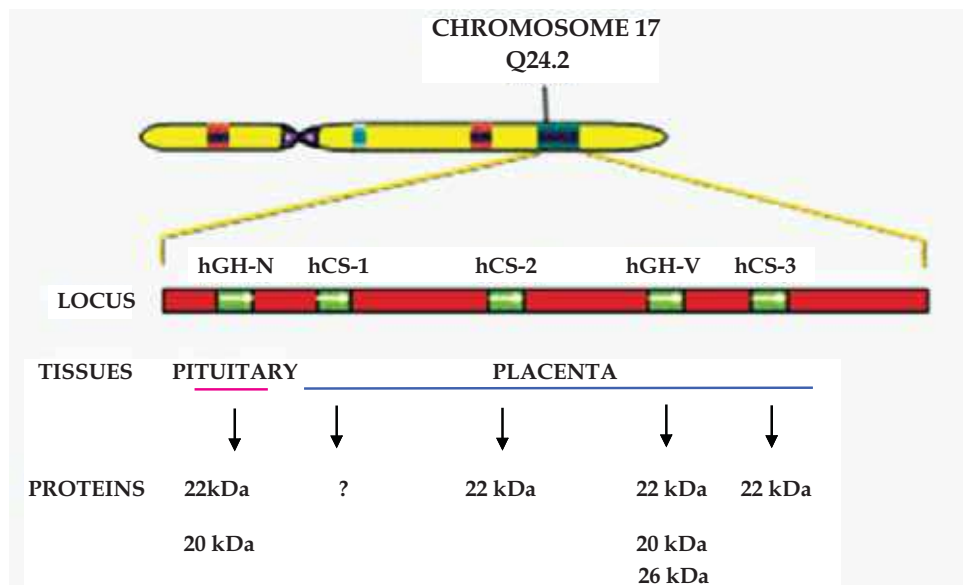
Fig. 2. HGH-HCS multigenic complex. Located on Chromosome 17, every gene is indicated; the tissue where they are expressed and the proteic isoforms that are produced are shown

Milk from cows treated with rBGH, does not differ from that of untreated cows (28, 29). The characteristics that have been evaluated include the freezing point, pH, thermal properties, susceptibility to oxygenation, and sensory characteristics, including taste; in fact all organoleptic properties are conserved. Also, differences have not been found in the properties necessary for producing cheese, including initial growth of the culture, coagulation, acidification, production and composition (29).

rBGH is administered subcutaneously and is dispensed as a long-acting suspension that is applied in a determined period of time. The taste of bovine meat and milk treated with rBGH is not altered, but the fat content is less.

3.2 Caprine growth hormone (CHGH)

For small ruminants there are studies in lactating goats in which the administration of rBGH increased milk production 23% and stimulated mammary gland growth more than in those that were frequently milked, with it being similar to prolactin (30). However, the production of recombinant CHGH, which is identical to ovine and thus can be used in both animals, had not been reported, until we achieve its expression on the methylotrophic yeast *Pichia pastoris*. (See section 7.2).

3.3 Equine growth hormone (ECGH)

With regard to horses, GH is used in the prevention of muscle wasting, in the repair of tendons and fractured bones, as well as for the treatment of anovulation in mares. Besides this, it is also used for repairing muscle tissue, to tonify and invigorate race horses, and for

improving physical conditions in older horses by restoring nitrogen balance. It can also stimulate growth and early maturity in young horses, increase milk production in lactating mares and promote wound healing, especially of bone and cartilage (31, 32), as occurred in the case shown in Figure 3.

Fig. 3. Uses of equine GH. The race horse "Might and Power" (right) became the winner of the Melbourne Cup in 1997. But in 1999, a tendon from one of its hooves was severely damaged. The horse was treated with ECGH, recovered and in 2000 was able to return to horse racing (32)

3.4 Canine growth hormone (CFGH)

With regard to the dog (*Canis familiaris*), each day there is more evidence of the role that its GH (CFGH) plays in bone fracture treatment, in which the hormone helps reduce the bone restoration period (33).

It is no less important in the treatment of obesity in dogs, thanks to the metabolism activation produced by the hormone in removing fatty acids, and in general, in counteracting symptoms related to the presence or absence of the same GH. Also, since this hormone is identical to pig GH (PGH) (33), its virtues are valid for the application of CFGH in the porcine industry, where it generates leaner meat (34), which is of greater value.

3.5 Feline growth hormone (FCGH)

Although there is very little literature on cat GH (FCGH), the benefits identified in other GHs apply to this feline species, since these animals present the symptoms mentioned before for dogs, which are caused by the absence or low concentration of FCGH (dwarfism and alopecia, among others). Also, as referred to in the literature, biological tests of adipogenic activity in culture cells use cat serum (which contains FCGH) instead of bovine serum, because FCGH lacks adipogenicity (17).

Therefore, recombinant production of this GH would be useful in the mentioned tests. It is important to point out the usefulness that recombinant FCGH would have in future research on the metabolic study and role of this hormone in this and other feline species, including of course, large wild cats in captivity.

4. Biological potential of GHs

4.1 Growth hormones of human origin

Although HGH22k is widely commercialized and more functions now have been recognized to it (Table 2), the same does not occur with the other proteins and isoforms from this family; essentially the 20 kDa isoform of HGH, HGHV, also the isoform of 20 kDa of the latter (HGHV20k), and lastly, HCS. Partly because of this, many of their functions and mechanisms of action are still unknown.

Immunization and healing	Mental function
- Resistance to common diseases - Ability to heal - Healing of old lesions - Healing of other lesions - Ulcer treatment	- Emotional stability - Memory - General aspect and attitude - Mental energy and clarity
Skin and hair	**Muscle strength and tone**
- Skin elasticity - Skin thickness - Skin texture - Growth of new hair - Disappearance of wrinkles - Skin hydration	- Increase in energy in general - Increase muscle strength - Promotion of muscle mass gain
Sexual factors	**Circulatory system**
- Duration of an erection - Increase in libido - Potential/frequency of sexual activity - Regulation and control of the menstrual cycle - Positive effects in the reproductive system - Increase in breast-milk volume	- Improvement in circulation - Stabilization of blood pressure - Improvement in cardiac function
Bone	**Fats**
- As treatment for bone fractures - Osteoporosis treatment - Increases flexibility of the back and joints	- Increases "good" cholesterol (HDL) levels - Reduces fat

(Taken from Elian y cols., 1999), (3).

