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Extensive Characterization of *Arapaima gigas* Follicle-Stimulating Hormone (ag-Fsh) Synthesized in HEK293 Cells

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Abstract

The cDNA sequences of the common gonadotrophic hormone α-subunit (ag-Gthα) and of the specific follicle-stimulating hormone β-subunit (ag-Fshβ) of the giant Amazonian fish *Arapaima gigas* have been previously isolated by our research group. A synthesis of ag-Fsh in HEK293 cells and its purification and preliminary characterization were also carried out. In the present work, ag-Fsh was obtained and purified from the same host cells, and an extensive physical chemical characterization was performed via RP-HPLC, HPSEC, and MALDI-TOF-MS. Ag-Fsh, when compared to human FSH (hFSH), showed a higher hydrophobicity by RP-HPLC and a higher molecular mass (MM) via HPSEC. The same higher MM was also confirmed via MALDI-TOF-MS: 35,353 Da for ag-FSH against 31,969 Da for hFSH. Its immunological activity was also confirmed via an hFSH ELISA, in comparison with the highly purified pituitary preparation of hFSH-AFP7298A, from the National Hormone and Pituitary Program (NHPP-USA), with a clear response that was, however, 1560-fold lower when compared to the purest preparation. Finally, an in vitro bioassay, based on the stimulated release of 11-ketotestosterone (11-KT) from immature *A. gigas* testis, quantified ag-Fsh biological activity in comparison with human chorionic gonadotropin (hCG) and with human pituitary FSH-AFP7298A, showing a potency clearly higher than that of hCG. This suggests that injections of ag-Fsh in *A. gigas* and ag-Fsh cDNA gene therapy applications could be used for improving the reproductive functions of this threatened species.

Keywords: *Arapaima gigas*; follicle-stimulating hormone; in vitro bioassay; immature testis; 11-ketotestosterone

Key Contribution: This work presents a detailed physicochemical and biological characterization of recombinant ag-Fsh from *Arapaima gigas*, underscoring its potential for enhancing reproductive biotechnologies aimed at conserving this endangered species.

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1. Introduction

Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are two pituitary gonadotrophic hormones that regulate reproductive processes such as gametogenesis and

follicular growth in all vertebrates, including fish [1–4]. These gonadotropins can, therefore, be employed to improve breeding in reproductive centers that could be established for endangered and overexploited species of fish, particularly important for alimentary and commercial purposes, like the giant Amazonian fish *Arapaima gigas*, called pirarucu in Brazil [5–8].

A. gigas belongs to the family of Arapaimidae and to the order of Osteoglossiformes; it is native to the Amazon river basin, present in Brazil, Ecuador, Colombia, Peru, and Bolivia, and can reach 3 m in length and weigh up to 250 kg [9]. This species is very important for human nutrition and extractivism in the region, but unfortunately it is in danger of disappearing because of human and industrial exploitation [5,10–12] and because of its limited reproductive capacity in captivity [6,7,13–16]. For these reasons, a study directed to the synthesis of *A. gigas* gonadotropins and also of *A. gigas* growth hormone and thyrotropin [17] can offer interesting applications concerning improvement in reproduction of this important species, also including a gene therapy approach.

Several recombinant fish Fsh and/or Lh have been produced in baculovirus systems for red seabream [18], Japanese eel [19], and goldfish [20,21]. Others have been produced in *Pichia pastoris* for tilapia [22,23], zebrafish [24], yellowtail kingfish [25], carp [26], and catfish [27]. Fish gonadotropins were also produced in CHO cells for Manchurian trout [28], Japanese eel [29], in the soil amoeba *Dichtyostelium discoideum* for African catfish [30], and in the insect cell line Sf9 for European sea bass [31]. It is important to note the studies carried out by Molés et al. [32–34] on Fsh extracted from European sea bass pituitaries and by Mazón et al. [35] concerning gene therapy application on the same sea bass. Direct applications of gene therapy, including electroporation, have been carried out in different fish species by Rambabu et al. [36], Lv et al. [37], and Iwaizumi et al. [38].

