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High Levels Production of Recombinant Human Activin A—Effect upon *in Vivo* Follicle Stimulation

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Abstract

Activin plays an important role in numerous physiological processes such as cell differentiation and remodeling, regeneration and repair of tissues from various organs, angiogenesis, morphogenesis of glandular organs, pluripotency and differentiation of stem cells, cell adhesion and apoptosis. It participates in reproductive processes like embryogenesis, in the expression of Follicle Stimulating Hormone and Luteinizing Hormone and maturation of ovarian follicles and therefore has application in the area of reproduction of vertebrates. Given the economic importance of activin, we develop an efficient and economical method for the production of recombinant human activin A (rACT), using as expression system the yeast *Pichia pastoris*. rACT showed biological activity as it induced, on submicromolar dose, the increase of ovarian mass and the ovulation process in a mammal model.

Keywords

Recombinant Human Activin, Yeast, Superovulation

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1. Introduction

Activins are homodimeric proteins which are present in three different isoforms [1]-[3]. Each isoform is constituted by two beta subunits (14 kDa) linked by disulphide bonds [1] [4]. They are members of the transforming growth fator-*β* (TGF-*β*) superfamily, a group of molecules with similar structure and functions. Activins are synthesized in various tissues and its expression levels are elevated in the hypothalamus, pituitary gland, adrenal and placenta [5] [6]. They participate in numerous physiological processes, such as cellular differentiation and remodeling [7]-[9], neural survival [10], angiogenesis [11], tissue repair [12], cell adhesion and apoptosis [6]. In reproductive process, activin A plays an important role in embryogenesis, gonadotropins expression (Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH)) and ovarian follicles maturation [13]-[16]. Activin A is distributed in spermatocytes, eggs and in various organs during embryogenesis [17] and is important to support granulosa cells survival and proliferation [18].

Some organisms are used to produce recombinant proteins in order to minimize potential problems related to antigenic reactions, and assist in the correct expression of heterologous proteins [19]. Among yeasts, *Pichia pastoris* secretory system permits posttranslational modifications such as proteolytic maturation, disulfide bond formation and glycosylation, being mostly N-linked mannose residues and few O-linked [20] [21]. This enables such compounds to be injected into the bloodstream without inducing antigenic reactions, a problem often found in highly glycosylated proteins.

In this work, a new protocol for the production and purification of recombinant human activin A was established. Recombinant protein produced is similar to the native and secreted into the culture medium, facilitating its purification. The recombinant protein showed to be biologically active since on submicromolar dose it induced the increase of ovarian mass and the ovulation process in a mammal model.

2. Material and Methods

2.1. Animals

Peripubertal female Wistar rats (31 - 34 days, 65 - 90 g, 7 each group) were kept under standard laboratory conditions with a 12-h light, 12-h dark regimen [22]. These animals were from the animal's house of University of Mogi das Cruzes. This project was approved by the local animal ethics committee.

2.2. Cloning of Activin

The cDNA fragment corresponding to mature activin was amplified using human genomic DNA and the oligonucleotides GAATTCGGCCTGGAGTGTGAC and GCGGCCGCCTATGAGCAACCA. The products were cloned into pGEM-T easy (Promega, USA). Clones with the correct sequence were sub-cloned into pPIC9 (Invitrogen) and incubated with BglII restriction endonuclease to linearization and integration into the AOX1 gene locus. The linearized plasmids were used to transform the yeast *Pichia pastoris* (SMD 1165 Invitrogen, USA) by electroporation (1500 V, 25 μF, 400 Ω).

