Introduction

The development of Aquatic Biotechnology has supported the application of experimental techniques to manipulate fish growth, as diets enriched in specific protein nutrients and administrating hormones like: prolactin, insulin and growth hormone (GH) [1].

GH is a single chain polypeptide of approximately 22 kDa, produced by the pituitary gland and with pleiotropic functions among vertebrates. It mainly regulates body growth, being also involved in reproduction, immunity and osmoregulation in teleost fish. In order to obtain the tilapia (Oreochromis hornorum) growth hormone (tiGH) in *P. pastoris* cells, its gene was cloned into expression vectors in both with and without a heterologous secretion signal. The tiGH, obtained either intracellularly or extracellularly in *P. pastoris* cells, was characterized showing its production as associated to the cellular rupture precipitate with an approximate molecular weight of 22 kDa; or being secreted with an approximate molecular weight of 18 kDa, respectively. The mass spectrometry analysis of the recombinant protein obtained in the culture supernatant corroborated the identity of the protein as tiGH but lacking 46 aminoacids of its carboxyl terminal sequence. The tiGH biological activity of *P. pastoris* intact cells producing this protein was carried out in tilapia larvae (Oreochromis sp.), showing, for the first time, that it is possible to stimulate fish growth by immersion baths with recombinant tiGH-producing yeast. On the contrary what was previously postulated for mammals, the evaluation of *P. pastoris* cells expressing the truncated variant of tiGH demonstrated that this protein is also able to stimulate growth and immune system in fish. This is the first report of a biologically active, truncated GH variant in fish.
Results and discussion

Obtaining the tilapia growth hormone intracellularly in \textit{Pichia pastoris} cells

The DNA fragment coding for tiGH was PCR-amplified from the pTTI plasmid, lacking its signal peptide for intracellular expression \cite{14}, and was inserted into the pNAO vector to obtain the pP-tiGH plasmid with the aim of obtaining a \textit{P. pastoris} strain producing tiGH intracellularly. It bears an integration unit composed of homologous regions to the AOX1 locus of \textit{P. pastoris} genome (5’AOX1 and 3’AOX1) for its integration by homologous recombination. The pP-tiGH was digested with the \textit{Cla} I endonuclease and used for transforming of \textit{P. pastoris} cells strain MP36 by electroporation. Southern-blot analyses of genomic DNA, isolated from five transformants showed a 2.8 kb band in two of them, indicative of double crossovers between the expression cassette and the endogenous AOX1 gene in the host genomes (gene replacement integration) \cite{28}. This event generates clones of slow methanol consumption (Mut) \cite{29}. The genomic DNA of the untransformed \textit{P. pastoris} strain MP36 was used as negative control of the assay, being detected the 5.5 kb-banding corresponding to the native AOX1 gene. Trans-formant C9 was denominated MP36/pP-tiGH and selected for further expression studies.

The expression of tiGH from the MP36/pP-tiGH strain was analyzed in the intracellular fraction under reducing conditions by 15% SDS-PAGE, showing a protein band of an approximate molecular weight between 21 and 31 kDa \cite{28}. Its molecular weight was similar to the 22 kDa of the tiGH molecule previously obtained in \textit{E. coli} cells. Protein identity was corroborated by Western blot analyses with an anti-tiGH-HRP monoclonal antibody (Figure 2B in reference \cite{28}). This recombinant protein was absent in analyzed samples of the native MP36 strain.

The intracellular tiGH obtained in \textit{P. pastoris} cells reached approximately 4.3% of the total protein content. This value was determined by optical densitometry from 15% SDS-PAGE gels (Figure 2A in reference \cite{28}). Moreover, the cellular density at the end of culture was approximately of 300 g/L as determined from the wet biomass weight. Recombinant tiGH expression levels were estimated by protein Dot blot, with the aid of a tiGH standard obtained in \textit{E. coli} of known concentration \cite{14}, reaching approximately 1.5 g/L of culture (data not shown).

Effect of tiGH from the MP36/pP-tiGH strain on growth of red tilapia (\textit{Oreochromis sp.}) larvae

An experiment was carried out to evaluate the biological activity of tiGH produced by the MP36/pP-tiGH \textit{P. pastoris} strain on stimulating growth of 3-days post-thatchling tilapia larvae. Intact yeast cells containing tiGH were administered by immersion baths, thrice a week for 6 weeks. Larvae body weight increased significantly four weeks after starting treatment with \textit{P. pastoris} tiGH-producing cells, compared to the negative control group treated with untransformed \textit{P. pastoris} cells (p < 0.0001) (Figure 4 in reference \cite{28}). Although the stimulatory effect of recombinant GH being administered by immersion baths has been previously demonstrated in fish \cite{18, 20}, this is the first report using \textit{P. pastoris} cells expressing a fish GH for that purpose. Previous demonstrations have only used diets enriched with GH-expressing yeast \cite{27,25}.