Table 2. New functions atributed to HGH22k

It is believed that some of the hormone's less abundant natural variants, such as HGH20k, could retain desirable properties of the principal hormone and lack some of its other undesirable effects, such as its diabetogenic effect, which occurs with prolonged use (35).

4.2 Growth hormones of animal origin

The biotechnological potential of GHs could be enormous, since besides its use in species of the same origin, it has been demonstrated that the GHs of mammals have activity in phylogenetically lower animals.

For example, BGH and porcine GH (PGH) have been used experimentally for the treatment of hypophyseal dwarfism in dogs (36) and cats (37).

Regarding farm animals, porcine, bovine, caprine and ovine livestock have been treated with exogenous GH to improve production, since it increases food conversion efficiency, growth rate, weight gain, and milk and meat production. What is surprising is the finding that BGH stimulates salmon growth, and even more interesting that bovine chorionic somatomammotropin (BCS) works even better (38).

5. Expression systems for growth hormones

5.1 The history of human recombinant GH

As previously mentioned, among the first cDNAs cloned and expressed in the bacteria *Escherichia coli* is precisely HGH (1). This expression system has been used since 1985 for the production of recombinant HGH by Genentech (protropin), which was later followed by Lily (humatrope), Biotech (biotropin), Novo Nordisk (norditropin), Serono (serostim), and others.

5.2 Different biotechnological hosts

Since the recombinant protein is frequently recovered from *E. coli* with undesirable modifications (extra methionine, incorrect folding, aggregated forms, etc.) and contaminated with highly pyrogenic substances, toilsome purification schemes are needed to obtain it with the desired purity, structure and biological activity. For this, subsequent efforts have focused on the search for better expression systems, with production being attempted with *Saccharomyces cerevisiae* (39), *Bacillus subtilis* (40), mammal cell cultures (41), as well as transgenic animals (42). Unfortunately, these expression systems do not offer a production level greater than that of *E. coli* and therefore in most cases they are not profitable.

In our laboratory, we succeeded in producing HGH22k in *E. coli* by fusing it with maltose binding protein (rHGH-MBP) in 1994 (unpublished results). However, due to the fact that to recover the hormone, whether from the periplasm or the cytoplasm, complicated strategies were needed, together with the limitations of the bacterial systems for folding and processing foreign proteins correctly, we proposed searching for an expression system that allows synthesizing the protein, purifying it with greater ease while retaining functionality. Thus, the evaluation of different expression systems was started in our laboratories, considering the methylotrophic yeast *Pichia pastoris* as the best (43).

5.3 *Pichia pastoris* as a biotechnological host for GHs

Yeasts offer the best of both prokaryotes and eukaryotes, since, in addition to performing some of the post-translational modifications that are common in superior organisms, they are easily grown in flasks and bioreactors, like bacteria, using simple and inexpensive culture media (44).

P. pastoris is a methylotrophic yeast (capable of growing in methanol as its only carbon source) that performs post-translational modifications, produces recombinant protein levels of one or two orders of magnitude above that of *Saccharomyces cerevisiae* (45), is capable of secreting heterologic proteins into the culture media (where the levels of native protein are very low), and in contrast with the latter, can be cultivated at cell densities of more than 100 g/L of dry weight (46).

6. Recombinant growth hormones

In our laboratories, we identified as a scientific objective and a technological advantage, the construction and evaluation of GH protein producing *P. pastoris* strains. This as a first step in evaluating its potential in medicine as well as in animal health and productivity; searching to develop both infrastructure and experience in producing, purifying, and testing its biological activity.

Also, as previously mentioned, mammalian GHs have activity in phylogenetically inferior animals, nevertheless potentially adverse reactions to heterologic GHs can be triggered, which is why having a GH specific-species would avoid these undesirable side effects.

Regarding human hormones, we proposed constructing productive strains for the HGH22k, the HGH20k, the HGHV, and the HCS. With regard to animal GHs, we channeled our efforts into building strains to produce GHs from bovines (BGH), caprines (CHGH), ovines (OGH), equines (ECGH), canines (CFGH), porcines (PGH) and felines (FCGH); all based on the *Pichia pastoris* yeast expression system.

For this, the following experimental strategy was proposed:

a. Obtain, clone, and manipulate cDNA from these hormones.
b. Construct and insert into the genome of *P. pastoris* the hormones' expression cassettes.
c. Develop the fermentation processes for each new strain.
d. Implement the purification schemes of the recombinant hormones.
e. Evaluate in vitro the bioactivity of the semipurified recombinant hormones.

As a result of this experimental work, we achieved the followings:

i. Using different methodological approaches (RT-PCR, mutagenic PCR, subcloning, etc.) we cloned the cDNAs of the hormones of interest.
ii. Through genetic engineering manipulations, we converted the cloned cDNAs into expression cassettes capable of functioning in *Pichia pastoris*.
iii. The respective expression cassettes were integrated into the *Pichia pastoris* genome by homologous recombination.
iv. Through an inducible (with methanol) expression system, we were able to overproduce and recover from the culture media each of the respective recombinant hormones (rGHs/rHCSs).

v. The data from the physicochemical and biological characterizations showed that the methodology described herein generates heterologous proteins that are identical to their natural counterparts and biologically active.

7. Technological platform for the production of recombinant GHs

7.1 Overall strategy

As depicted in figures 4, the following are the two stages of the overall strategy in which the work was divided:

a. Construction of *P. pastoris* strains carrying the hormones´ expression cassettes producing rGHs/HCSs.
b. Production and characterization of the recombinant hormones.

