Physico-chemical and Biological Characterization of Glycosylated Human Prolactin Produced at IPEN

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BACKGROUND

Human prolactin hPRL is a 199 aminoacid protein hormone (MM ~23.000 Da) with a wide spectrum of biological activities being, however, best known for its stimulation of lactation and development of mammary gland. This protein contains only one potential asparagine-linked glycosylation site which is partially (~10%) occupied when the protein is synthesized in eukaryotic cells. Although the biological activity of glycosylated hPRL (G-hPRL) has been found ~ 4-fold lower compared to that of non-glycosylated hPRL (NG-hPRL), its physiological function is not well defined yet, and the carbohydrate moiety seems to play an important role in the biosynthesis, secretion, biological activity, and plasma clearance of glycohormones.

Cycloheximide is an inhibitor of the elongation step of protein synthesis. The observed increase in glycosylation site occupancy upon addition of cycloheximide is consistent with the current opinion that the initial glycosylation event occurs co-translationally during a limited time period. Cycloheximide may extend this time period by reducing elongation rate and thus allowing an increased glycosylation level.

RESULTS

In the present study, G-hPRL obtained from CHO after cycloheximide addition was purified for comparison with a control product obtained without inhibitor and accurately quantified via High-Performance Size-Exclusion Chromatography (HPSEC).

Effect of cycloheximide concentration on prolactin glycosylation

G-hPRL secretion was increased ~4-fold and the mass fraction of this isoform present on total prolactin was also greatly enriched, facilitating its purification and characterization (Fig. 1).

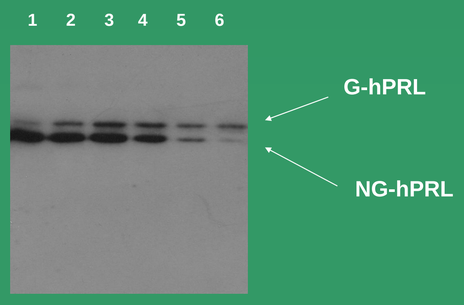
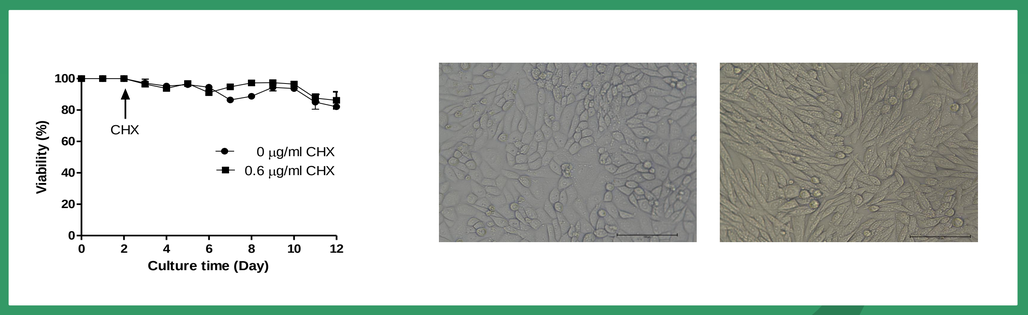


Fig. 1. Effect of cycloheximide on prolactin glycosylation. Confluent cell cultures incubated with α-MEM medium without serum (37 C, 5% CO2) were collected daily and analysed by western blotting. Lane 1, cultivation without cycloheximide. Lane 2-6, cultivation with 0.02 - 0.06 – 0.2 – 0.6 – 2.0 µg cycloheximide mL-1, respectively.

Effect of cycloheximide concentration on CHO cell growth

G-hPRL-secreting confluent cultures were maintained during the 10 days of production by replacing the medium every 24 h. The confluence and cell viability were not affected by the presence of cycloheximide in the culture medium (FIG. 2).



Purification

A two step purification process was developed. SP-Sepharose Fast Flow was found to be a practical concentration/purification step (Fig. 3). The HPSEC column proved to be a flexible tool useful for both the final purification and the qualitative and quantitative analysis of purified G-hPRL. It also was capable of separating the two isoforms in a run of < 30 min (Fig. 4).

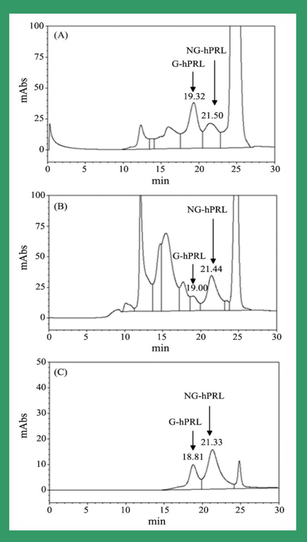


Fig. 3. HPSEC analysys of partially purified hPRL obtained after SP-sepharose FF chromatography. (A) Product obtained after culturing CHO cells in the presence of 0.6 µg/mL cycloheximide. (B) Control product obtained without cycloheximide addition. (C) Chemical Reference Standard (CRS) of hPRL from WHO.

