HIGH LEVEL SYNTHESIS OF RECOMBINANT HUMAN PROLACTIN IN CHO CELLS AND IDENTIFICATION OF THE 22 kDa FRAGMENT hPRL $_{11\text{-}199}$

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Abstract

Two eukaryotic, human prolactin (hPRL) expression vectors, based on a selectable dihydrofolate reductase (dhfr) marker, were used to transfect dhfr⁻ Chinese hamster ovary (CHO) cells. One vector, p658-hPRL, contains the hepatitis-B virus-X cDNA coding for a viral transactivator and sequences mediating dhfr mRNA degradation. The other, pEDdc-hPRL, carries the encephalomyocarditis virus leader sequence, coupled to hPRL cDNA, to provide high level protein expression, possibly via a mechanism of internal translation initiation. Without methotrexate (MTX) amplification, p658-hPRL-transfected, stable cell lines, secreting up to ~10μg hPRL/10^6 cells/day, could be rapidly obtained; production by pEDdc-hPRL-transfected cells was about 10-fold lower. However, a three-step MTX amplification of the latter led to clones secreting up to ~30μg hPRL/10^6 cells/day. A pilot production in a hollow-fiber bioreactor provided a highly concentrated medium, up to 150μg hPRL/ml/day. SDS-PAGE analysis indicated that rec-hPRL contained approximately 10% glycosylated PRL. Chromatographically purified non-glycosylated and glycosylated rec-hPRL had bioactivities of 34.7 and 16.5 IU/mg, respectively (Nb2 cell bioassay). This is the first report describing production and purification of rec-hPRL from CHO cells secreted at levels higher than so far reported for eukaryotic systems.

1. Structure of expression vectors

Fig. 1 A

pEDdc-hPRL is derived from pEDdc kindly provided by the Genetics Institute (Cambridge, MA), and carries the insertion of the encephalomyocarditis (EMC) virus leader sequence between the gene of interest and the selectable gene in a dicistronic assembly.

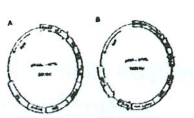

Fig. 1 B

p658-hPRL is derived from p7055 (Sanofi Recherche, Labege-Innopole, France) and carries the 3'-untranslated region of GM-CSF, that mediates selective dhfr mRNA degradation.

2. Levels of rec-hPRL secretion

TABLE 1 Prolactin expression level of p658-hPRL and pEDdc-hPRL transfected CHO dhfr- cells without MTX amplification

clone number	p658-hPRL (μg/10^6 cells/day)*	pEDdc-hPRL (μg/10^6 cells/day)*
1	2.04	0.50
2	0.15	0.32
3	1.13	0.37
4	3.04	0.05
5	8.71	0.91
6	0.03	0.50
7	1.10	0.51
8	0.03	0.56
9	0.12	0.45
10	0.05	0.37
11	0.15	0.72
12	4.33	0.60
13	1.26	0.05
14	0.02	0.30
15	0.03	0.50
16	10.44	0.28
17	0.03	0.85
18		0.43
19		0.03
20		[illegible]
21		0.47
22		0.27
23		0.4[illegible]
24		0.72

* hPRL radioimmunoassay determination

TABLE 2 Statistical analyses of prolactin expression levels of the best two clones obtained with p658-hPRL without amplification and of pEDdc-hPRL clones with MTX amplification

Assay number	p658-hPRL (μg/10^6 cells/day)*		pEDdc-hPRL (μg/10^6 cells/day)*	
	Clone 5	Clone 16	Clone 5	Clone 18
1	9.5	9.4	28.6	21.4
2	10.3	12.6	44.3	31.3
3	9.0	8.5	33.0	22.4
4	7.6	13.1	22.6	19.8
5	10.4	6.8	25.0	27.6
6	8.8	9.9	30.1	21.2
7	9.6	10.8		
average	9.3	10.2	32.1	24.4
CV	10.4%	[illegible]	20.1%	17.[illegible]%

* hPRL radioimmunoassay determination

Two pEDdc-hPRL-transformed clones (#5 and #18 from TABLE I) were treated with increasing concentrations of metrotrexate (MTX), i.e. 20nM, 50nM and 100 nM MTX (TABLE 2).

