Workshop 5: Protein production in animal and plant systems **Poster 4**

Analysis and characterization of different preparations of recombinant human follicle stimulating hormone (hFSH) and of its subunits

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Background

Human follicle stimulating hormone (hFSH), synthesized by the human pituitary gland, is a biologically active glycoprotein composed of two noncovalently bound α- and β- subunits and is critically involved in the maturation of ovarian follicles and in spermatogenesis. Considerable heterogeneity associated with different hFSH preparations has been reported, mainly related to the presence of different glycoforms [1]. The characterization of preparations of hFSH utilized as a therapeutic agent in reproductive medicine is therefore very important, especially considering that no specific monography has yet been published by the main pharmacopoeias.
In this work four hFSH preparations were analyzed, two of them being natural (pituitary- and urinary-derived) and the other two recombinant (CHO-derived). Studies were conducted to assess and compare hydrophobicity, molecular weight, charge heterogeneity and purity of the natural and recombinant heterodimeric preparations. These characteristics were examined by reversed–phase high performance liquid chromatography (RP-HPLC), matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF), isoelectric focusing and size-exclusion high performance liquid chromatography (HPSEC).

Results

RP-HPLC analysis indicated a significant difference ($p<0.005$) between the retention time (t_R) of the pituitary and of the two recombinant FSH preparations. Urinary-derived hFSH was found more heterogeneous than the other three preparations. HPSEC analysis showed a significant difference ($p<0.001$) between the t_R of the urinary preparation and that of the pituitary or of the recombinant preparations. Urinary-derived hFSH presented the lowest HPSEC t_R, in agreement with the highest molecular mass more accurately determined by MALDI-TOF mass spectrometry. The relative molecular mass (Mr) for the heterodimeric form of urinary, pituitary and recombinant hFSH preparations was 32527, 29176 and 28536 respectively.
An efficient subunit dissociation process (dissociation yield of 95%) was also set up by incubating pituitary- and CHO-derived FSH preparations with 3M acetic acid, overnight, at 37°C. Yields of approximately 52% and 48% for the β and α subunit respectively were obtained via RP-HPLC, in agreement with theoretical yields based on the mass determined in this work via MALDI-TOF mass spectrometry (53% and 47%). The Mr of the individual subunits determined by this methodology for pituitary- and CHO-derived hFSH were respectively 14467 and 14082 for the α subunit and 16509 and 16067 for the β subunit. The urinary preparation presented a Mr of 15139 for the α subunit and of 17196 for the β subunit (see Table 1). All subunits, when analyzed on RP-HPLC, presented retention times significantly different from the retention time of the heterodimer ($p<0.01$) and between them ($p<0.001$). The mean relative retention times ($t_{RR} = t_R$ subunit/t_R heterodimer), though, were found highly constant, 1.100 ± 0.004 (CV=0.4%) and 1.517 ± 0.023 (CV=1.5%), respectively for the β- and α-subunit of the three preparations (see Table 2)

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Table 1. Relative molecular mass (Mr) of the heterodimer (α+β) and related subunits of different hFSH preparations, determined by Maldi-Tof mass spectrometry

Preparation	α-subunit	β-subunit	Heterodimer		Calc/Exp
			Experimental	Calculated α+β	
p-hFSH	**14467**	**16509**	**29176**	**30976**	**1.06**
r-hFSH	**14082**	**16067**	**28536**	**30149**	**1.06**
u-hFSH	**15139**	**17196**	**32527**	**32335**	**0.99**

Table 2 Retention times of heterodimeric hFSH before dissociation, of α-and β-subunits after dissociation and relative retention times (t_{RR}) of the α and β subunits with basis on heterodimeric hFSH, determined on RP-HPLC (n = 2).

SAMPLE	heterodimer t_R	β-subunit t_R	α-subunit t_R	β-subunit t_{RR}[a]	α-subunit t_{RR}[a]
p-hFSH	24.43 ± 0.156	26.98 ± 0.160	36.63 ± 0.198	1.104	1.499
r-hFSH Gonal F	25.19 ± 0.129	27.62 ± 0.235	38.86 ± 0.214	1.096	1.543
r-hFSH Puregon	25.29 ± 0.070	27.85 ± 0.131	38.16 ± 0.127	1.101	1.509

[a]t_{RR}, (relative retention time) = t_R subunit / t_R heterodimer

Conclusions

Different isoforms were observed, by RP-HPLC , in the analysis of hFSH preparations of different origins (CHO, urinary and pituitary-derived). While the recombinant and pituitary hFSH preparations presented one main peak, the urinary-derived hFSH presented two major isoforms, one of which equivalent to the major form of the other preparations. The other form could be an oxidized form of FSH present in this urinary preparation in high amount, as reported [2].The RP-HPLC characterization of the hFSH heterodimer and of individual subunits revealed differences in hydrophobicity in the following order: α-subunit > β-subunit > heterodimer. For the first time a quite satisfactory separation of the heterodimer from the dissociated β-subunit was attained.

Urinary-derived hFSH showed a higher Mr (11 -14%) when compared with pituitary and recombinant hFSH, while pituitary hFSH showed a slightly higher Mr (~ 2%) in comparison with the recombinant preparation.

