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Role of environmental conditions on the expression levels, glycoform pattern and levels of sialyltransferase for hFSH produced by recombinant CHO cells

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Abstract

A recombinant CHO cell line in which the expresison of human follicle stimulating hormone (hFSH) was under the control of the β actin promoter was maintained in steady state perfusion cultures on a protein free medium. The level of expression of the hFSH was controlled by varying the steady state level of dissolved oxygen (10–90% of air saturation) and of sodium butyrate (0–1.5mM). Under these conditions, the specific productivity of hFSH (q_{FSH}) varied from 0.7 to 4.8 ng hFSH/10^6 cells/h. As the specific productivity of hFSH increased, there was a shift in the FSH isoforms to the lower pI fractions, corresponding to increased sialic acid content. As the specific productivity of hFSH increased, shifting the isoform distribution towards the lower pI isoforms, that the sialyltransferase enzymic activity also increased.

Introduction

There has been considerable conjecture to date as to whether the rate of expression of a glycoprotein by a recombinant mammalian cell affects the glycoform pattern of the secreted protein. The role of the host cell and the environmental conditions on the glycoform distribution has been described (e.g. Goochee and Monica, 1990). However, the question as to whether with the same cell line under basically the same environmental conditions, the distribution of isoforms is a function of the rate of recombinant glycoprotein expression has, to date, been unanswered.

In this paper, the role of expression rate on the glycoform distribution of human follicle stimulating hormone (hFSH) being produced by recombinant CHO cells is reported. Follicle stimulating hormone (hFSH) is a heterodimeric molecule with 2 N-glycosylation sites on each peptide chain. hFSH is secreted by the anterior pituitary gland and is a member of the gonadotropin class of peptide hormones, sharing a common alpha subunit with hCG, hTSH and hLH (Pierce and Parsons, 1981; Chappel *et al.*, 1983). Recombinant CHO and B cell lines producing hFSH have been used in our group as model systems to study heterologous glycoprotein production. (Gebert and Gray, 1994; Gray *et al.*, 1992).

In this study, CHO cells in which the r-hFSH expression was under the control of the actin promoter were grown in steady state perfusion cultures. By varying the steady state level of dissolved oxygen in the cultures, it was possible to vary the specific productivity (q_{FSH}) of the hFSH. Further increases in q_{FSH} were obtained by adding sodium butyrate to the perfusion medium. As the expression levels increased, there was an increase in the proportion of acidic isoforms in the expressed hFSH.

There is a close relationship between the isoform pI and the sialic acid content of hFSH. (Ulloa – Aguirre *et al.*, 1988). Accordingly the levels of sialyl transferase, the enzymes responsible for sialic acid addition in the terminal stages of hFSH synthesis were studied as a function of the rate of expression. Data are presented which shows that sialyl transferase activity increased as the specific productivity of hFSH increased.

Materials and methods

Cell line

The Darren cell line used in this study had the c-DNA coding for hFSH alpha and beta subunits under the control of the β actin promoter (Gebert and Gray, 1994). The cells were grown until confluent on Cytodex 2 microcarriers in DMEM:F12 medium containing 10% FCS. Once confluent, the medium was replaced with DMEM: F12 protein free production medium. All other growth conditions and assays were as previously described (Gray *et al.*, 1990).

Bioreactor and associated equipment

The bioreactor was a 3 litre vessel (Applikon) with an operating volume of 1.5 litres. The vessel was controlled by an FC-4 computer linked biocontroller (Real Time Engineering). The cells growing on microcarriers were retained in the bioreactor by a rotating stainless steel screen with a 75 micron mesh size. Fresh sterile medium was added to the bioreactor at the rate of 0.3 volumes per 10^6 cells per day; the exit pump was set at a slightly higher flow rate than the inlet pump and scavenged medium from inside the spin filter down to the level set by the height of the exit pipe.