2.3. Fermentation

Transformed *Pichia pastoris* were picked up from solid YPD medium (1% yeast extract, 2% peptone, 2% glucose) plates and inoculated into 300 mL of MGY medium (1.34% yeast nitrogen base, 1% glycerol; 4×10^{-5} % biotin) to grow at 30˚C under shaking (200 rpm) in a 1 L shake flask for 24 h (until OD_{600}), when the medium was transferred to a bioreactor (Bioflo 110, New Brunswick, USA) containing 3 L of glycerol plus salts medium [23] and 13.2 mL of trace mineral medium (PTM1). When glycerol was consumed (36 h), a solution containing 50% glycerol and PTM1 (4.4 mL/L) was added in a flow rate of 1 mL/min/L, for 48 h, until yeast wet weight reached 208 g/L. To induce protein expression the glycerol solution was replaced by a mixture containing 100% methanol and PTM1 (12 mL/L) which during the first 24 hours was added in a flow rate of 2 mL/h/L and increased to 3 mL/h/L for the next 48 hours when the fermentation was completed. The biomass (yeast) was separated of the supernatant using the CFP-2-E-4MA (Amersham Biosciences Corp, USA) column in the hollow fiber system. The biomass was discarded and the supernatant transferred to a hollow-fiber system with a UFP-5-C column (GE Healthcare, USA) for tangential filtration and dialysis against 0.15 M NaCl. The protein concentration was estimated using Proteoquant reagent (Proteobras, Brazil) according to the method described

previously [24].

2.4. Gel Filtration Chromatography

A sample of protein from tangential filtration was loaded on a Biogel P-30 (1 × 60 cm, Bio-Rad, USA) column equilibrated and eluted with sodium phosphate buffer 50 mM, pH 7.0, at a flow rate of 1 mL/min. Fractions of 1 mL were collected and protein elution was monitored at 280 nm.

2.5. Western Blotting

Protein samples were heated (95˚C; 5 min), centrifuged and electrophoresed on 12.5% polyacrylamide gel containing 0.4% sodium dodecyl sulphate (SDS-PAGE). Proteins were transferred to a nitrocellulose membrane (Millipore, Ireland) using a Trans-Blot turbo system (Bio-Rad, Singapore) (20 mA, 7 min). The membrane was incubated with 35 mM sodium phosphate buffer containing 150 mM NaCl (PBS) and 0.3% tween-20 (PBST) for 1 hour and washed (PBST; 0.05% tween). Then the membrane was incubated with anti-activin A antibody (R & D Systems Inc., USA) for 2 hours, washed with PBST and incubated with anti-rabbit IgG peroxidase-conjugate (Merck, Germany) for 1 hour. The revelation was performed using the Luminata Forte Western HRP Substrate (Millipore, USA).

2.6. Biological Activity

The purified protein was injected daily (intraperitoneally) in prepubertal female rats [22], during 3 days, at doses of 1.0, 0.5 or 0.1 µg per day. Animals from control group received saline (0.15 M NaCl). The animals were weighed, the ovaries (left and right) were excised and weighed after removing the water excess. The effect of treatment on the development of the ovaries was evaluated by determining the relationship between ovarian mass and animal body mass.

2.7. Histological Analysis

Ovaries were stained by hematoxylin/eosin and viewed at 40× and 100× magnification. The degree of maturational development of ovarian follicles was observed comparing the slides of ovaries treated with activin with the control.

2.8. Statistical Analysis

The relative mass of experimental and control groups were analyzed using the Student's t test and are presented as mean ± standard error of the mean (SEM), considering significant p values < 0.05. GraphPad Prismversion 5.00 for Windows, GraphPad Software (San Diego California, USA).

3. Results

3.1. Expression of Recombinant Human Activin A

The supernatant from fermentation (4 L) was dialyzed and concentrated in the hollow fiber system to 0.5 L, the protein content estimated (0.42 mg/mL) and one sample loaded on a 12.5% SDS-PAGE (**Figure 1**). The selected clone expressed one protein with approximately 14 kDa (lanes 2 and 3). Two additional bands ranging from 17 to 19 kDa appear with higher intensity. Another bands around 28 - 32 kDa appear with lower intensity (lane 3). A commercial activin (R & D systems, USA) was loaded on the lane 4. From commercial sample, two bands, a weak of 14 kDa, and one of 23 kDa are in the region of the molecular mass of activin. A sample of the recombinant activin, containing 5 µg of protein was electrophoresed and blotted against a commercial anti-activin antibody. After blotting three immunoreactive bands with 14, 18 and 30 kDa were observed (**Figure 2**).