Acknowledgements

Supported by FAPESP and CNPq

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[1]. E Loumaye, M Dreano, A Galazka, C Howles, L Ham, A Munafo, A Eshkol, E Giudice, E De Luca, A Sirna, F Antonetti, CE Giartosio, L Scaglia, C Kelton, R Campbell, S Chappel, B Duthu, S Cymbalista, P Lepage: **Recombinant follicle stimulating hormone: development of the first biotechnology product for the treatment of infertility.** *Hum Reprod* 1998, **4**: 862-881

[2]. C Bergh, CM Howles, K Borg, L Hamberger, B Josefsson, L Nilsson, M Wikland: **Recombinant human follicle stimulating hormone (r-hFSH; Gonal-F) versus highly purified urinary FSH (Metrodin HP): results of a randomized comparative study in women undergoing assisted reproductive techniques.** *Hum Reprod* 1997, **12**: 2133-2139

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Microbial Cell Factories 2006, **5(Suppl 1)**:P90

Background: X-box binding protein 1 (XBP-1), a key regulator for the cellular secretory pathway, is essential for the differentiation of plasma cells and the unfolded protein response. In the XBP-1 knock-out B primary cells, a profound depression in synthesis and secretion of immunoglobulin M was observed, clearly demonstrating the importance of XBP-1 in protein secretion. There are two protein isoforms of XBP-1, XBP-1S and XBP-1U. The spliced form of XBP-1, XBP-1S, functions as a transcription activator and upregulates many genes associated with protein secretion and biosynthesis of endoplasmic reticula (ER), whereas the unspliced XBP-1U is transcriptionally inactive. Since the production of some recombinant proteins is widely believed to be limited by the secretory capacity of the host cell, we reason that an increase in protein productivity may be achieved by overexpressing XBP-1S in cells. However, XBP-1S is only synthesized when UPR is initiated. To constitutively express XBP-1S in cells, but not XBP-1U, we generated a specific expression plasmid which contains the spliced XBP-1S cDNA. Effects of overexpression of XBP-1S on the productivity of human erythropoietin (hEPO) in CHO-K1 cells were examined.
Results: We hypothesized that protein secretion may become a determinative factor when the production of recombinant proteins exceeds the secretory capacity of host cells. To simulate the saturated condition, CHO-K1 cells were transiently transfected with a hEPO expression vector. 2- to 3-fold increase in hEPO titre was observed when XBP-1S was ectopically expressed in the hEPO-saturated cells. Our findings suggest that the putative saturation of secretory capacity can be alleviated and protein production can be further enhanced by overexpression of XBP-1S.
Conclusion: XBP-1S could be an ideal gene target to improve productivity of recombinant proteins by modulating cellular secretory pathways.

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Conclusion: Different isoforms were observed, by RP-HPLC, in the analysis of hFSH preparations of different origins (CHO, urinary and pituitary-derived). While the recombinant and pituitary hFSH preparations presented one main peak, the urinary-derived hFSH presented two major isoforms, one of which was equivalent to the major form of the other preparations. The other form could be an oxidized form of FSH present in this urinary preparation in high amount, as reported [2]. The RP-HPLC characterization of the hFSH heterodimer and of individual subunits revealed differences in hydrophobicity in the following order: α-subunit > β-subunit >

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Characterization of *Medicago truncatula* cell suspension cultures producing valuable recombinant proteins

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Microbial Cell Factories 2006, **5(Suppl 1):**P92

Background: Nowadays, the use of plants for large-scale production of recombinant proteins is gaining wider acceptance because of their many practical, economic and safety advantages, compared with traditional microbial and animal production systems. However, production systems that use whole plants to express recombinant proteins may lack several of the intrinsic benefits of cultured cells, such as the precise control over growth conditions. Plant cell cultures may combine the merits of plant systems with those of microbial and animal cell cultures [1]. Optimization of environmental conditions in culture could be used to enhance foreign protein synthesis and stability, reducing the total cost of protein production. Moreover, a great advantage of synthesizing recombinant proteins in plant cell cultures is the very simple procedure of product purification, especially when the product is secreted into the liquid culture medium. As well as the potential commercial benefits, in vitro systems also represent an important tool for studying the process of foreign protein synthesis, assembly, secretion and turnover in plant cells and tissue.

Results: Recently, we have proposed the legume model plant *Medicago tuncatula* as a promising production system [2]. In this work, specifically, several suspension cell lines were established from transformed *M. truncatula* plants expressing two valuable recombinant proteins from different sources, human Erythropoietin (EPO) and fungal phytase. For both proteins two versions were available, one where the protein is secreted and the other where it is retained in the Endoplasmic Reticulum (ER).

Callus induction was achieved through incisions using a sharp razor blade (perpendicular to the mid-vein of the folioles) and the abaxial side of the folioles was maintained in contact with the medium with appropriate growth regulators. Calli were kept at 23°C in the dark on solid media for approximately two months. When calli reached the appropriate size, cells were transferred to Erlenmeyer flasks with shosen medium, kept with agitation in the dark at 24°C, and subcultured to fresh medium every week (A.S. Pires, unpublished results).

Identification of recombinant EPO or phytase was performed by Western blot analysis, showing that different cell suspension lines exhibit different expression levels of recombinant protein. Interestingly, we observed that in some cell lines with higher expression levels of the secreted version of recombinant protein, part of it is not being secreted to the medium. On the other hand, in suspension cells generated from transgenic plants engineered to produce recombinant protein targeted to the ER, the proteins are not being totally retained. These results are probably related with stress imposed to the cell by high expression levels of a foreign protein.

Preliminary immunolocalization analysis, using electron microscopy, revealed that in those cell lines with different expression levels, the subcellular localization of the recombinant protein is significantly different as well as the subcellular structure itself. As we can see in Figure 1, in phytase higher expressor cells, anti-phytase signal is located on unidentified structures under the cell wall but in the lower expressor cells the labelling is concentrated in apparently lytic vacuoles. We hope these studies will help us to answer the following question: is it the cell morphology that determines the subcellular fate of the product or the opposite?

In order to compare the obtained results with other plant based systems, leaves from transgenic *M. truncatula* original plants and transgenic BY2 Tobacco Suspension Culture expressing the same recombinant proteins were also analysed.