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Enhanced erythropoietin heterogeneity in a CHO culture is caused by proteolytic degradation and can be eliminated by a high glutamine level

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Abstract

The molecular heterogeneity of recombinant human erythropoietin (EPO) increased during the course of a batch culture of transfected Chinese hamster ovary (CHO) cells grown in serum-free medium. This was shown by both an increased molecular weight and pI range of the isolated EPO at the end of the culture. However, analysis of the N-glycan structures of the molecule by fluorophore-assisted carbohydrate electrophoresis (FACE) and HPLC anion exchange chromatography indicated a consistent pattern of glycosylation. Seven glycoforms were identified, the predominant structure being a fully sialylated tetra-antennary glycan. The degree of sialylation was maintained throughout the culture. Analysis of the secreted EPO indicated a time-dependent increase in the molecular weight band width of the peptide consistent with proteolytic degradation. A high glutamine concentration (16–20 mM) in the culture decreased the apparent degradation of the EPO.

Introduction

Erythropoietin (EPO) is a 165 amino acid glycoprotein with two disulfide bonds and a molecular weight of 39 kDa (Jelkmann, 1992). Three N-linked and one O-linked oligosaccharide chains are attached to EPO polypeptide backbone and comprise 40% of the molecular weight of HuEPO (Sasaki et al., 1987).

The function of EPO is to stimulate the proliferation and differentiation of erythroid progenitor cells (Goldwasser and Kung, 1968). EPO is produced by the kidneys and is targeted to bone marrow (Jacobson et al., 1957). EPO affects early red progenitor cells through specific binding to one or more receptor molecules (Eridani, 1990). Recombinant EPO has been used for treatment of anemia associated with chronic renal failure, as well as some non-renal anemias. Treatment with EPO can restore normal hematocrit, eliminate the need for transfusions and improve the quality of life of its recipients (Ridley et al., 1994).

Large quantities of human EPO are required to satisfy the clinical demand for therapeutic use. Since primary cells produce only low concentrations of EPO, the human gene has been isolated, expressed and amplified in various mammalian cell lines (Jacobs et al., 1985; Lin et al., 1985). These transfected cell lines can form the basis of a production process. Product quality is extremely important for a molecule to be used therapeutically and any heterogeneity of a recombinant protein can be a serious problem.

Analysis of the effects of selective glycan removal from EPO has shown that the carbohydrate groups are essential for bioactivity in vivo (Dordal et al., 1985; Dubé et al., 1988; Narhi et al., 1991; Takeuchi et al., 1990; Tsuda et al., 1990). Of particular significance is the finding that the lack of terminal sialic acid (Neu5Ac) on the oligosaccharide structure results in high activity by in vitro bio-assays but low activity in vivo (Delorme et al., 1992; Narhi et al., 1991). This is explained by the presence of membrane receptors in the liver that can remove asialo-EPO from the circulatory system.

An important criterion for the development of a suitable production processes is to ensure the synthesis of a biologically active product that shows a consistent profile of heterogeneity. The heterogen-

eity of cell-secreted recombinant glycoproteins arises from two sources – variable peptide size and variable oligosaccharide structures. The heterogeneity of oligosaccharide structures can arise from variable intracellular processing as a result of various culture parameters, only some of which have been characterised. The culture parameters that may affect the variability of glycosylation include glucose depletion (Gershman and Robbins, 1981; Rearick et al., 1981), ammonia accumulation (Borys et al., 1994; Thorens and Vassalli, 1986), lipid composition (Castro et al., 1995; Jenkins et al., 1994), protein content (Castro et al., 1995) and pH (Borys et al., 1993).

Further heterogeneity is caused by proteases and glycosidases released from cells into the culture medium. These enzymes may degrade peptides and oligosaccharides in the culture medium. Thus a prolonged residence time of a secreted glycoprotein may result in some degradation which gives rise to further molecular heterogeneity (Gramer and Goochee, 1993; Gramer et al., 1995; Lind et al., 1991). It has been shown that the level of glycosylation can decrease over time in a batch culture as the chemical environment of the cell changes (Curling et al., 1990). Proteolytic degradation of the N-terminus of interferon-γ was shown to occur during its production in culture and this reduced its anti-viral activity (Hogrefe et al., 1989; Ichimori et al., 1987).