Obtaining an extracellular variant of tiGH in \textit{Pichia pastoris} cells

The PCR-amplified 0.8 kb DNA fragment from the pTTI plasmid \cite{14} that codes for tiGH was cloned into the pPS7 vector in frame between the \textit{Saccharomyces cerevisiae} suc signal peptide sequence and the GAP terminator, generating the construct pPS7-tiGH. This vector bears an AOX1 homologous integration unit similar to that described above for the pP-tiGH construct.

The pPS7-tiGH construct was digested with the \textit{Sph} I restriction endonuclease and used for transforming of \textit{P. pastoris} cells strain MP36 by electroporation. Southern blot analysis of genomic DNA showed four out of five transformants with a DNA band of approximate molecular weight between 28.7 and 29 kDa indicating the homologous integration by double crossover of the expression cassette (integration by AOX1 gene replacement) \cite{28}. These clones showed the slow methanol consumption (Mut) \cite{29} phenotype. The 5.5 kb band corresponding to the native host AOX1 gene was observed in the genomic DNA of the control, untransformed \textit{P. pastoris} MP36 strain. The transformant corresponding to lane 1 in figure 1 \cite{30} was selected for expression studies, being denominated MP36/pPS7-tiGH.

The Western blot analysis of culture supernatants from 15% SDS-PAGE under reducing conditions showed a protein band immunoreactive to an anti-tiGH monoclonal antibody. Its molecular weight was between 15 and 25 kDa (Figure 1), lower than both the protein banding of an approximate molecular weight between 21 and 31 kDa (Figure 3 in reference \cite{28}) and the recombinant tiGH extracellular fraction.

Figure 1. Western blot analysis of tiGH gene expression by the MP36/pPS7-tiGH transformant strain in 15% SDS-PAGE gels under reducing conditions. Lane 1: MP36/pPS7-tiGH intracellular extracts. Lane 2: Supernatants of native MP36 strain. Lanes 3 and 4: MP36/pPS7-tiGH culture supernatants. MWM: Molecular weight marker.
22 kDa native tiGH [14] and the intracellular tiGH produced in P. pastoris cells [28]. The recombinant protein was absent in culture supernatants of the native untransformed MP36 strain. Its expression levels were estimated by Dot blot, comparing the previously mentioned tiGH standard obtained in E. coli [14], reaching 40 and 80 mg/L (data not shown). Mass spectrometry analyses corroborated the identity of the protein as tiGH but lacking the 46 carboxyl A similar experiment to evaluate the intracellular tiGH effect on growth of tilapia larvae was carried out, but using this time culture supernatants from the MP36/pPS7-tiGH strain containing the truncated tiGH to stimulate growth of 3-days post-hatching tilapia larvae. Treatment was applied by immersion baths thrice a week for 45 days. As for the intracellular variant, larvae body weight increased significantly four weeks after starting treatment with culture supernatants from MP36/pPS7-tiGH cells, compared to the negative control group treated with supernatants from untransformed P. pastoris cells (p < 0.0001) (Figure 2), reaching a 2.2-fold increase after 7 weeks (p < 0.0001) (Figure 2). These results demonstrated that the truncated tiGH variant lacking the 46 carboxyl terminal aminoacids from the native tiGH is biologically active.

Comparison of biological activities of the cellular lysate and culture supernatant from the respective intracellular and truncated tiGH variants-expressing cells

The stimulatory biological effects of both tiGH variants (intracellular complete tiGH or secreted truncated tiGH) on the innate immunity and growth of tilapia larvae were evaluated in of 3-days post-hatching larvae. Treatment was applied by immersion baths thrice a week for 45 days. Larvae were analyzed at 21 and 45 days after the start of the experiment for growing parameters and innate immune functions (lysozyme and hemmaglutination activities).

Growth stimulating activity

A significant increase in body weight was observed in day 21 for tilapia larvae treated with culture supernatants containing the truncated tiGH, compared to both the larvae control group (p < 0.001) and the group treated with lyses of the untransformed native P. pastoris strain (p < 0.05) (Figure 4 in reference [30]). Moreover, larvae treated with intracellular tiGH-containing P. pastoris cell lyses also significantly increased body weight compared to the untreated control group (p < 0.001) (Figure 4 in reference [30]).

Larvae treated with supernatants containing the truncated tiGH showed the best growing results 45 days after treatment. They had a 3.6-fold increase of body weight compared to the untreated group (p < 0.001), a 2.1-fold increase treated with untransformed P. pastoris culture supernatants (p < 0.001), 2.9-fold in the group treated with untransformed P. pastoris cell lyses (p < 0.001) and 1.6-fold in the group treated with intracellular tiGH-containing cell lyses (p < 0.05) (Figure 4 in reference [30]). Likewise, the group treated with the intracellular tiGH-containing lyses showed a 2.2- and 1.8-fold increase in body weight compared to the untreated control group (p < 0.001) and the group treated with untransformed P. pastoris cell lyses (p < 0.001), respectively (Figure 4 in reference [30]).