Given the extensive experience of our research group with human pituitary gonadotropins, in previous work we cloned and characterized the cDNA of the α-subunit (GTHα) of *A. gigas*, which is common to the two gonadotropins [39] and both hormone-specific β-subunits (ag-Fshβ and ag-Lhβ) [40]. These subunits form, together with the α-subunits, the two heterodimeric, non-covalently bound, glycoprotein hormones. In the latter work, ag-FSH was also synthesized in human embryonic kidney 293 (HEK293) cells [40], the alpha and beta subunit cDNA being separately inserted into a pcDNA ™ 3.4- TOPO® vector and preliminarily purified and characterized. We have chosen HEK293 cells for our ag-Fsh production because we needed a type of host cell that could reliably and efficiently synthesize a glycoprotein hormone, according to our experience with human pituitary hormones. The CHO alternative was, in our case, normally less efficient. In the present work, purified ag-Fsh was extensively characterized via reversed-phase high-performance liquid chromatography (RP-HPLC), high-performance size-exclusion chromatography (HPSEC), matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS), immunoassay (ELISA), and, in particular, by an in vitro bioassay. In this bioassay, the biological activity of the ag-Fsh preparation was determined via release of 11-KT from immature *A. gigas* testis in comparison with hCG [41] and with the International Reference Preparation of pituitary hFSH (AFP7298A from NHPP).

A schematic figure representing the main signaling pathways in the control of *A. gigas* reproduction involving *A. gigas* growth hormone (ag-Gh), ag-Fsh, and ag-Lh was reported in our previous work [42], adapted from Migaud et al. [43].

Purified ag-Gh, ag-Fsh, and ag-Lh cDNAs can be used for preliminary gene therapy applications directed to fertility studies and to improving reproduction in captivity of this important species of fish. Ag-Gh cDNA will be particularly useful, considering also its action on somatic growth and muscle mass increase.

2. Materials and Methods

2.1. Qualitative and Quantitative Analysis of Purified ag-Fsh

Purification of ag-Fsh, derived from conditioned medium of transfected HEK293 cells, was performed by two chromatographic steps, RP-HPLC and HPSEC, as previously described, obtaining a total of 400 µg of purified material (~80 mg/L) [40]. A sample of purified material (50 µL) was then analyzed via RP-HPLC on a Vydac C4 column (300 Å pore size, 5 µm particle size, 25 cm × 4.6 mm I.D.) from Grace-Vydac (Fisher Scientific, Waltham, MA, USA). The elution and gradient conditions were specifically set up for maintaining the FSH heterodimer undissociated [44]. The same quantity of ag-Fsh solution (50 µL) was applied to an HPSEC G200 column (125 Å pore size, 10 µm particle size, 60 cm × 7.5 mm I.D.) from Tosoh Bioscience (Tokyo, Japan), eluting as described [45]. The mobile phase was 0.15 M NaCl, sodium phosphate buffer (pH 7.0; 0.02 M) with a flow rate of 1.0 mL/min. In both analyses, a reference preparation of human pituitary FSH-AFP7298A from the National Hormone & Peptide Program (NHPP, Torrance, CA, USA) was used.

2.2. MALDI-TOF-MS

The MM (molecular mass) of the ag-Fsh preparation was determined (using approximately 30 µg of glycoprotein) by MALDI-TOF-MS on an UltrafleXtreme equipment (Bruker Daltonics, Bremen, Germany), operated in the linear positive ion mode. A diluted glycoprotein sample (1:5; 1:10, and 1:20 from a 1 mg/mL solution) was mixed 1:1 with MALDI matrix solution (sinapinic acid 7 mg/mL in 0.1% trifluoroacetic acid and 50% acetonitrile) and spotted directly onto the MALDI plate (1 µL). The analysis was performed in linear positive mode in the range of 5000–40,000 Da. The Open Source Mass Spectrometry tool data processing software was used for increased resolution analysis in the range of 17,000–19,000 Da.