Serum contains specific protease inhibitors which can reduce product degradation in serum-supplemented cultures. However, the disadvantages in using serum in production processes, have led to the widespread use of low protein serum-free formulations for the growth of producer cell lines such as those transfected for synthesis of specific glycoproteins. In these cultures a reduction in proteolytic activity during cultivation of the cells may be achieved by addition of a protease inhibitor. However, these inhibitors are expensive and cause difficulty in product recovery (Schlaeger et al., 1987; Teige et al., 1994).

Other culture parameters may affect protease activity in the medium. It was reported that amino acid starvation (glutamine, asparagine, aspartate and serine) induced protease activity (Cartwright, 1994).

In the present study the heterogeneity of human EPO is determined during the batch culture of a transfected CHO cell line. The effect of glutamine concentration on this heterogeneity is also determined. Glutamine is an essential amino acid, a major energy source in proliferating mammalian cells and it serves as both a carbon and the nitrogen source (McKeehan, 1982; Reitzer et al., 1979). Glutamine in culture medium can prevent hybridoma and murine plasmacytoma cell apoptosis (Singh et al., 1994). Moreover, glutamine is involved in the biosynthesis of purines, pyrimidines and amino sugars, and the efficiency of recombinant protein glycosylation (Castro et al., 1995; Nyberg et al., 1999).

Materials and methods

Cell line and cultures

A stable CHO-K1 cell clone (EPO-81) transfected with the human EPO gene was provided by Cangene Corp. for this work. The cells were maintained in culture flasks in a humidified incubator at 37 °C and 10% carbon dioxide. Cell growth was in the serum-free medium designated CHO-SFM2.1. Cells were inoculated at 1×10^5 cells ml^{-1} into 7 ml medium containing different concentrations of glutamine in 25 cm^2 T-flask. Cells were detached from the growth surface by trypsin (0.05%/3 min.). Viable cell concentration was determined by haemocytometer counting from each culture sample to which an equal volume of trypan blue (0.2%) was added. Culture supernatants were harvested and stored in –20 °C until analysis. For EPO purification, cells were inoculated at 1×10^5 cells ml^{-1} into 100 ml medium in 150 cm^2 T-flask. After 4 or 7 days culture, supernatants were collected and stored frozen.

Determination of culture concentrations of glucose, lactate, glutamine and ammonia

The glucose was determined by a kit from Sigma (Glucose Trinder 315-100). Glutamine was measured by a specific glutaminase assay based on a method previously described (Lund, 1985). Lactate was determined by a spectrophotometric assay using lactate dehydrogenase (Gutmann and Wahlefeld, 1974). Ammonia was measured by a gas-sensing electrode (Orion, model 95-12).

EPO determination by ELISA

EPO concentration in culture supernatant was determined by a sandwich ELISA as described previously (Yang and Butler, 2000). Briefly, polyclonal anti-human EPO (Sigma) was coated onto microtiter plates and then incubated with serial dilutions of EPO standard or culture supernatant samples. EPO was detected

with a monoclonal mouse anti-human EPO antibody (5F12 AD3) (produced and purified in our laboratory), followed by an alkaline phosphatase (AP) conjugated anti-mouse IgG (adsorbed with rat serum protein) (Sigma). For detection of the antigen-antibody reaction, p-nitrophenyl phosphate (Sigma) was added as a substrate. The optical absorbance at 405 nm was measured by a multi-well plate reader (Molecular Devices).

Deglycosylation of EPO

Samples (50 μl) of desalted and concentrated culture supernatant containing EPO were denatured by boiling for 3 min with 1 μl of 10% SDS, 1 μl of 2-mercaptoethanol, 5 μl of 12.5% NP-40 and 40 μl of 50 mM $NaPO_4$ pH 7.2. Each sample was incubated at 37 °C for 16 h with 4 U/ml recombinant peptide-N-glycosidase F (PNGase F) (Boehringer); 20 mU/ml *Clostridium perfringens* neuraminidase (sialidase) (Sigma); and 25 mU/ml *Diplococcus pneumoniae* O-glycan-peptide hydrolase (O-glycosidase) (Boehringer). After enzyme digestion, samples were ready for Western blot analysis.