Effects of tiGH variants on innate immunity

Lysozyme activity

Lysozyme activity was undetectable in larvae homogenates 21 days after starting treatment. They had a 3.6-fold increase of body weight compared to the untreated group (p < 0.001), a 2.1-fold increase treated with untransformed P. pastoris culture supernatants (p < 0.001), 2.9-fold in the group treated with untransformed P. pastoris cell lyses (p < 0.001) and 1.6-fold in the group treated with intracellular tiGH-containing cell lyses (p < 0.05) (Figure 4 in reference [30]). Likewise, the group treated with the intracellular tiGH-containing lyses showed a 2.2- and 1.8-fold increase in body weight compared to the untreated control group (p < 0.001) and the group treated with untransformed P. pastoris cell lyses (p < 0.001), respectively (Figure 4 in reference [30]).
containing truncated tiGH compared to the untreated control group ($p < 0.05$) (Table 1 in reference [30]).

Hemagglutinating activity
There were no statistically significant differences 21 days after beginning treatment. Consistently, after 45 days, the highest hemagglutinating activity was obtained in the group treated with truncated tiGH-containing supernatants ($1024 \pm 1$), statistically significant when compared to the untreated control group ($128 \pm 1$; $p < 0.001$) and groups treated either with untransformed *P. pastoris* culture supernatants ($512 \pm 1$; $p < 0.01$), untransformed *P. pastoris* cell lysates ($256 \pm 1$; $p < 0.001$) or intracellular tiGH-containing cell lysates ($32 \pm 2.5$; $p < 0.001$) (Figure 5 in reference [30]). All the treatments stimulate haemagglutinin activity compared to non-treated group (Figure 5 in reference [30]). No differences among groups were found at day 21 (data not shown). This was the first report linking GH administration to lectin activation. In this sense, we cannot exclude a synergic or individual influence on this parameter of some factors present in culture supernatants activating innate immunity in fish, coming either from *P. pastoris* cells (intracellular and/or secreted) or even from culture medium components.

Effect on growth and survival of goldfish larvae (*Carassius auratus*)
The effect of truncated tiGH-containing supernatant on growth and survival of goldfish larvae was evaluated from 5-days post-hatching larvae. Treatment was applied by immersion baths thrice a week for 75 days. Treated larvae showed a 3.1-fold increase in body weight compared to the untreated group ($p < 0.0001$; Figure 3) after 45 days of started treatment, that remained two-fold after 75 days ($p < 0.0001$; Figure 3), also showing increased coloration (Figure 3B). Besides, truncated tiGH-containing supernatant also enhanced survival 22% after 75 days, compared to the animals of the control group.

Effect on growth of larvae and juvenile carp (*Cyprinus carpio*)
The truncated tiGH-containing supernatant was also evaluated in different developmental stages of trout (*Cyprinus carpio*). For this purpose, 5 days posthatching larvae and juveniles (1.2 ± 0.2 g) were treated by immersion baths thrice a week. Larvae were sampled from 15 to 30 days after starting the experiment, showing a 3.5-fold body weight increase compared to the control group ($p < 0.0001$) at day 15, which remained 2.2-fold ($p < 0.0001$) after 30 days (Figure 4).

Trout juveniles were sampled 0, 15 and 28 days showing significant increased body weight compared to the control group ($p < 0.05$), which was 1.5-fold after 28 days ($p < 0.0001$) (Figure 5).

Effect on growth and anatomical parameters of angelfish (*Pterophyllum scalare*) juveniles
The effect of truncated tiGH-containing supernatant on growth and quality-related parameters of trout was studied on 45 and 75 days of treatment. Consistently, after 45 days of treated, bars represent mean weight ± standard deviation (n = 30). Data were statistically analyzed by a Student’s t test with and without Welch’s correction on days 45 and 75, respectively. Asterisks (***) indicate $p < 0.0001$. B) Photograph showing the phenotype of treated and control fish after 75 days.

ornamental fish was also evaluated in angelfish juveniles (3.1 ± 0.6 g) receiving immersion baths thrice a week. Fish showed gained weight 1.6-fold after 30 days than those of the untreated group (p < 0.01, Figure 6). Besides, their body length (p < 0.01), width (p < 0.01), and length of the dorsal fin (p < 0.001) significantly increased in the treated group (Table 1).

These results demonstrate that treatment with truncated tiGH-containing supernatants not only increases growth, but also stimulates quality-related body parameters in ornamental fish. It was also demonstrated its action in larvae and juvenile fish developmental stages.

**Conclusions**

For the first time, these findings provide differences of the hormone receptor interaction and the structure-function relationship between fish and mammals, as well contribute to understand the interactions of GH with its receptors in fish. Moreover, because the truncated tiGH variant being supplied in *P. pastoris* culture supernatants showed to be a potent...
enhancer for growth, survival, quality of larvae and immune parameters, it can be used in aquaculture mainly for ornamental fish production. It allows to have treated fish of increased growing rates and more resistant to stress conditions and infections, and reducing production costs.

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<tr>
<th>Table 1. Effect of truncated tGHI-containing P. pastoris supernatants on body parameters of scalar juvenile fish</th>
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<tr>
<td>Body length (mm)</td>
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* Data are expressed as the mean ± standard deviation (n = 10).
** Statistical significance by Student’s t test with Welch’s correction for p < 0.01.
*** Statistical significance by Student’s t test with Welch’s correction for p < 0.001.