7.2 Construction of propagating GH cDNA plasmids (pBS-XGHs)

Oligonucleotides for GHs cDNAs amplification by PCR were designed based on consensus nucleotide sequences of GHs (mature region) of related mammals. Extra restriction sites were added on their flanks (*Xho*I and *Avr*II) to facilitate insertion of the amplicon into the expression vector. With these, each of the hormones' cDNAs was amplified from plasmids previously constructed in our laboratory carrying the respective nucleotide sequences. Each amplicon was cloned into propagating plasmid such as the pBS II KS plasmid (+) and subsequently subcloned into the yeast expression vector pPIC9 at its multicloning site, between the restriction sites *Xho*I and *Avr*II (after previous purification of the corresponding fragment and vector), thereby giving rise to each of the pPIC9-XGH expression plasmids.

In CHGH's case, which differs from BGH in a single aa residue, a different strategy was implemented. Site-directed mutagenesis was used relying on a primer to convert codon 130 of BGH cDNA into one corresponding to CHGH. A 345 bp region containing the mutated GH cDNA was thus amplified, which was cloned in pBS and later transferred into pPIC9-BGH to converted it into pPIC9-CHGH (49).

7.3 Construction of expression plasmids (pPIC9-XGHs) for each hormone

Preparative digestions of pBS-XGH and pPIC9 with the enzymes *Xho*I and *Avr*II were performed for all GHs except for CHGH. For CHGH, *Apa*I and *Xma*I (natural site) enzymes, which release a 133 bp fragment containing the mutagenized codon for CHGH, were used. This was purified and linked into the previously digested pPIC9-BGH vector in the same sites, replacing the fragment to originate the pPIC9-CHGH expressor vector The ligation reactions between pPIC9 and each cDNA were used to transform competent Ca++ cells of XL1-Blue *Escherichia coli*. PCR was used to verify that the resulting tranformants indeed carried each pPIC9-XGH, where "x" corresponds to each of the sequences of the hormone in question. The candidate clones produced by PCR with AOX1 primers for an amplicon of 1050 bp, since the expression cassette for each hormone is flanked by long regions of the AOX1 gene. While strains that were not integrated into the "cassette" gave rise to an amplicon of only 500 bp.
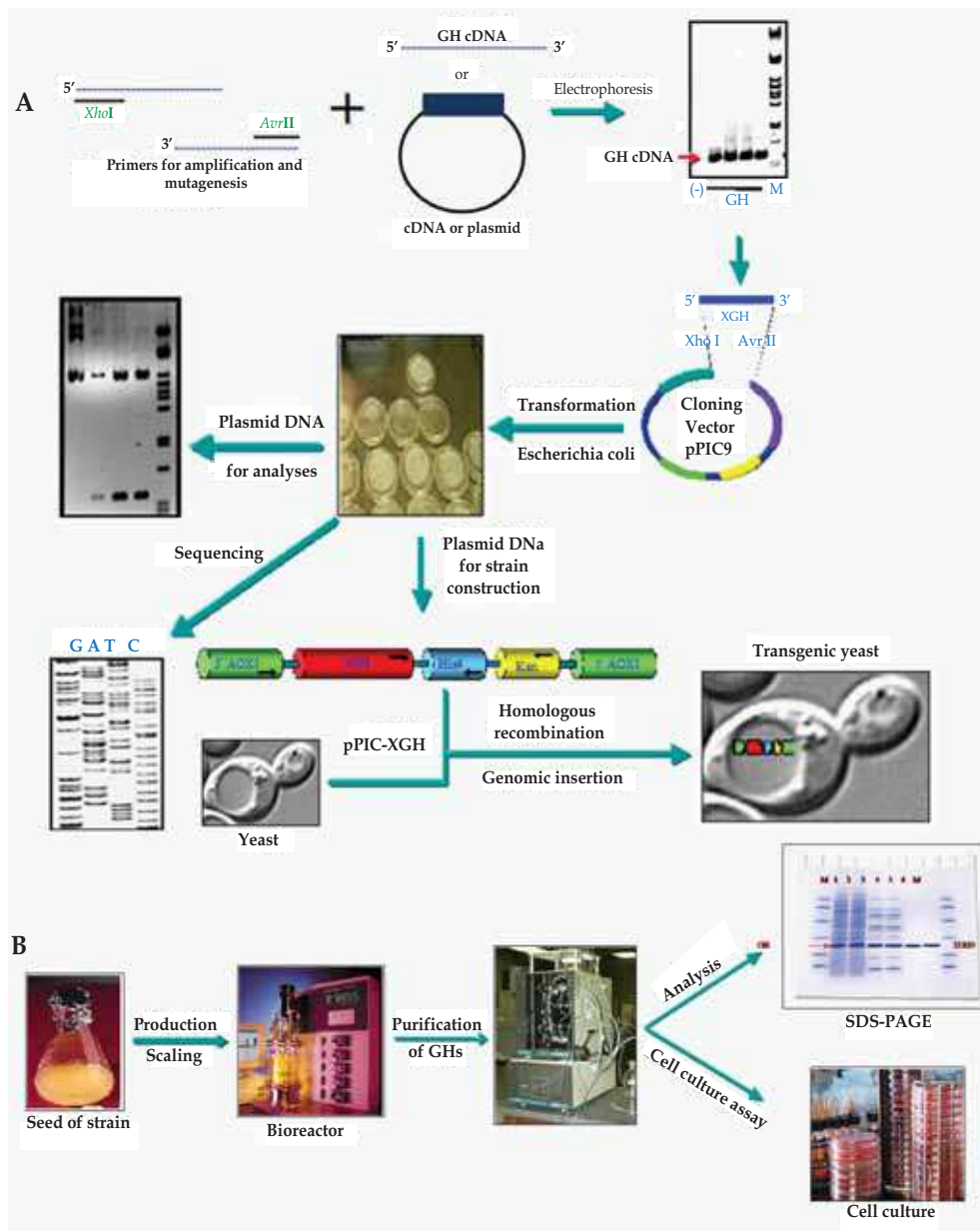
Fig. 4. General strategy for strain construction and recombinant hormones production. (A) Genetic engineering phase. The steps followed to construct and characterize new strains of GHs and HCSs producing *Pichia pastoris* are shown. Protocols followed were based in different techniques (47, 48). (B) Biotechnology phase. The steps followed for the production and scaling, semipurification and bioassay of each of the recombinant hormones are shown
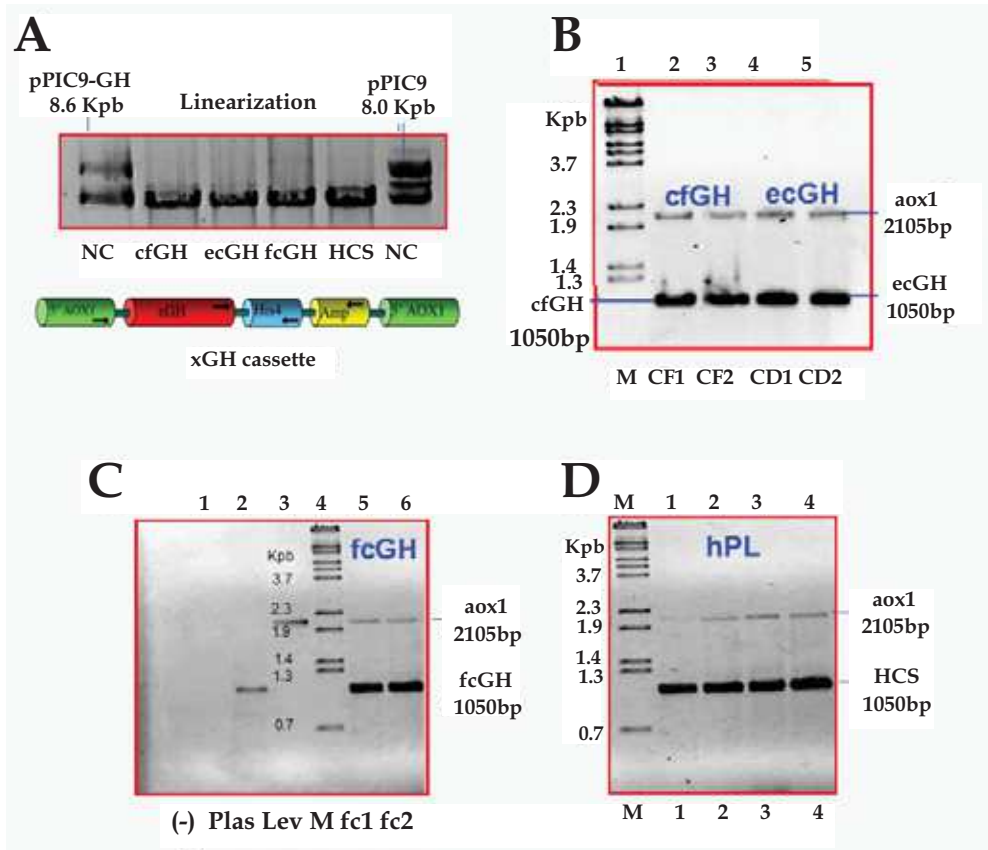
Fig. 5. Detection of the expression "cassettes" of cfGH, ecGH, fcGH and hCS in P. pastoris genome. Analysis by PCR with AOX1 primer yeast strains transfected. In each case, the 1050 bp corresponds to the expression cassette of the recombinant hormone in question, while the 2105 bp to the AOX1 gene of the yeast itself. A) The diagram shows the linearized "cassette" of XGH and the gel products (which transfected into *Pichia pastoris* integrate the "cassette" into the genome) with *Sac*I enzyme: cfGH = dog GH, ecGH = horse GH, fcGH = cat GH and HCS = human CS; in lane 1 NC-GH = uncut plasmid pPIC9 and in lane 6 NC = uncut pPIC9 plasmid. B) CF (1 and 2) = dog GH lanes 2 and 3 respectively, and CD (1 and 2) = horse GH lanes 4 and 5, respectively. C) (-) = negative PCR lane 1, Plas= amplification positive control lane 2, Lev = *Pichia pastoris* genomic DNA lane 3, M= pb marker lane 4 and fc (1 and 2) = cat GH lanes 5 and 6 respectively. D) Lanes 1 to 4 correspond to strains with the HCS "cassette". M = marker-bp λ *Bste*II. The gels correspond to 1% agarose