3. Estimation of glycosylated prolactin (G-hPRL) in conditioned culture medium

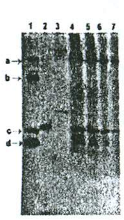

Fig. 2

SDS-PAGE analysis under non-reducing conditions for the estimation of G-hPRL in conditioned culture medium. Protein bands were visualized by silver nitrate staining. Lane 1: molecular mass markers: a, BSA (66 kDa); b, ovalbumin (45 kDa); c, rec-hGH (22 kDa); d, myoglobin (17 kDa). Lane 2: pituitary hPRL, 200 ng. Lane 3: internal reference preparation of G-hPRL. Lanes 4-7: serum-free culture medium (= MLM) conditioned by hPRL-secreting CHO cells, at various dilutions (1:1, 1:2, 1:4, 1:8).

The average proportion of G-hPRL obtained in 9 densitometric determinations on 7 different culture media of pEDdc-hPRL-transfected CHO cells, was 10.3% (CV=19.2%).

4. SDS-PAGE analysis of a typical purification process

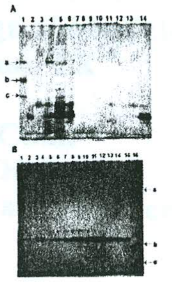

Fig. 3

SDS-PAGE analysis under non-reducing conditions, of various rec-hPRL purification steps.

A. Lane 1: molecular mass markers: a, BSA (66 kDa); b, ovalbumin (45 kDa); c, carbonic anhydrase (30 kDa). Lane 2: pituitary hPRL, 120 ng. Lane 3: internal reference preparation of G-hPRL. Lane 4: culture medium. Lane 5: SP Sepharose Fast Flow column eluate (rec-hPRL mixture). Lane 6: Sephacryl S-100 column eluate (rec-hPRL mixture). Lanes 7-10: SP-Sepharose High-Performance (HP) washing eluates. Lanes 11-13: SP Sepharose HP eluted fractions corresponding to G-hPRL. Lane 14: SP-Sepharose HP eluted fraction corresponding to NG-hPRL.

B. Lanes 1-13: Sephacryl S-100 elution profile corresponding to the region of immunoreactive prolactin. Lane 14: pool of Sephacryl S-100 eluted fractions. Lane 15: pituitary hPRL (600 ng). Lane 16: molecular mass markers: a, BSA (66 kDa); b, rec-hGH (22 kDa); c, myoglobin (17 kDa).

5. Bioactivity of purified G-hPRL and NG-hPRL (Nb2 lymphoma cell in vitro bioassay)

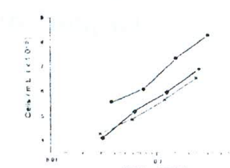

Fig. 4

Growth responses of Nb2 lymphoma cell cultures to NG-hPRL (●) and G-hPRL (•), quantified by radioimmunoassay, and the International Standard of pituitary hPRL, WHO 84/500 (■). Data are means of results from duplicate cultures.

Results from three independent bioassays showed that the CHO cell-produced NG-hPRL had a specific activity of 34.7 IU/mg (CV=24.1%), whereas the G-hPRL had a specific activity of 16.5 IU/mg (CV=32,5%).

6. Pilot production of rec-hPRL in a hollow-fiber bioreactor

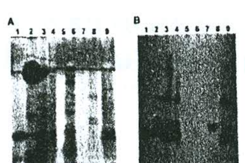

Fig. 5

SDS-PAGE (A) and Western blot (B) analysis, under non-reducing conditions of different purification steps of rec-hPRL secreted by CHO cells in the extracapillary medium of a hollow fiber bioreactor at various purification steps. Lane 1: pituitary hPRL, 200ng. Lane 2: CHO cells culture medium, containing 10% bovine fetal serum from petri dish. Lane 3: CHO cells serum free culture medium from the extracapillary space of a hollow-fiber bioreactor. Lane 4: Sephacryl S-100 eluate. Lane 5-7: SP-Sepharose HP washing eluates. Lane 8: SP-Sepharose HP eluate containing G-hPRL. Lane 9: SP Sepharose HP eluate containing NG-hPRL.

Cells were cultured in the extracapillary space (7mL), in low protein, serum-free medium (CHO-S-SFM II). It was thus possible to increase rec-hPRL production to ~150μg/mL/day. A byproduct, identified as glycosylated and non-glycosylated hPRL$_{11\text{-}199}$, was also obtained.

Conclusion

1. High-level synthesis of rec-hPRL was obtained for the first time in CHO cells.
2. The proportion of G-hPRL was estimated quite accurately.
3. G-hPRL and NG-hPRL were obtained at a high purity level.
4. The bioactivity of G-hPRL was about half of that of NG-hPRL.
5. The utilization of a hollow fiber bioreactor led to a substancial increase in the production of rec-hPRL. Under these conditions a byproduct, identified as hPRL$_{11\text{-}199}$, was also obtained.