2.3. Immunoenzymatic Assay Based on hFSH ELISA

The immunological activity determination of purified ag-Fsh was carried out utilizing a human FSH ELISA kit (FSH SYM, Vyttra Diagnostics, São Paulo, Brazil) following the manufacturer's instructions. The kit standard curve was based on six doses determined in duplicate, and the absorbance reading was carried out at 450 nm in a 96-well plate reader (Multiskan EX, Thermo Electron Corp., Vantaa, Finland). The doses provided were 0, 5.0, 10.0, 25.0, 50.0, and 100.0 IU/L, while the calculated standard curve equation was Yabs = 0.029 XIU/L + 0.557 (n = 6; r = 0.924; $p < 0.01$). Based on WHO protocols defining the potency of highly purified hFSH as 16,000 IU/mg, 1 ng of ultrapure hFSH was found to correspond to 0.016 IU. This relation was, therefore, taken into account when carrying out immunoassay curves based on the commercial standard of the kit or on the pituitary hFSH reference preparation from NHPP. The negative control corresponded to the conditioned medium that was submitted to the same transfection procedure with the expression vectors not containing the ag-Fsh α- and β-subunit cDNAs and used at different dilutions (1:100, 1:500, and 1:1000).

2.4. In Vitro Bioassay Based on the Release of 11-Ketotestosterone (11-KT) from Immature A. gigas Testes

Testes from three *A. gigas* (two-year-old) were dissected, and the immature stage was confirmed according to Nuñez and Duponchelle [46]. The use of homogenized testes aimed to reduce variability from individuals and from the testes functional regionalization, thus providing a more representative profile of *A. gigas* immature steroidogenic activity. The dissected testes were ground using a scalpel, uniformly homogenized, and kept frozen at

−80 °C until assays were performed. For assays, 20 mg aliquots of testes were pre-incubated for 1 h at 20 °C with 300 µL of solution containing 0.88 mg/mL of Dulbecco's Modified Eagle Medium (Gibco®, Life Technologies™, Grand Island, NE, USA), 0.5 mg/mL of glucose (Equi-plex®, Aparecida de Goiânia, Brazil), and 1.0% *w*/*v* antibiotics with antimycotics (Gibco®, Life Technologies™, Carlsbad, CA, USA). After pre-incubation, this medium was pipetted off and changed with 300 µL of fresh medium containing various concentrations of the hormones under analysis: ag-Fsh (produced in-house), 1, 2.5, 5, 25, and 100 ng/mL; pituitary hFSH, 0.1, 0.5, 1.0, and 4.0 ng/mL; and hCG (Sigma Aldrich CG5-1VL, St. Louis, MO, USA), 10, 20, 40, 100, and 500 ng/mL. These incubations continued for 24 h at 20 °C. Negative controls were performed with medium alone or with different concentrations of Bovine Serum Albumin-BSA (Sigma Aldrich 5470-1G, St. Louis, MO, USA). After incubation, the medium was collected and stored at −80 °C until ELISA analysis. For this analysis 50 µL of medium were used in order to determine the 11-KT concentration according to the ELISA kit protocol (Cayman Chemical Inc., Ann Arbor, MI, USA), reading the microplates at 405 nm using an ELX808 reader (Biotek, Swindon, UK). The 11-KT ELISA assay was previously validated for *A. gigas* by Torati et al. [16]. Results were back calculated in pg per 100 mg of testes tissue. Dose–response curves, relating the logarithm of the applied hormonal dose (ln X, ng/mL) versus the amount of released 11-KT (Y, pg of 11-KT/100 mg testes tissue), were thus obtained.

2.5. Statistical Analysis

Curve equations were generated by fitting the data to a quadratic relationship. The quadratic, linear, and independent coefficients, calculated from the different experimental groups, were compared via the F-test method included in the Prism 5.0 package (GraphPad Software Inc., La Jolla, CA, USA). Means and curves were considered to be statistically different, representing distinct treatment effects, when the *p*-value was <0.05.

3. Results

3.1. Qualitative and Quantitative Analysis of ag-Fsh by RP-HPLC and HPSEC

Figure 1 shows the qualitative and quantitative analysis of purified ag-Fsh. The asymmetric peak of ag-Fsh obtained by RP-HPLC (Figure 1B) and the higher amount of material when compared to the HPSEC peak (Figure 1D) may indicate the presence of different glycosylated isoforms, recalling that this purified ag-FSH obtained from HEK293 cells does not necessarily have a glycosylation pattern similar to pituitary hFSH. The same chromatograms also indicate a higher hydrophobicity of ag-Fsh compared to hFSH. HPSEC exhibited two perfectly symmetric peaks of either hFSH or ag-Fsh; considering its retention time, the latter should have a larger MM. From Figure 1D, showing the presence of 4 µg in the ag-Fsh peak, we could calculate a total yield of 8 mg/L obtained from the HEK293 culture.