8. Construction of GHs producing *Pichia pastoris* strains

The *Pichia pastoris* GS115 strain has a mutation in the histidinol dehydrogenase (his4) gene, which prevents it from synthesizing histidine. The class of plasmids used to transform it contains this gene (his4). The transformants are selected for their ability to restore growth in

a medium lacking histidine. The plasmid vectors of the pPIC series and those constructed to express the GHs are of this class.

8.1 Insertion of GHs expression cassettes into the genome of *P. pastoris*

Each pPIC-XGH vector was linearized with the enzyme *Sac*I, transformed into the yeast previously made competent for transformation and left exposed to the homologous regions in the yeast genome necessary for recombination.

After incubating the DNA with competent cells, transformation reactions were plated to recover clones needing no histidine to grow (HIS[+] transformants). Then transformants were analyzed on their genomic DNAs by PCR using AOX1 primers to verify the presence of the transgenic hormone expressing cassette.

Verification of integration into the yeast genome of the expression cassette of the hormones was achieved in agarose gel by confirming that the amplification reaction rendered a prominent band of 1050 bp, which corresponds to the expression cassette of the hormone involved in each case and another of 2105 bp corresponding to the endogenous gene AOX1 of the yeast genome (Figure 5). In addition, each hormone "cassette" was subjected to nucleotide sequencing to verify that all they corresponded to the expected growth hormones.