3.2. Purification of Recombinant Activin A

The purification of recombinant activin was performed on liquid chromatography FPLC system (Amersham Biosciences Corp, USA) using a biogel P-30 column. From the concentrated supernatant 2.0 milligrams protein were loaded onto column, eluted with 50 mM sodium phosphate buffer, pH 7.0 at 1 mL/min flow rate, moni-

tored at 280 nm. Fractions of 1 mL were collected. The chromatographic profile presenting two peaks is in **Figure 3**. Fractions of each peak were pooled and loaded on a SDS-PAGE. Activin eluted in the first peak presenting only one 14 kDa band (**Figure 4**). From this chromatography it was recovered 1 mg of the recombinant protein.

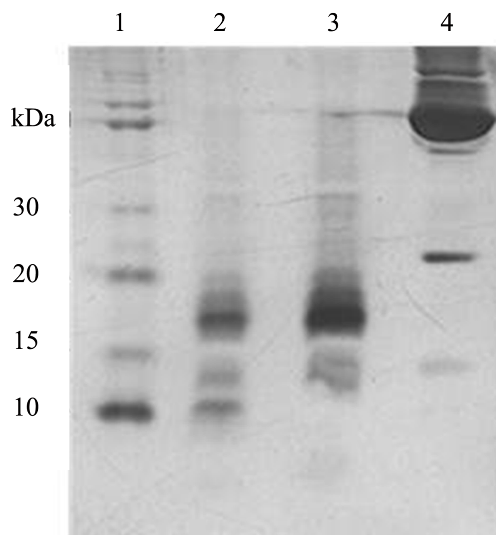

Figure 1. Polyacrylamide gel electrophoresis (12.5%) of fermentation products. Lane 1: Bench mark protein ladder (Life Technologies, USA); lanes 2 and 3: 1 and 2 μg of recombinant protein; lane 4: 1 μg commercial activin (RD Systems).

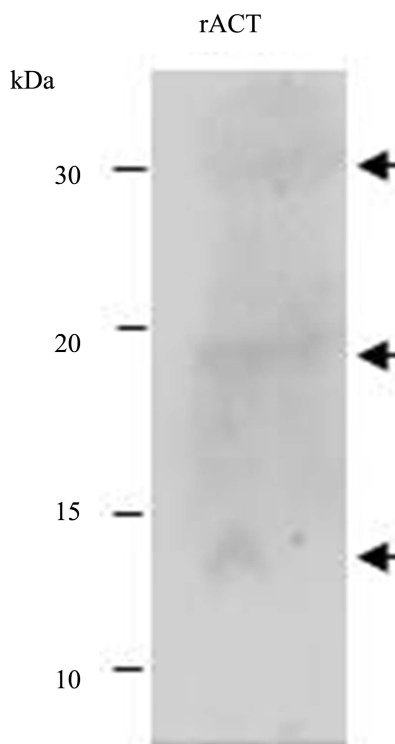

Figure 2. Immunoblotting of the fermentation products using anti-activin antibody (RD Systems). Arrows indicate the immunoreactive bands.

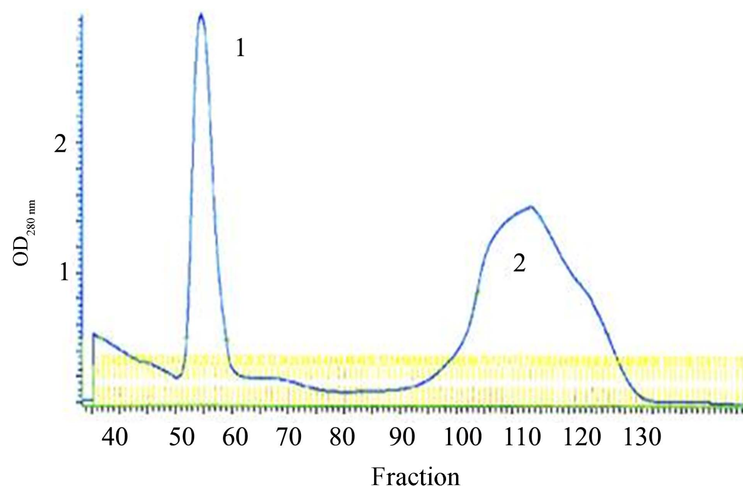

Figure 3. Crude recombinant activin elution profile using biogel P-30 column (1 × 60 cm) sodium phosphate (50 mM), pH 7.0, was used for elution, 1 mL fractions were collected and the optical density (OD) monitored at 280 nm.