Western blot analysis

SDS-PAGE and Western blot was performed as described earlier (Yang and Butler, 2000). Briefly, glycosylated EPO in culture supernatant or deglycosylated EPO were separated by 14% polyacrylamide gel. The protein was transferred to a nitrocellulose membrane (NC) and detected by monoclonal mouse anti-human EPO antibody (5F12 AD3). A standard non-glycosylated EPO sample produced from *Streptomyces* was kindly provided by D. Stewart (Binnie et al., 1997).

2-Dimensional electrophoresis

Isoelectric focusing gels were made in glass capillary tubes. The gel mixture contained 9.2 M urea, 4% acrylamide, 2% Triton X-100, 2% ampholyte (preblended pH 4–6), 0.01% ammonium persulfate and 0.1% TEMED. The mixed solution was drawn into each gel tube and allowed to polymerize. After polymerization, the tubes were connected to a Mini-protein II 2-D Cell (Bio-Rad).

Samples for analysis were prepared by adding an equal volume of sample buffer (9.5 M urea, 2% ampholyte (preblended pH 4–6), 5% 2-ME, and 2% Triton X-100). The mixture was incubated at room temperature for 10 min. Samples (40–60 μl) were loaded into the sample reservoir connected to the capillary tube gel. The sample was overlaid with 30 μl overlay buffer (9 M urea, 1% ampholyte and 0.0025% Bromphenol blue). The cathode reservoir was filled with the cathode electrode solution (100 mM NaOH) and the anode reservoir was filled with the anode electrode solution (10 mM H_3PO_4). Electrophoresis was performed at 750 V for 3.5 h at room temperature.

After the first dimension, the tube gel was removed by the tube gel ejector and equilibrated in a buffer (0.062 M Tris HCl pH 6.8, 2.3% SDS, 5% 2-ME and 10% glycerol) for 10 min. The tube gel was then placed on top of the slab gel for electrophoresis in the second dimension. SDS-PAGE and immunoblot were performed as previously described. The pI was calculated according to the markers for 2-dimensional electrophoresis (Sigma).

Immunoaffinity purification of human EPO

Culture supernatant (1 l) was loaded on to a column consisting of purified monoclonal anti-EPO antibody bound on to the affi-prep 10 matrix (10 ml) (Bio-Rad). After loading, the column was extensively washed with PBS (40X bed volume) and 10 mM sodium phosphate/0.5 M NaCl (pH 7.4) (5X bed volume). The bound EPO was eluted with 3 M KSCN, 3 mM EDTA and 20 mM Tris pH 7.0. The eluate fraction was dialyzed against (0.1X) PBS/0.1% $Tween_{20}$ and concentrated by centrifugal filter units (Millipore).

Oligosaccharide preparation for analysis

Purified EPO was denatured and incubated with PNGase F at 37 °C for 16 h. The released N-glycans were separated from the EPO polypeptide and detergent by the addition of 3 volume of cold 100% ethanol and incubation on ice for 10 min. The precipitated protein was removed from each sample by centrifugation for 5 min. The remaining supernatant containing glycan was dried with a Speed-Vac. For HPLC analysis the sample clean up procedure was followed by addition to a GlycoClean S Cartridge (Oxford Glyco-Systems). Non-glycan material was eluted with 96% acetonitrile (5×1 ml) and the glycans were eluted with water (3×0.5ml). The glycan samples were dried in preparation for 2-aminobenzamide (2-AB) labeling.

N-linked oligosaccharide profile analysis by fluorophore-assisted carbohydrate electrophoresis (FACE)

Released and cleaned N-linked glycans were labeled with the fluorophore 8-aminonaphthalen-1, 3, 6-trisulfonic acid (ANTS) at the reducing terminus by reductive amination according to the Bio-Rad's instructions (Jackson, 1994; Hu, 1995). Labeling was performed by incubation at 37 °C for 16 h. After labeling, samples were dried in a Speed-Vac and one-fifth of the sample was loaded onto the polyacrylamide gel. Fluorescently-labeled oligosaccharides were electrophoretically separated in an N-linked oligosaccharide gel using the Mini-Protein II electrophoresis cell. The electrophoresis buffer was obtained from Bio Rad. Polyacrylamide gels were subjected to an electric field at 15 mA/gel at 4 °C. Images were acquired with the Glyco Doc imager and analyzed by Glyco Doc analytical Software (Bio-Rad). Glycan standards, asialo-agalacto-biantennary with core fucose; asialo biantennary with core fucose; asialo triantennary and asialo tetraantennary were purchased from Oxford Glyco-Systems.