8.2 Analysis of new *Pichia pastoris* strains´ phenotypes

Pichia pastoris strains were grown and the biomass was adjusted to low cell density (0.5 u at 600 nm). These were transferred to induction medium with 0.5% methanol and grown for

(A)   (B)
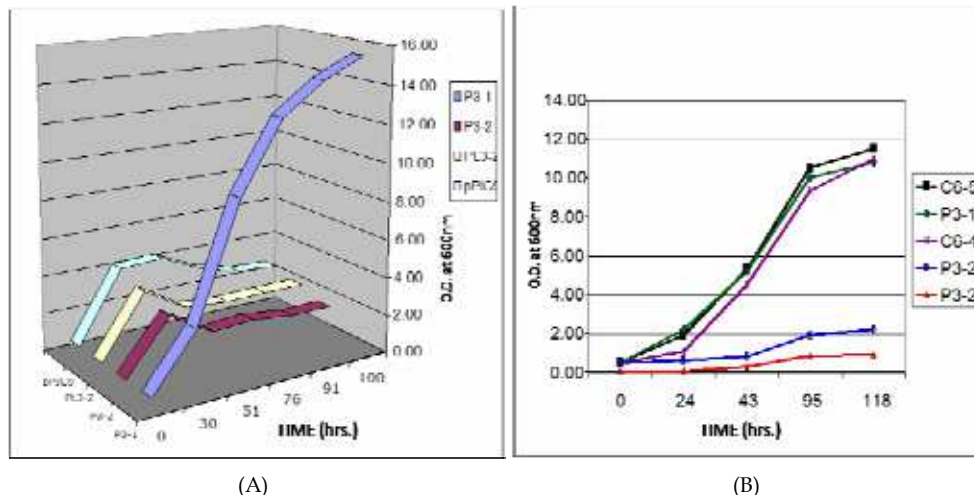
Fig. 6. Mut phenotype characterization in *Pichia pastoris* strains. Growth kinetics in minimal medium using methanol as sole carbon source. Induction was started at the density of 0.5 U and ended after about 100 hrs. (A) Plot of the samples P3-1 and 2 = dog GH 1 and 2 strains, CS3 CS-2 = 2 and strain human strains pPIC9 = "mock" with the pPIC9 plasmid. (B) Graph of the C6-5T samples = horse GH, P3-1T =dog GH, C6-4T = horse GH, GH P3-Q2 = P3-dog and dog-2B = GH

100 hours with the addition of methanol every 24 hours to compensate for its evaporation. Biomass growth was analyzed under methanol as the sole carbon source. The Mut$^+$ phenotype strains metabolize methanol more rapidly, achieving significantly higher cell densities than their Mut^s counterparts that metabolize more slowly, appreciating a slight increase in biomass under the same fermentation conditions.

An analysis of the growth of the strains after 100-hour fermentation with 0.5% methanol identified the Mut phenotype of each strain.

After fermentation, the strains found to be Mut$^+$ reached about 15 optical units at 600 nm, while those that were Mut^s did not exceed 2 units (Fig. 6). In the strains that had been built previously, their Mut phenotype was inferred when these were fermented in the bioreactor.

9. Production and analysis of recombinant hormones in the flask

To test the fermentation of strains, a biomass was generated in a flask. This was inoculated with a colony of each strain in 25 ml of biomass producing culture medium (BMGY) (50). This was incubated at 30°C at 250 rpm for 24 to 48 hours for the first stage of growth until a biomass with an OD of 600 nm of 10 was reached.

For the second stage, which is the induction of the recombinant hormone production, yeasts were harvested by centrifugation and the packed cells were washed with 30 mL sterile water, then these were pelleted and resuspended in fresh cassette induction medium (with methanol) (BMMY) (50). The induction was maintained by adding methanol every 24 hours to a final concentration of 1% to compensate for loss by evaporation. The experiment lasted 96 hrs. Figure 7 shows the process that was followed.

When analyzing the polyacrylamide gels of proteins from the culture media of each strain, we observed that all constructions produced and directed the secretion into the medium of the recombinant hormone in question to a greater or lesser extent. For the particular case of CFGH it was observed that a strain of Mut^s phenotype displayed better production of recombinant protein than its counterpart Mut$^+$. Strain of HGH-V proved to be the least productive (Fig. 8).

Figure 9 shows the gel with the results of the production of all recombinant strains generated in *Pichia pastoris*. They all produced different amounts of their respective hormone at the level of 22 kDa, except for the HGH20k, which, migrated below the rest of the recombinant hormones.

The percentage of each recombinant hormone in the culture medium was estimated by densitometry of each gel. For this we used the Gel-Doc software by BIO-RAD (Hercules, CA. EUA) and the ImageJ program (51). The results of estimation of the percentage of each hormone in relation to background proteins from *Pichia pastoris* were: HCS = 65%, CFGH = 60%, HGH22k = 30%, ECGH = 30%, BGH = 25%, FCGH = 25%, CHGH = 25%, HGH20k = 12% and HGHV = 8%.

Production kinetics was carried out for CHGH strain in a flask with a volume of 50 ml of rich medium. Samples were taken at 24, 48, 72, 96 and 120 hours of induction with methanol with restitution every 24 hours of 1% methanol. Bradford protein determination showed that the production of total protein secreted into the culture medium was 20µg/mL by densitometry and 60% represented CHGH giving us 12µg/mL of production of the recombinant hormone.
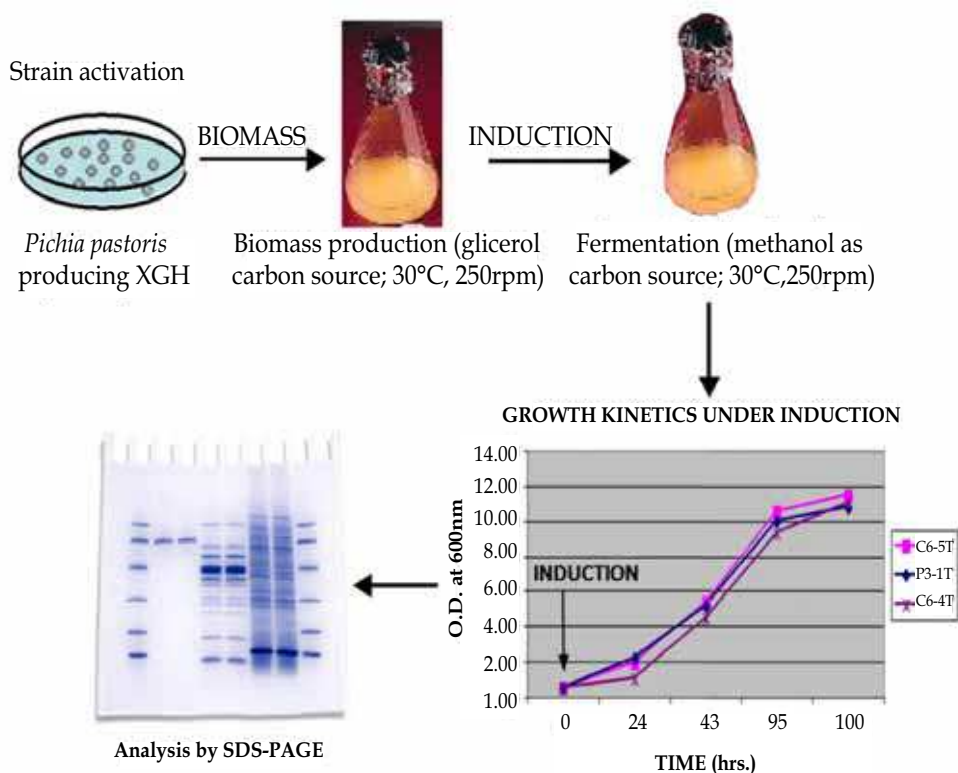
Fig. 7. Outline of the fermentation process. General procedure for the biotechnological production of recombinant hormones by fermentation of each strain. Strains were plated to activate them, incubated in liquid medium to generate a biomass flask, and the induced transgene expression by adding methanol. The culture medium was analyzed by SDS-PAGE in search of the hormones that are migrating around 22 kDa