3.2. MALDI-TOF-MS of ag-FSH, in Comparison with the hFSH Standard

Figure 2 compares a MALDI-TOF-MS analysis for the pituitary hFSH-NHPP standard to our preparation of ag-Fsh. In Figure 2A the two α- and β-subunits of hFSH are well separated, providing a heterodimeric form of 31,969 Da, while the MM of a similar pituitary hFSH preparation, determined in previous work, was 30,976 Da [44]. In Figure 2B the same analysis for ag-Fsh shows that the two α- and β-subunits exhibit very similar MM (17,609 Da and 17,743 Da). The MM of the heterodimeric form is therefore 35,352 Da. Again, this significant difference is surely due to different glycosylation patterns, since the MM of the protein backbones of hFSH and of ag-Fsh are 22,689 Da and 22,322 Da, respectively. Considering, moreover, that the identities of the amino acid compositions

between hFSH and the ag-Fsh α- and β-subunits have been found to be 60.0% and 45.1%, respectively [40], we hypothesized that an anti-hFSH polyclonal antibody acting in a traditional immunoassay could also have some affinity for ag-Fsh.

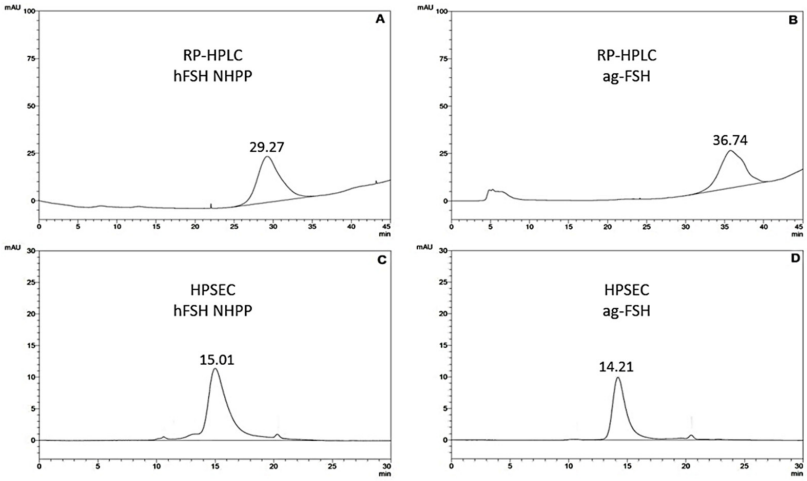

Figure 1. Qualitative and quantitative analysis of the final product (ag-Fsh) after two purification steps. (**A**) Reference preparation of pituitary hFSH from NHPP, 5 µg/5 µL, via RP-HPLC. (**B**) Analysis of the final product, ag-FshH (4.8 µg/50 µL), via RP-HPLC. (**C**) Reference preparation of pituitary hFSH from NHPP, 5 µg/5 µL, via HPSEC. (**D**) Analysis of the final product of ag-Fsh (4 µg/50 µL), via HPSEC.