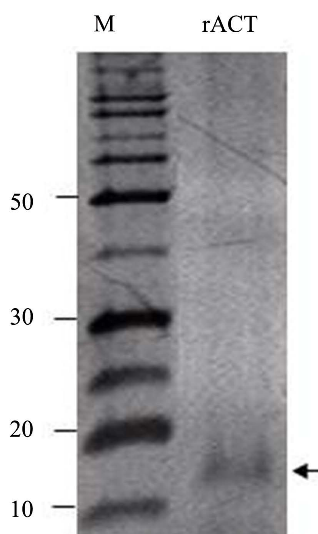

Figure 4. The electrophoretic pattern of purified recombinant activin in 12.5% SDS-PAGE. M, BenchMark protein ladder (Life Technologies, USA) and A, 1 μg sample from biogel P-30 chromatography.

3.3. Biological Activity

The purified recombinant protein was injected in prepuberty female Wistar rats at 1, 0.5, 0.1 μg/animal/day doses (n = 7 for each group). After three doses (three days injection), the rats were sacrificed and the body and ovarian mass determined. **Figure 5** shows the results obtained after 0.1 μg dose. There was a significant increase in ovarian relative mass of rACT-treated group by comparing with the ovarian relative mass of control group.

3.4. Histological Analysis

Histological results are shown in **Figure 6**. It can be observed, in the slides ovaries of 0.1 μg treated animals, the presence of corpus luteum (**Figure 6(A)**), indicating that the recombinant protein has the capacity to interfere with follicular maturation even when administered at submicromolar doses. The ovaries of the control group had many developing follicles (**Figure 6(B)**).

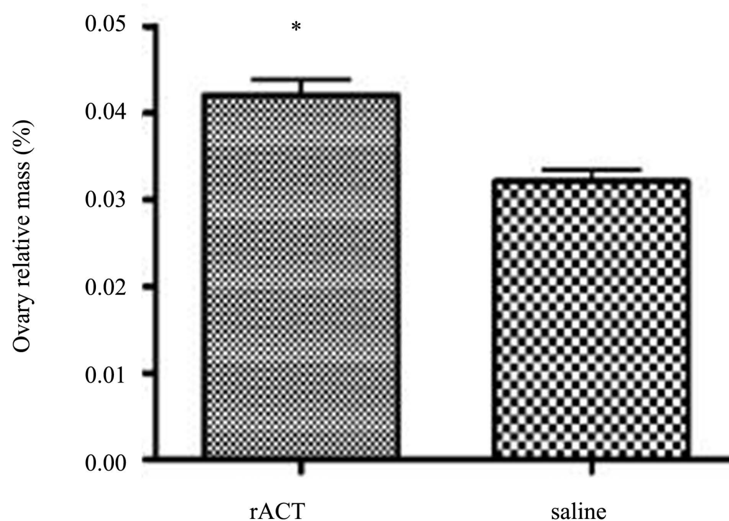

Figure 5. Effect of 0.1 µg of purified protein (rACT) and saline on the weight of the ovaries.

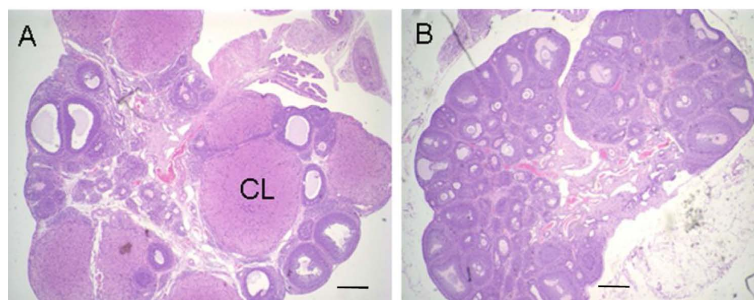

Figure 6. Histological section of rat ovary treated with purified recombinant protein (0.1 µg rACT) (A) or saline (B). CL = corpus luteum. Magnification bar (horizontal blackline) = 50 µm.