10. Production scaling in the bioreactor

When passing to a bioreactor and increasing the scale, it is possible to obtain protein concentrations 20 to 200 times greater than in flasks. In the fermentor *Pichia pastoris* reaches high cell densities greater than 100 g/L of dry weight (46).

The fermentor was Bioflo 3000 (1 liter) of New Brunswick Scientific (NBSC) (NJ. EUA). The type of fermentation conducted was in fed-batch. The parameters monitored were scheduled addition of substrates to the fermentor, pH, percentage of dissolved oxygen, agitation, temperature, and aeration. The process involved three basic steps: 1) obtaining high densities of biomass, 2) induction of the cassette expression of each hormone with methanol and 3) harvest of biomass and culture medium containing the recombinant protein. Figure 10 shows the steps followed for the recombinant production of each hormone.
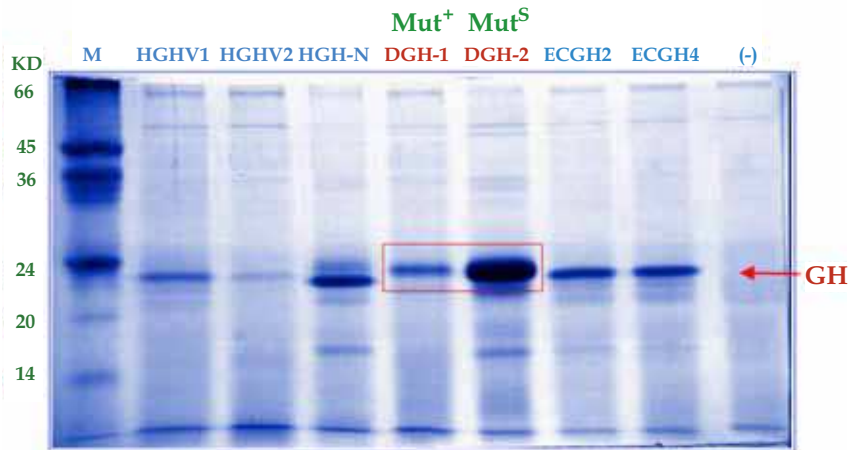
Fig. 8. Production in *Pichia pastoris* of recombinant GHs (CFGH, HGH, HGH-V and ECGH) at flask level. The bands correspond to the GHs proteins resolved at the level of 22 kDa that come from the culture media induced with methanol. The lanes are: M = molecular weight marker, HGHV1 = HGH variant strain 1; HGHV2 = HGH variant strain 2; HGH = normal pituitary HGH of 22 kDa; DGH-1 = Dog GH strain 1; DGH-2 = dog or *Canis familiaris* GH strain 2; ECGH-2 = horse GH strain 2, ECGH-4 = horse GH strain 4 and the last lane identified as (-) = negative control of PCR. Mut+= methanol utilization plus; Muts = "methanol utilization slow". Note the prominent band of the DGH-2 corresponding to the Muts phenotype, compared to the lower intensity of Mut+. The samples correspond to 500 µL concentrates of the original media. Gel corresponds to one of 15% polyacrylamide-SDS stained with Coomassie blue
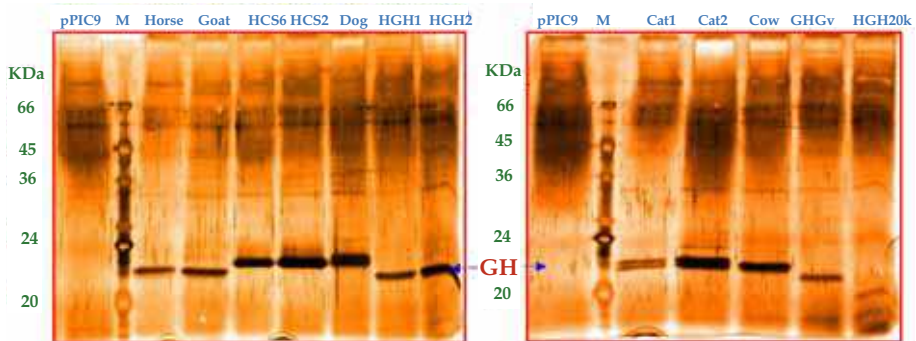