The level of dissolved oxygen in the bioreactor was controlled by the addition of oxygen to the vessel, and carbon dioxide addition was used to control pH. For each steady state, three to four volumes were allowed to pass through the bioreactor, then samples were taken to ensure steady state conditions existed. Several samples were taken over the next day and these data points used as the steady state data.

Preparation of microsomal membrane fraction

Microsomal membrane preparations of the CHO cells were prepared and used in the determination of sialyl transferase activity. Approximately $1–2\times10^8$ CHO cells were collected by centrifugation at 1,000 rpm for 10 mins, washed with phosphate buffer saline and recentrifuged. Cells were suspended in homogenization buffer (0.1MTris-HCl, 0.25mM sucrose, 1mM EDTA, 10mM mercaptoethanol, 0.5mM PMSF) homogenized by a Potter Elvehjem homogenizer, then centrifuged at 10,000 rpm for 30 mins. The supernatant was collected and centrifuged at 30,000 rpm for 60 mins. The microsomal pellet obtained from the second centrifugation was dissolved in solubilization buffer (Levart *et al.*, 1990; Kaplan *et al.*, 1988). Protein concentrations were determined by the Bradford assay.

Sialyltransferase activity determination

Total sialyltransferase activity in the microsomal preparation was assayed using asiolofetuin as an exogenous acceptor. The reaction mixture contained 800 μg asialofetuin, 12.5 μmol sodium phosphate buffer pH 6.8, 0.5% triton X-100, 55 nmol CMP-(^{14}C) and enzyme preparation (100 μg protein) to a final volume of 100μL. Incubation was carried out for 1 hr at 37°C and terminated by the addition of 0.5 ml of 15% trichloroacetic acid containing 5% phosphotungstic acid. Precipitates were filtered under suction through 2.4 cm glass fibre filters (Whatman 934-AH) and washed with 10 ml 5% trichloroacetic acid three times. Radioactivity in the discs was determined in 10 ml Ecolite with a Beckman LS-250 Scintillation Counter. Replicates of reactants without the added acceptor were used as blanks (Chu and Walker, 1986).

Purification and quantification of hFSH

Samples were prepared for assay and characterisation using the following methods. The harvested medium was loaded onto a Protein G affinity column (1.5 × 2.0 cm). The Protein G Sepharose was bound with anti-FSH (78/24) according to the method of Harlow and Lane (1989). The FSH bound on the column was eluted with 0.1M Glycine pH 2.5. The FSH in the sample and after purification was quantified by Enzyme Linked Immunosorbent Assay (ELISA).

The ELISA used in this study was performed by using purified hFSH(80/1) as a standard. The monoclonal anti-FSH(79/7) was coated onto the plate, which was then exposed to the sample containing the FSH which then bound to the antibody. The bound FSH was detected by the conjugated biotin-anti FSH(78/24) and Streptavidin-horse radish peroxidase (HRP). The colour was developed by ABTS(2,3-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid diammonium salt) in 0.04M citric acid/0.06M phosphate buffer pH 4 and determined at 410 nm.

Isoelectric focusing (IEF) gel and Blotting

Immunoaffinity purified samples 0.5μg obtained under the different conditions were run on IEF gels, pI 3–9 (Phastgel System, Pharmacia). The gel was then

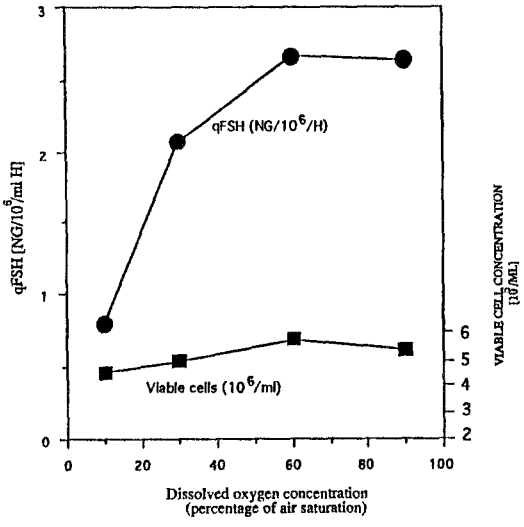

Fig. 1. Steady-state specific productivity of hFSH (q_{FSH}) and viable cell concentration as functions of the dissolved oxygen concentration.