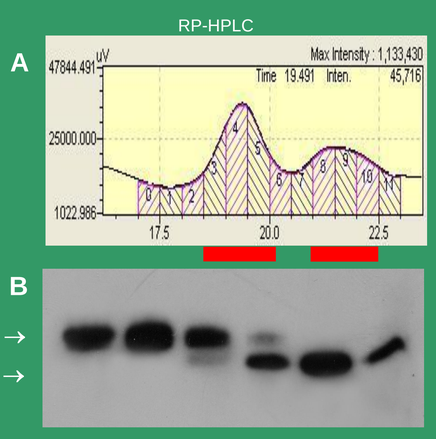


Fig. 4. HPSEC purification of G-hPRL obtained in fraction #29 from SP-Sepharose FF (Fig. 3B). Fractions eluted from HPSEC (A) and respective western blot analysis (B).

Table 1. Recombinant hPRL purification table, starting from 1 L of CHO conditioned medium.

Purification step	- cycloheximide			+ cycloheximide		
	Total protein(1)	NG-hPRL(2)	G-hPRL(2)	Total protein(1)	NG-hPRL(2)	G-hPRL(2)
	mg					
medium	178.1 ± 12.1	1.35	ND	112.2 ± 14	0.22	0.287
SP-Sepharose FF	12.2 ± 1.0	1.29	ND	3.6 ± 0.6	0.08	0.259
HPSEC	ND	0.86	ND	ND	0.065	0.14
yield		63,7 %			30 %	48.8%

(1) = BCA determination (n=3)
(2) = RP-HPLC determination
ND = not determined

Characterization

Purified G-hPRL was characterized by HPSEC and western blot (Fig. 5). MALDI-TOF-MS, allowed identification and relative molecular mass determinations of the two isoforms, confirming previously reported values (Fig. 4). The same mass spectrum showing the position of NG-hPRL also indicated the presence of two major forms of G-hPRL (Mr = 24640 and 25891) in the same range of those (Mr 24864-25588) that were determined via electrospray ionization mass spectroscopy for recombinant C127 cell-derived G-hPRL by the manufacturer. When analysed by MALDI-TOF-MS, purified G-hPRL obtained from a different production batch exhibited a slightly different form (Mr = 25346), though still within the same mass range. Two different in vitro bioassays were utilized to assess the biological activity of purified G-hPRL, confirming a potency 4-5 fold lower in comparison with NG-hPRL.

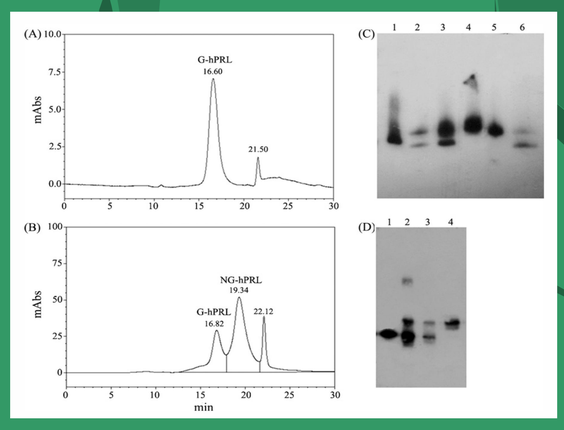


Fig. 5. HPSEC analysis of final purified G-hPRL (A) and of the Chemical Reference Standard of hPRL (WHO-CRS) (B). (C) Western blot analysis of G-hPRL from different purification steps. 1, internal reference preparation of hPRL from *E. coli*; 2, CHO conditioned medium; 3, fraction eluted from SP-Sepharose FF; 4, purified G-hPRL eluted from HPSEC; 5, 1 st Reference Reagent for G-hPRL (WHO 98/580); 6, WHO-CRS, showing the presence of G-hPRL and NG-hPRL (D) N-glycosydase F digestion of G-hPRL.1, hPRL from *E. coli*; 2, WHO-CRS; 3, glycosydase-treated G-hPRL; 4, untreated G-hPRL.

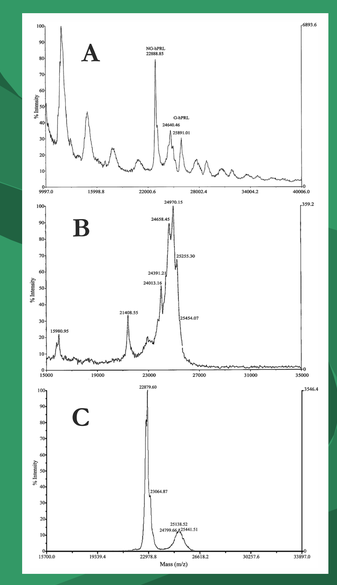


Fig. 6. MALDI-TOF-MS analysis of the partially purified fraction eluted from SP-Sepharose FF and containing G-hPRL and NG-hPRL (A), of purified G-hPRL (B) and of the Chemical Reference Standard–WHO containing G-hPRL and NG-hPRL (C).

CONCLUSION

Our results show that cycloheximide can be an important tool for G-hPRL production, facilitating the purification and characterization of this potentially important isoform of prolactin, whose physiological action need to be studied and better defined. While by MALDI-TOF-MS NG-hPRL presented a relative molecular mass (Mr) of 22,888 (-0.04% compared to the theoretical value), G-hPRL presented a range of Mr between 24,640 and 25,891, quite similar to that reported for G-hPRL obtained from C127 cells: 24,864 – 25,588.

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