Fig. 9. Flask production in all strains yielding recombinant hormones. In all cases a prominent band is seen (except for HGH20k) at the 22 kDa level for each hormone. They are seen in their respective lane in each case indicated by their name. The lanes of the left side gel show the proteins from the culture media with recombinant hormones: horse = ECGH, goat = CHGH, HCS (6 and 2) = Human chorionic somatomammotropin clones 6 and 2, dog = CFGH and HGH (1 and 2) = cloned human GH 1 and 2. In the right gel lanes: cat (1 and 2) = GH 1 and 2 from cat; cow = BGH; HGHv = HGH placental variant and HGH20k = isoform of 20 kDa of hGH. The pPIC9 lane refers to the "mock" strain of *Pichia pastoris*. SDS-PAGE 15% gels are silver stained
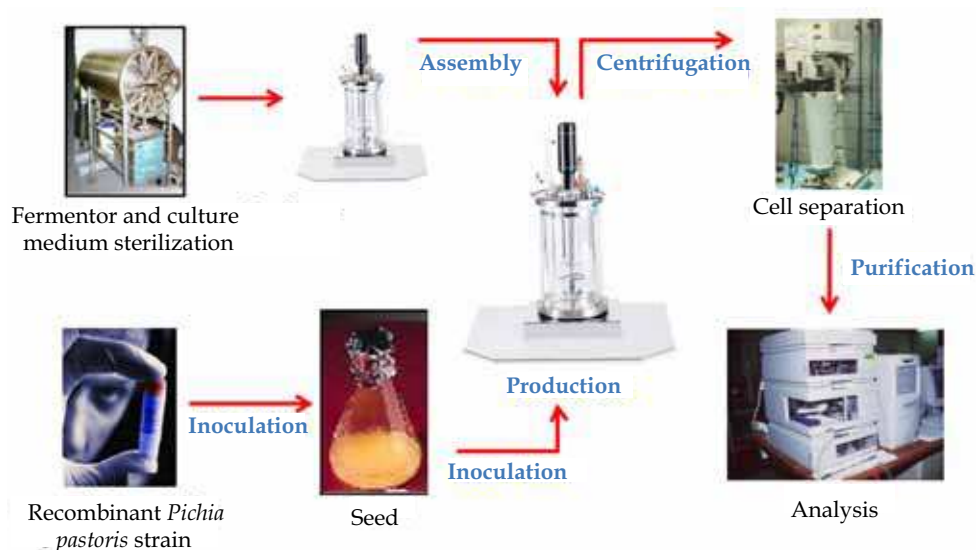
Fig. 10. Recombinant hormone production bioreactor. This illustrates the stages of the biotechnological process of production, from the preparation of the fermentor and the medium, to the analysis of proteins in the fermented culture medium
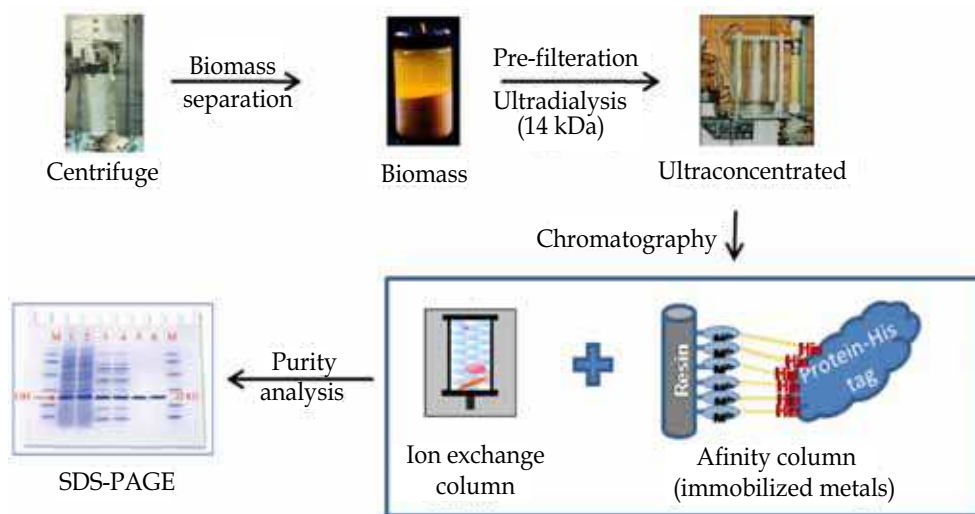

Fig. 11. "DownStream" Process for recombinant hormones. The medium was separated from the biomass by centrifugation. This was pre-filtered with a 0.45μm membrane, and ultra-dialyzed in a cutoff membrane of 10 kDa. It was then passed through an anion exchange column (FF-QS) and/or in an affinity column by immobilized metal (IMAC). Purity was analyzed in PAGE-SDS, quantified, and finally lyophilized and stored for later use

11. Semipurification of recombinant hormones

Figure 11 shows the process of purification or "downstream" that was followed for each recombinant hormone produced.

Each culture medium containing the recombinant hormone was ultra-dialyzed and ultraconcentrated. The pore membrane used was 14 kDa. At the same time it was ultradyalized. The ultraconcentrate obtained was lyophilized to preserve the samples and all the powder was recovered in 50 mL plastic tubes and weighed. Total protein was quantified in each lyophilized culture medium with the Bradford method (52). Samples were stored at -20°C until use.

Each sample was prepared for loading into a chromatographic column. Five mg of the total protein from each sample was adjusted to the conditions of the loading buffer. A column was loaded with Q-Sepharose fast flow anion exchange resin; column filling with resin was carried out by gravity flow. After passing the sample, washing was done with 10 mL of loading buffer. To recover the proteins from the column, 15 mL of elution buffer (loading buffer plus NaCl) was passed with an ionic strength increased sequentially with NaCl. Total protein was measured by the Bradford method (52) for each of the fractions recovered to see what percentage of the total they represented.

The collected fractions were visualized on discontinuous polyacrylamide gel concentrations of 4-15% under denaturing conditions (SDS-PAGE), and stained with Coomassie and silver techniques (53). The fractions were subjected to lyophilization, the powder was recovered and weighted, total protein was measured by the Bradford method. In addition, using PAGE-SDS the percentage of purity of the monomer was determined with ImageJ software. The samples were stored at -20°C until used for further analysis or to determine their biological activity.

12. Testing the biological activity of recombinant hormones

12.1 Lactogenic activity bioassay

The biological activity of hormones produced in *Pichia pastoris* recombinant was determined by their ability to promote proliferation of the Nb2 cell line, which comes from rat lymphoma (54, 55). GHs were also tested for their somatogenic activity in the adipocyte differentiation model based on the glyceraldehyde 3-phosphate dehydrogenase (GPDH) assay.