transferred onto a polyvinylidene difluoride membrane (PVDF Millipore). The rhFSH on the PVDF membrane was detected by rabbit anti-FSH and mouse anti-rabbit conjugated with alkaline phosphatase, respectively. The colour was developed by 15mg of 5-bromo-4 chloroindolylphosphate and 30 mg of nitroblue tetrazolium in 100ml of 0.1M carbonate buffer/ 1mM $MgCl_2$ pH 9.8, and the isoform patterns on the membrane were scanned and quantified by the Bio-image gel scanner (Millipore).

Results

The results of steady-state perfusion cultures at a range of different dissolved oxygen levels are shown in Figure 1. Increasing the level of dissolved oxygen increased the specific productivity of FSH (q_{FSH}), from a value of 0.7 at 10% DO to a value of 2.6 ng/10^6cells/h at 90% DO. The number of viable cells was relatively constant, ranging from 4.5–5.7 × 10^6 cells/ml over the dissolved oxygen levels studied. Figure 2 shows the variation in isoform distribution within a pI range of about 4.5 to 8.6 at the selected DO levels. The data is presented as the percentage of isoforms (determined by the percentage of integrated intensity for the bands on an IEF gel) which have a pI less than the pI value plotted on the abscissae. At the lowest level of dissolved oxygen studied (DO of 10%), 65% of the

Table 1.

Conditions	Specific acctivity of Sialyl transferase (n mole/mg protein)/hour
Level of Dissolved Oxygen	
10%	1.0
30%	1.2
60%	2.05
90%	4.9
Concentration of Sodium Butyrate	
0.5 mM	2.8
1.0 mM	2.9
1.5 mM	3.85

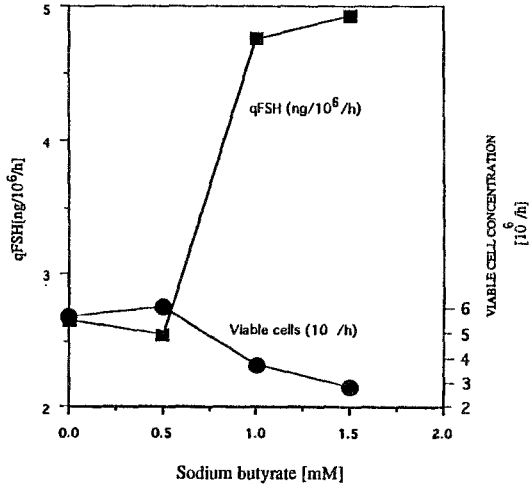

Fig. 3. Steady-state specific productivity of hFSH (q_{FSH}) and viable cell concentrations as functions of the sodium butyrate concentration.

FSH isoforms had a pI of 4.5 or lower. As the level of dissolved oxygen increased, the percentage of FSH isoforms at the lower pI's increased, with the highest level of dissolved oxygen studied, 90%, having 86% of the FSH bands occurring at a pI of 4.5 or lower.

The levels of the sialyltransferase activity observed at the different levels of dissolved oxygen are shown in Table 1. The sialyl transferase activity determined correlated positively with the dissolved oxygen level.