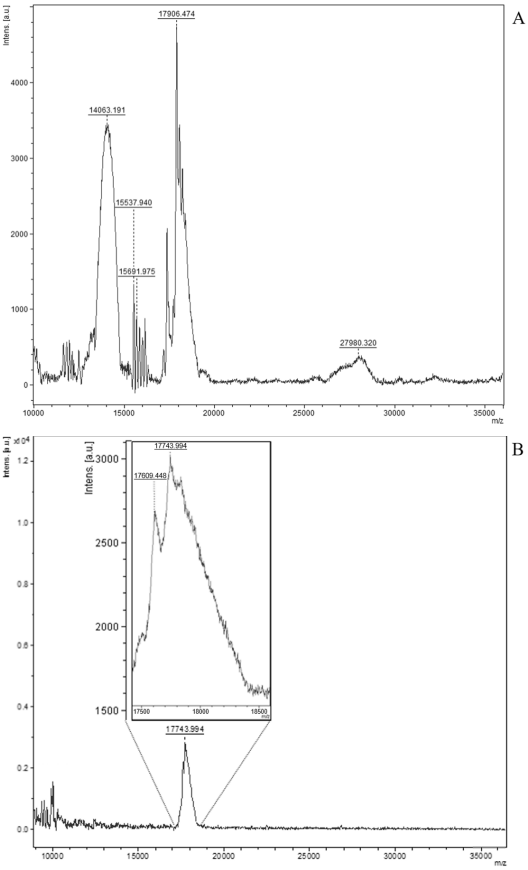

Figure 2. (**A**) MALDI-TOF-MS analysis of pituitary hFSH reference preparation (FSH-SIAFP-B-3 from the National Hormone & Peptide Program, NHPP, Torrance, CA, USA): hFSH, MM = 31,969 Da and (**B**) ag-Fsh, MM= 35,352 Da. In (**B**) the peaks of interest are also shown at a higher resolution.

3.3. Immunological Activity Determination of ag-Fsh

Indeed, it was possible to demonstrate the immunological activity of ag-Fsh against an anti-hFSH polyclonal antibody (Figure 3). The standard kit preparation, as expected, was much less active than the highly purified NHPP reference preparation of pituitary hFSH. The pituitary hFSH reference preparation from NHPP presented the highest immunological potency (slope of the curve = 0.277), while ag-Fsh, though still active, had a substantially lower potency when compared to hFSH-NHPP.

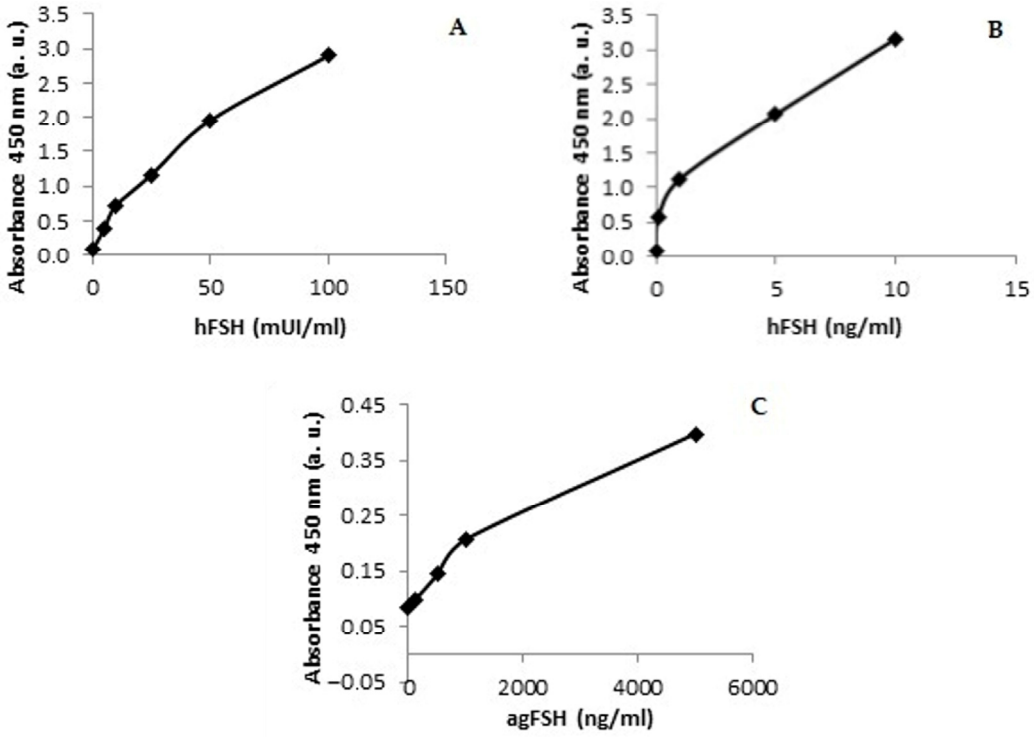

Figure 3. Dose–response curves based on the human FSH ELISA kit applied to immunological activity determination of purified ag-Fsh. (**A**) Standard curve based on ELISA kit reagents: YAbs = 0.029 XIU/mL + 0.557 (n = 6; r = 0.924; $p < 0.01$), i.e., 9.5-fold less active than hFSH-NHPP. (**B**) hFSH reference preparation from NHPP, used also as a quantitative standard: $Y_{Abs} = 0.277\ X_{ng/mL} + 0.501$ (n = 5; r = 0.971; $p < 0.01$). (**C**) purified ag-Fsh: $Y_{Abs} = 0.177.10^{-3}\ X_{ng/mL} + 0.004$ (n = 5; r = 0.999; $p < 0.001$), i.e., ~1560-fold less active than hFSH-NHPP.