The cell-free culture medium was dialyzed and each hormone was quantified by gel densitometry. Dilutions of each recombinant hormones in the culture medium dialyzed were tested. Cell proliferation was determined by tetrazolium salt assay (MTT) (56) and was expressed as the average of three repetitions, in comparison with the positive control recombinant rat prolactin (rRPRL) and the negative control (culture medium without hormone). The concentrations tested were 0.001, 0.01, 0.1, 1, 10 and 50 nM of the following hormones, CFGH, ECGH, FCGH, HGH, HCS and RPRL. Upon completion of the testing time, we proceeded to measure the effect of hormones on cell proliferation by MTT assay (56). The activity was evaluated by color generation based on the reduction of tetrazolium salt (methyl 3- [4,5-dimethylthiazol-2-yl] -2,5-diphenyl tetrazolium) from yellow to purple forming crystals by Nb2 cell metabolism. An increase of living cells is reflected by increased

metabolic activity. This increase directly correlates with the formation of absorbance monitored formazan crystals; i.e., the greater the number of cells, the greater the increase in cell metabolism with greater formazan formation and greater biological activity.

The bioassay was carried out in triplicate in a humid atmosphere with 5% CO_2-95% air at 37°C. After the incubation period of 3 days, we added 10μL of MTT (to a final concentration of 0.5 mg/mL) to each well. Samples were incubated for 4 hrs under the atmospheric conditions mentioned above. After this time, we added 100 uL of formazan solubilizing solution (10% SDS in 0.01 M HCl) to each of the wells. These were left incubating overnight under the same atmospheric conditions. It was verified that the precipitate of formazan purple crystals had completely dissolved and absorbance was measured at 590 nm using an ELISA plate reader.

12.2 Somatogenic bioassay

To test the biological activity of goat GH, we assayed fibroblasts from cell line 3T3-F442A (pre-adipocytes) and with the glyceraldehyde 3-phosphate dehydrogenase (GPDH) assay as described in (18, 57). Cells were exposed to different concentrations of CHGH in the medium. The positive control was 10% fetal bovine serum (FBS) (v/v) the negative control was 0.25% FBS (v/v). The cells were incubated for 12 days, were harvested and proteins extracted; the supernatants were frozen in aliquots for subsequent tests. Specific activity of GPDH was measured in cell extracts by NADH oxidation measured in a spectrophotometer at 340 nm; 40 μg of protein was used. CHGH demonstrated biological activity in the essay. The same was doing with the others hormones showing biological activity.

13. Contributions

These are the first reports in the literature of the production by biotechnology of the recombinant GHs described here using the *Pichia pastoris* methylotrophic system. The new strains of *Pichia pastoris* constructed with GH cassettes such as canine-porcine, equine, feline, and caprine, complemented our strains previously constructed for GHs of 22 y 20 kDa, HCS and bovine GH (unpublished results), making our collections of GH clones the largest one for this protein family in the world as far as we know.

All hormones were efficiently produced, processed, and secreted into the culture media. Each hormone constituted the main proteic band among proteins secreted by *Pichia pastoris* analyzed by SDS-PAGE. The phenotypes Mut[s] resulted the best for producing GHs, as was the case for CFGH (58) and for HCS (Ascacio-Martínez y Barrera-Saldaña, unpublished results).

All our constructed strains had correct processing of their heterologous secretion *Saccharomyces cerevisiae* alpha mating factor signal peptide in the maturation of the recombinant GHs. They were secreted into the culture media in their native and bioactive form (49, 59, 60).

Using a bioreactor increased the production of the recombinant proteins 10 to 20 times compared to an Erlenmeyer flask. Ionic chromatography was a good option in all cases for semipurification of rGHs and HCS in this system. All hormones showed biological activity in the Nb2 essay, showing that human GH had more activity than animal GHs. The same

happened in the pre-adypocite system (3T3), concluding that *Pichia pastoris* produces, processes, and secretes rGHs in the bioactive form.

Biotechnological platforms were developed that made possible to move from the construction of the producer clones to the bioassay of the semipurified recombinant protein. With the technology here developed we have acquired the capacity to advance in scaling the production of veterinary and livestock rGHs for their field tests.

14. Perspectives

We have developed an efficient expression system and laboratory fermentor-scale production biotechnological platform with which to partner with the productive sector to produce virtually any GH of human and animal origin with acceptable quantity, quality and activity to start field evaluations. Doing that, would allow us investigate their full potential in animal biotechnology, to then offer them as an option in veterinary treatments and to stimulate cattle production and the health of competition animals. In addition to the obvious veterinary or livestock application, their availability will also allow the discovery of unexpected biological activities for animal wellness.

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16. References

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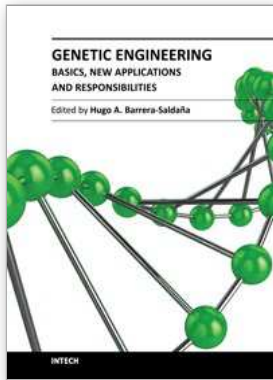
Genetic Engineering - Basics, New Applications and Responsibilities
Edited by Prof. Hugo A. Barrera-Saldaña

Leading scientists from different countries around the world contributed valuable essays on the basic applications and safety, as well as the ethical and moral considerations, of the powerful genetic engineering tools now available for modifying the molecules, pathways, and phenotypes of species of agricultural, industrial and even medical importance. After three decades of perfecting such tools, we now see a refined technology, surprisingly unexpected applications, and matured guidelines to avoid unintentional damage to our and other species, as well as the environment, while trying to contribute to solve the biological, medical and technical challenges of society and industry. Chapters on thermo-stabilization of luciferase, engineering of the phenylpropanoid pathway in a species of high demand for the paper industry, more efficient regeneration of transgenic soybean, viral resistant plants, and a novel approach for rapidly screening properties of newly discovered animal growth hormones, illustrate the state-of-the-art science and technology of genetic engineering, but also serve to raise public awareness of the pros and cons that this young scientific discipline has to offer to mankind.

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