In Fig. 3, the effect of increasing levels of sodium butyrate in the medium on the q_{FSH} and on viable

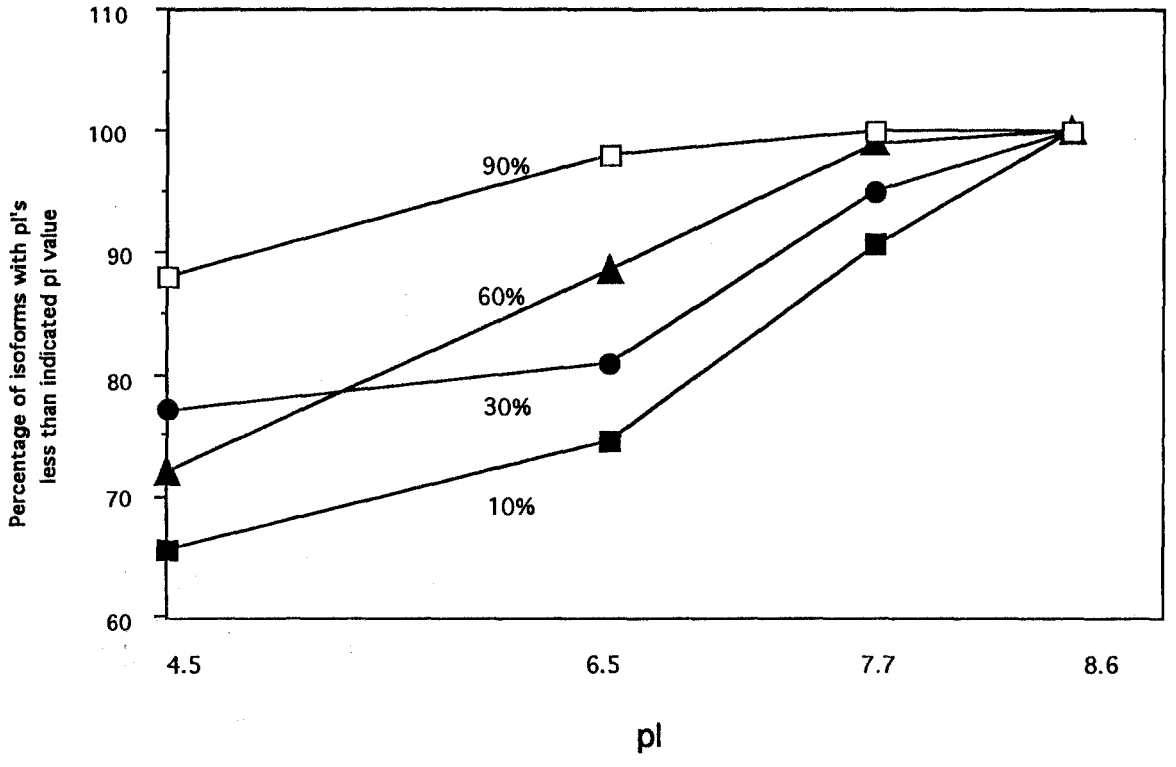

Fig. 2. Distribution of pI's of r-hFSH isoforms as a function of the steady-state dissolved oxygen concentration.