The standard kit component is presenting about 10-fold less activity than the NHPP reference preparation because its purity/potency is usually much lower than that of a highly purified preparation used as an international standard. The kit standard, moreover, is expressing its potency in international units and not in ng.

3.4. In Vitro Bioassay of ag-Fsh, Based on the Stimulated Release of 11-KT from Immature A. gigas Testes

Figure 4 presents the curves obtained in the in vitro bioassay, based on the stimulated release of 11-KT from immature *A. gigas* testes, induced by different hormones. Considering the slopes of the regression lines, ag-Fsh exhibits a slightly higher logarithmic dose–biological response profile than pituitary hFSH and hCG (1.51-fold and 1.39-fold greater slope, respectively). However, pituitary hFSH AFP7298A (NHPP) is biologically active at substantially lower hormone concentrations, indicating that it is probably the purest product, besides being a natural human pituitary hormone whose carbohydrate moiety is certainly quite different from ag-Fsh, which has been produced by human embryonic kidney cells (HEK293).

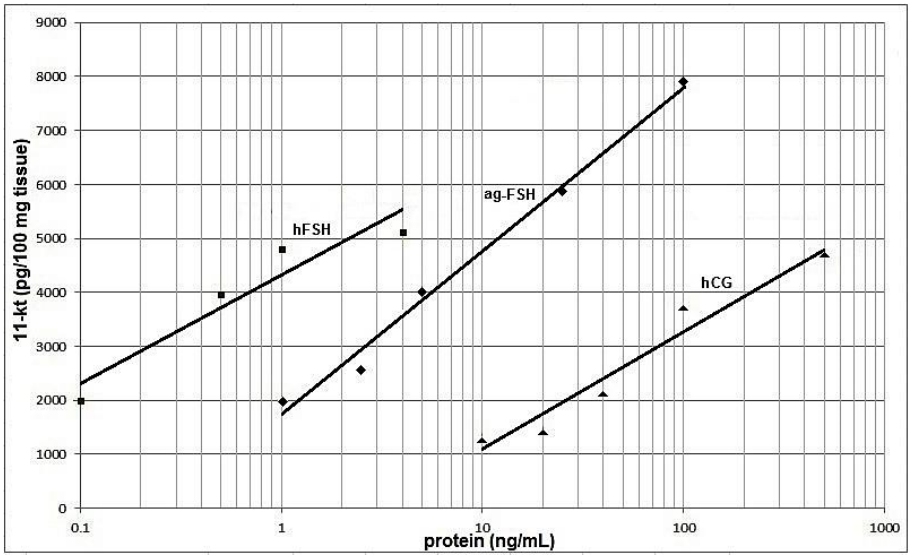

Figure 4. Dose-dependent 11-KT secreting activity of hFSH, ag-Fsh, and hCG in immature *Arapaima gigas* testis. hFSH: Y = 869.7 lnX + 4321 (n = 4; r = 0.952; $p < 0.05$); ag-Fsh: Y = 1318.5 lnX + 1752 (n = 5; r = 0.994; $p < 0.001$); hCG: Y = 947.3 lnX–1096 (n = 5; r = 0.972; $p < 0.01$).

4. Discussion

An extensive characterization of purified ag-Fsh obtained in HEK293 cells has been carried out via physicochemical, immunological, and biological methodologies. As far as we know, concerning fish, only Japanese eel Fsh and Lh, have been synthesized in HEK293 cells [47]. As already observed, our ag-Fsh production in HEK293 cells was about 8 mg/L of highly purified hormone. Much higher yields have been reported in the literature, such as in the case of Japanese eel gonadotropins, produced by baculovirus in silkworm (Bombyx mori) larvae, where up to 5.6 mg/10 mL and 3.5 mg/10 mL of partially purified Fsh and Lh, respectively, were obtained [19].

The literature data indicate that it is possible to simplify the production process and increase production efficiency, as approximately 50% of the cases reported involved fish Fsh and Lh obtained as a single chain [21–24,26,28,35,37]. In our case we preferred to synthesize the ag-Fsh alpha and beta subunits, introducing them separately into the TOPO ® vector in order to obtain the heterodimeric protein more similar to the native one.