Fluorescent labeling of glycans with 2-aminobenzamide (2-AB)

The labeling reagent was prepared by dissolving 10 mg 2-AB in 200 μl of 70% DMSO/30% glacial acetic acid (v/v). This mixture (100 μl) was added to a tube containing 6.2 mg of sodium cyanoborohydride and vortexed until the sodium cyanoborohydride was totally dissolved. The pure glycans were dissolved in 5 μl of the labeling reagent (Bigge et al., 1995). The glycan solution was incubated at 65 °C for 2 h. After incubation, labeled glycans were recovered by GlycoClean S Cartridge with the procedure as described above. The 2-Ab labeled samples were then dried and reconstituted in 120 μl distilled water

Analysis of 2-AB labeled sialylated N-glycans by HPLC

Anion-exchange HPLC was carried out using a GlycoSep C column (4.6×100 mm; Oxford Glyco-Systems). Each sample (100 μl) was injected into the column by an autoinjector (Shimadzu SIL-9A). Buffer A was 20% acetonitrile: 80% water and buffer B was 20% acetonitrile: 80% 250 mM ammonium acetate, pH 4.5. The solvent gradient was 0–40% B in 18 min, followed by 40–100% B over the next 16 min. Oligosaccharide elution was monitored with a fluorescent detector (Pharmacia LKB). The excitation filter was 330 nm and the emission was monitored with a cut off filter at 408 nm. By this procedure, neutral oligosaccharides eluted in the void volume. Negatively charged glycans were separated into mono-, di-, tri-, or tetra-sialylated peaks by the gradient. Oligosaccharide standards used in HPLC analysis were 3′ sialyl-lactose (Sigma); di-sialylated-galactosylated-biantennary and tri-sialylated galactosylated triantennary (Glyko).

Specific rates of consumption or production

The specific rates of consumption of substrates (Q_S), production of EPO (Q_{EPO}) or production of metabolic by-products (Q_P) were calculated from a plot of substrate or product concentration against the integral values of the growth curve (Renard et al., 1988). Data were obtained from the supernatant collected every 24 h during cell growth.