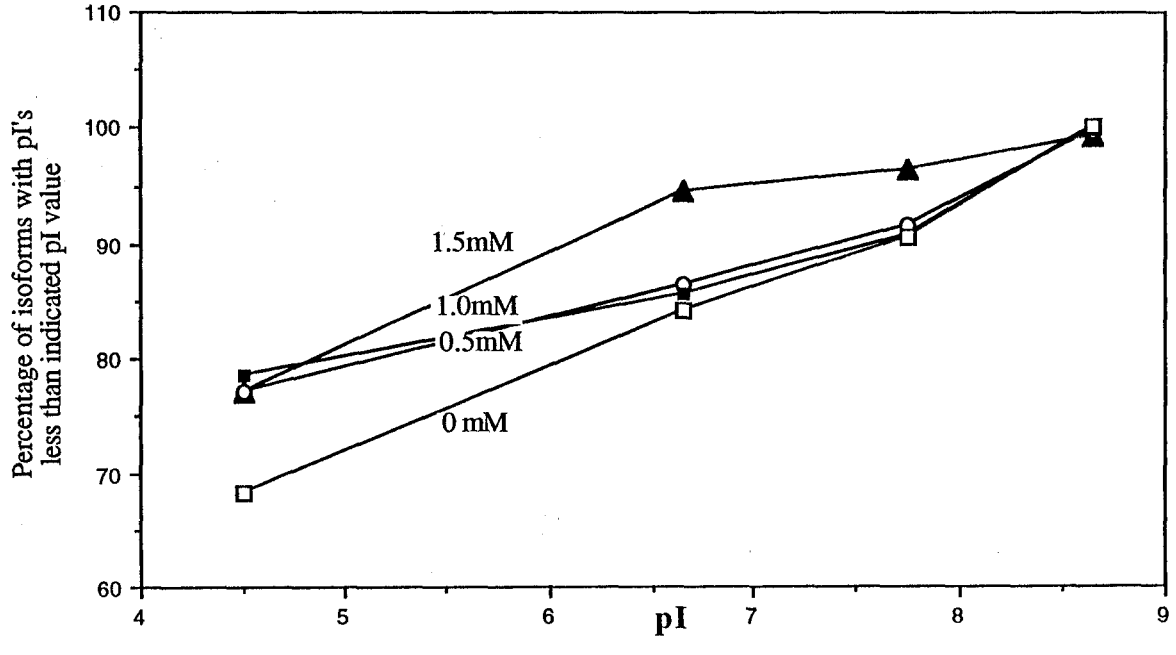

Fig. 4. Distribution of pI's of r-hFSH isoforms as a function of the steady-state sodium butyrate concentration (□, 0mM; ■, 0.5mM; ○ 1.0mM; ▲, 1.5mM)

cell numbers is shown. These experiments were carried out at a dissolved oxygen concentration of 60%. The addition of sodium butyrate resulted in further increase in the specific productivity, from 2.6 up to 4.8 ng/10^6cells/h at 1.5mM sodium butyrate. Higher levels of sodium butyrate are toxic to the cells, and although the levels used in these experiments were not high enough to have a major impact on viability, there was some reduction in cell numbers, from 5.7 $\times$ 10^6 cells/ml down to 2.8 $\times$ 10^6 cells/ml at the 1.5mM level. Figure 4 shows that the increases in specific productivity of FSH caused by the added butyrate were accompanied by further shifts in the isoform profile to lower pI's.

Table 1 shows that there were modest increases in the sialyl transferase activity in the experiments with added butyrate.

Conclusions

By altering the concentrations of dissolved oxygen and sodium butyurate in a perfusion culture of a recombinant CHO cell line expressing hFSH, it was possible to vary the specific productivity, q_{FSH}, over a seven fold range. At the lowest value of q_{FSH}, 65% of the FSH isoforms had a pI of 4.5 or less; at the highest value of q_{FSH}, 90% of the FSH isoforms had a pI of 4.5 or less. It was also observed that as the q_{FSH} increased there was an increase of approximately four fold in the total sialyltransferase activity. The observation that the q_{FSH} had higher content of sialic acid at high expression rates, means that as the expression rate increased that at least one enzyme on the glycosylating pathway, the sialyl transferase, did not become limiting, in fact the inverse occurred.

The results obtained in this study clearly demonstrated that the rate of expression of a glycoprotein by a recombinant mammalian cell affects the glycoform pattern of the secreted protein, in this case FSH. Steady state perfusion cultures were used to ensure that the only environmental variation the cells were experiencing was the change in the single parameter viz dissolved oxygen level or the concentration of sodium butyrate used to regulate expression levels. Different environmental conditions and modes of bioreactor operation (batch, fed batch etc) will produce glycoproteins with differing sets of glycoform distribution (e.g. Watson *et al.*, 1994). Steady state continuous operation offers the possibility of maintaining constant environmental conditions to produce glycoproteins of constant glycoform distribution.

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