Considering that one of our goals is the application of gene therapy to *Arapaima gigas*, we note that this methodology has mostly been applied to juvenile fish, such as sea bass [35], orange-spotted grouper [37], and Japanese flounder [38]. This is quite important, as applying gene therapy via electroporation to giant adult *A. gigas* presents significant challenges. However, an interesting study by Mazón et al. [35] approached gene therapy in juvenile European sea bass using two intramuscular injections of single-chain Lh plasmid, collecting samples on days 0 and 3 and weekly until day 30, without electroporation. This methodology allowed them to reach Lh plasma levels exceeding 100 ng/mL (negative control ~10 ng/mL). Their observations indicate that electroporation, used only in adult fish, yielded ~9 ng/mL (negative control ~0.84 ng/mL) but resulted in a higher percentage of fish responding to DNA. We conclude that, in some cases, avoiding such stressful methodology may be beneficial.

An important study by Molés et al. [32] focused on the purification and characterization of Fsh from sea bass (*Dicentrarchus labrax*) pituitaries. The molecular masses of the heterodimer (28.5 kDa), α-subunit (12.6 kDa), and β-subunit (13.6 kDa) were 8–17% lower than their human counterparts [44]. As expected, sea bass Fsh activated the Fsh receptor

expressed in HEK293 cells, stimulating the release of E2 from sea bass ovary and 11-KT from testicular tissue cultured in vitro.

Previous work [40] demonstrated that the α-subunit (ag-Gthα) protein backbone has a molecular mass of 10,489 Da, while the ag-Fsh β-subunit has 11,833 Da. For heterodimeric ag-Fsh, this corresponds to a total protein backbone of 22,322 Da. Given that ag-Fsh was synthesized in human HEK293 cells, its glycosylation is undoubtedly qualitatively and quantitatively different from that of natural pituitary *A. gigas*-Fsh. We considered, however, that an in vitro biological activity, even higher than that of hCG, confirmed that a clearly different carbohydrate composition could not totally suppress the biological activity of our synthesized ag-Fsh.

Characterization via RP-HPLC and HPSEC revealed that the heterodimeric protein differs from human hFSH in terms of hydrophobicity, exhibiting a longer retention time in RP-HPLC and a higher molecular mass in HPSEC. Both methodologies proved suitable for preliminary identity testing and qualitative and quantitative analysis of ag-Fsh.

Accurate molecular mass determination via MALDI-TOF-MS yielded a value of 31,969 Da for pituitary hFSH from NHPP, which is 3.2% higher than the 30,976 Da reported in previous work for a similar pituitary-extracted hormone [44]. The molecular mass of ag-Fsh, derived from the sum of its α- and β-subunits, was 35,352 Da, representing a 10.6% difference compared to hFSH-NHPP. Considering the protein backbone (22,322 Da), our synthesized ag-Fsh is estimated to have a carbohydrate moiety of 58.4%, whereas hFSH-NHPP (protein backbone = 22,689 Da) has a carbohydrate moiety of 40.9%.

The second characterization was based on the hypothesis that ag-Fsh should be recognized by anti-hFSH polyclonal antibodies. This was confirmed in the experiment shown in Figure 3, where the purest preparation of hFSH (hFSH-NHPP) exhibited 9.5-fold higher immunoactivity compared to the kit standard preparation of hFSH. In contrast, its immunopotency was ~1560-fold higher when compared to purified ag-Fsh, which remained immunoreactive against anti-hFSH antibodies. This serves as an additional, more specific identity test.

When evaluating a hormone and its potential applications, in vivo or in vitro bioassays provide the strongest evidence of identity and relative potency. The results of this bioassay, presented in Figure 4, are based on the release of 11-KT by immature *A. gigas* testes, stimulated by human CG, human FSH, or recombinant ag-Fsh.