4. Discussion

The *P. pastoris* yeast is an efficient expression system for the production of a variety of heterologous proteins. The use of *P. pastoris* for the production of recombinant activin was already described by others [25] [26]; however, the protocol presented here is less consuming time with better yield. The expression of activin A was performed using similar protocols described previously [27] [28] with some modifications. Hollow fiber system was used for concentration and dialysis of the protein. The product was purified in just one chromatographic step, using gel filtration on FPLC system. Some authors [25] [29] used affinity chromatography which makes the process much more laborious and expensive. The activin A process of production and purification presented in this work consists of a fast, efficient and cost-effective methodology for the production and purification of the protein. Higher capacity columns may be used to facilitate the purification procedure. Taking into account the results from SDS-PAGE, we may conclude that the recovery of the activin in the biogel P-30 chromatography was around 70%. The SDS-PAGE for the crude material showed one strong band around 18 kDa, the same molecular mass of one immunoreactive by using the commercial anti-activin antibody. We believe that this protein may be an aggregate involving the activin and an activin degradation product. Taking into account these data, around 100 mg of pure activin can be obtained by our protocol, which finishes with 4 L fermentation. To determine the biological activity of the rACT, it was used different doses of the protein in the crude and purified conditions; however, it is presented only data obtained with the purified protein. All animals of rACT-treated group presented increased ovarian mass, including that treated with crude protein at higher doses (5 µg/animal/day) with similar results (data not shown). These results evidence that rACT produced is in the active form since it

stimulated the development, growth and maturation of ovarian follicles and thus the increase in ovarian mass. This increase seems to be related to the accumulation of follicular fluid during follicle maturation process in folliculogenesis. This process is mediated primarily by gonadotropic hormones, local development and growth factors that participate in autocrine and paracrine regulation in these events [30]-[32]. Activin is one of these growth factors. The increase in ovarian mass after treatment with activin A was observed in rodents and showed their autocrine and paracrine role in the structural and functional development of follicles [31] [33] [34]. Another evidence came from histological analysis of ovaries. The results showed the presence of corpus luteum, indicating that ovulation had previously occurred whereas rats of control group showed no ovulation. The premature ovulation in female rats after three days of treatment with rACT showed that some follicles started early ovulation, in agreement with previously result [33]. The mechanism by which activin A promotes premature ovulation is unknown. A hypothesis is related to the ability of activin A to increase the expression levels of FSH and LH hormones during folliculogenesis [35] [36]. The FSH actions are involved in development and growth of follicles, estrogen production stimulation and oocytes maturation. The specific activity of the FSH beta chain promoter is mainly regulated by activin and also presents a key role in the transcription activation of FSH gene encoding [37]-[39]. Activin A maintains FSH expression levels increased and maintains FSH receptors active *in vitro* even in absence of this hormone [18].

Activin A also promotes increase in LH expression and may regulate the production of LH, controlling LH beta subunit expression [40] [41]. The main function of LH refers to the maturation of ovarian follicles after ovulation, corpus luteum formation and progesterone synthesis. FSH and LH levels increment induced by activin A, *in vivo*, could explain the increase in premature ovulation observed in rodents [33]. Another hypothesis is that premature superovulation caused by activin A is a direct action within the formation follicles. *In vivo* experiment [42] showed that systemic administration of activin A in hypophysectomized rats stimulated growth of small follicles indicating that this event occurred in the absence of FSH. Therefore, FSH alone failed to cause granulosa cells proliferation in hypophysectomized rats and activin directly organized follicular development and increased estradiol production. These studies showed that activin acts not only in the pituitary but also in the ovarian tissue. The effect of FSH on the growth of the ovarian follicle *in vivo* appears to be not direct but mediated by paracrine and autocrine ovarian factors [43] [44]. These studies suggest that activin can be such factor. This proves the activin ability to stimulate follicles production in different *in vivo* and *in vitro* studies, featuring paracrine and autocrine regulation of this protein in process of folliculogenesis [40] [44]. Studies have shown that activin participate not only in the early development of the follicle, but also in all stages of follicular development and luteal activity [45]. The activin A transcripts and receptors are expressed in all follicular stages and in the corpus luteum [46]. These data reinforce the regulatory role of the protein since the process of folliculogenesis until luteal activity.

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