Androgen release from immature testicular interstitial cells, leading to spermatogenesis initiation, serves as the basis for various homologous and heterologous in vivo and in vitro bioassays for measuring fish gonadotropin activity [41,48–53]. In this study, we developed a homologous in vitro bioassay using *A. gigas* immature testis tissue and compared the results with another homologous bioassay measuring aj-Fsh activity in *Anguilla japonica* immature testis tissue. Notably, eels do not mature spontaneously in captivity and require exogenous gonadotropin administration to induce gonadal maturation [41]. In contrast, *A. gigas* reaches final oocyte maturation but fails to ovulate under suboptimal captive conditions [15], so future assays should evaluate the effects of ag-FSH on the ovulation and production of maturation-inducing steroids (MIS) in the ovaries of adult fish, especially from captive populations for which this reproductive dysfunction remains.

By converting the hCG doses (IU) to nanograms, according to Kamei et al.'s [41] data on rjeFsh versus 11-KT according to a known highly purified preparation (1 IU = 50 ng) [54], we observe that approx. 150 ng/mL of pure hCG (~3 IU/mL) should stimulate the release of ~3500 pg 11-KT/100 mg of *A. japonica* testis tissue. Similarly, in the corresponding *A. gigas* testis bioassay, ~3700 pg of 11-KT/100 mg of *A. gigas* testis tissue was released by ~150 ng/mL of hCG, indicating that both species exhibit similar bioactive responses upon hCG stimulation. However, their responses to ag-Fsh or aj-Fsh stimulation may differ.

Further validation of the inter-assay and inter-laboratory precision and accuracy of the proposed bioassay is required to ensure reliable comparisons between different recombinant ag-Fsh productions or *A. gigas* pituitary extracts. Nevertheless, recombinant ag-Fsh cDNA represents an important tool for gene therapy applications aimed at improving the reproductive success of *A. gigas* in captivity.

5. Conclusions

Arapaima gigas Fsh, synthesized in HEK293 cells, has been purified and characterized via physicochemical and immunological assays and via an in vitro bioassay based on the stimulated release of 11-ketotestosterone from immature *A. gigas* testes. We consider the latter bioassay fundamental, since ag-Fsh could also be compared to other well-known bioactive hormones: hFSH and hCG. Since *A. gigas* has limited reproductive capacity in captivity, injections of purified ag-Fsh and especially ag-Fsh cDNA gene therapy applications will be used in collaboration with important fishing stations to improve the reproductive capacity of this important species.

Author Contributions: Conceptualization, L.S.T., P.B. and C.N.P.; methodology, E.R.L., T.C.F., J.E.O., T.C.A.S. and M.F.S.; software, E.R.L. and M.F.S.; validation, E.R.L. and J.E.O.; formal analysis, E.R.L., T.C.A.S. and L.S.T.; investigation, E.R.L., J.E.O. and M.F.S.; resources, L.S.T. and P.B.; data curation, L.S.T., P.B. and C.N.P.; writing—original draft preparation, L.S.T., P.B. and C.N.P.; writing—review and editing, L.S.T., P.B. and C.N.P.; visualization, E.R.L. and J.E.O.; supervision, P.B.; project administration, P.B.; funding acquisition, L.S.T. and P.B. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The animal study protocol was approved by the Ethics Committee for the Use of Animals (CEUA) of the National Research Center on Fisheries, EMBRAPA Fisheries and Aquaculture (Palmas, TO, Brazil), under protocol No. 09, dated 25 February 2016.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data supporting the findings of this study are openly available in the following repositories: Biomolecules at https://doi.org/10.3390/biom13010158; PLoS ONE at https://doi.org/10.1371/journal.pone.0183545; and Fish Physiology and Biochemistry at https://doi.org/10.1007/s10695-012-9730-1.

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Conflicts of Interest: The authors declare no conflicts of interest. Author Dr. Lucas Torati was employed by the company EMBRAPA. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

Abbreviations

ag-Fsh	*Arapaima gigas* Follicle-Stimulating Hormone
ag-Gh	*Arapaima gigas* Growth Hormone
ag-Lh	*Arapaima gigas* Luteinizing Hormone

hCG	human Chorionic Gonadotropin
RP-HPLC	Reversed-Phase High-Performance Liquid Chromatography
HPSEC	High-Performance Size-Exclusion Chromatography
MALDI-TOF-MS	Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry
ELISA	Enzyme-Linked Immunosorbent Assay
11-KT	11-Ketotestosterone
HEK293	Human Embryonic Kidney 293 cells
NHPP	National Hormone and Pituitary Program
MM	Molecular Mass

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