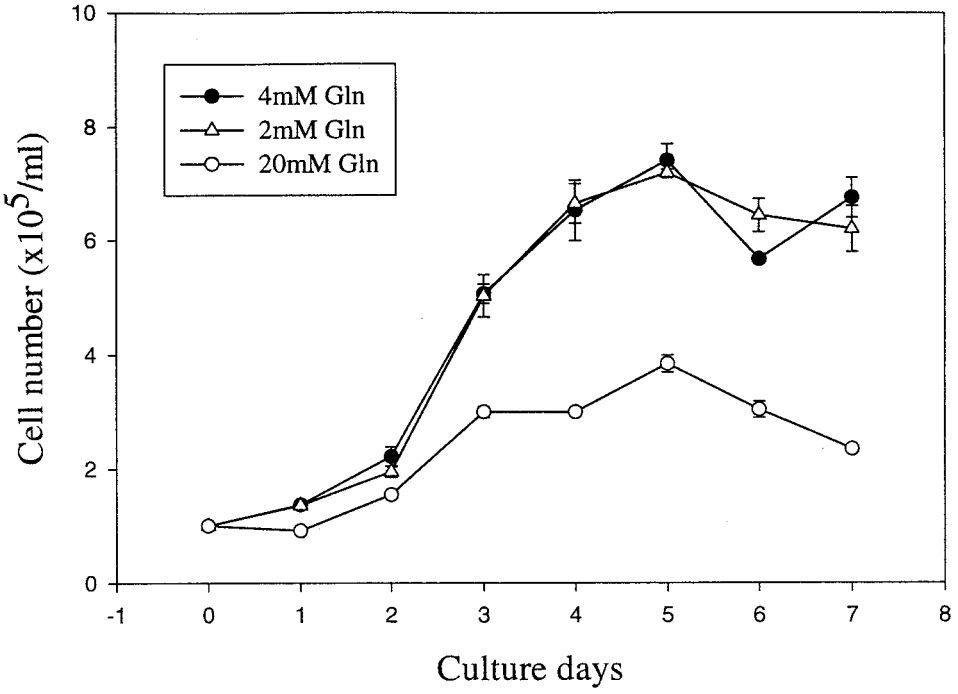
Results

1. Effect of glutamine on CHO cell growth and EPO production

The effect of a high (20 mM) or a low (2 mM) glutamine concentration on the growth and productivity of the transfected CHO cells was compared to that of the standard serum-free medium containing 4 mM glutamine. The cells were inoculated at 1×10^5 cells/ml into 7 ml culture medium in a 25 cm^2 T-flask. Viable cell number and EPO concentration were determined daily in each culture from day 1 to day 7 (Figure 1). Under standard conditions (4 mM gln) cells grew with a maximum specific growth rate of (μ= 0.042 h^{-1}) for 5 days after which there was a decline in the viable cell concentration. There was no significant difference in the growth profile in the culture at 2 mM glutamine. However, the maximum specific growth rate of cells in the culture with a high glutamine concentration was 0.032 h^{-1} and the final yield of cells in this culture was about 50% lower.

The highest EPO concentration was attained in the low glutamine culture at 250 U/ml followed by the standard and high glutamine cultures (Figure 1B). Although the EPO concentration in the high glutamine culture was relatively low, the cell specific productivity of EPO was 161 U/10^6 cell-day which was higher than the values calculated from the other cultures

(A)



(B)

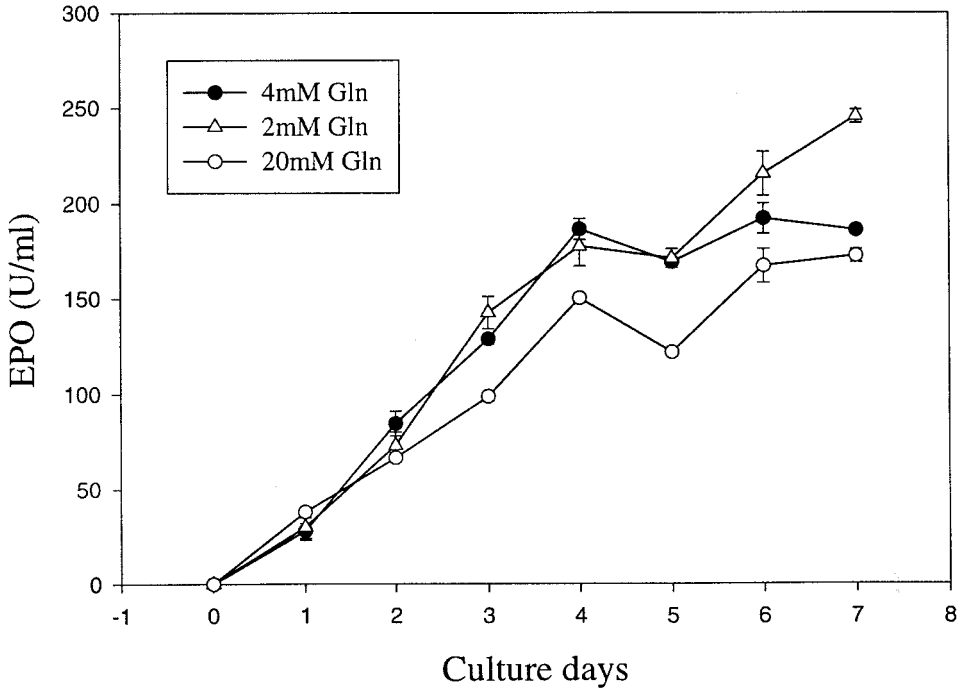


Figure 1. Cell yield and EPO concentration in cultures with different glutamine levels. CHO cells were inoculated at 10^5 cells ml^{-1} into 7 ml in standard (4 mM gln), low gln (2 mM) or high gln (20 mM) media in 25 cm^2 T-flask and cultured for 7 days. Viable cells and EPO concentrations were determined from day 1 to day 7. Values are means ± SE of duplicate cultures.

Table 1. Specific consumption and production under different glutamine concentration (U or μmole/10^6 cells/day)

Initial Gln	qEPO	qGln	qNH_3	qGlc	qLactate	qAmm/qGln	qLac/qGlc
(mM)	(U/10^6 cell/d)	(μmole/10^6 cell/d)					
2	130	1.13	0.74	13.0	15.4	0.65	1.18
4	132	1.85	1.18	11.0	14.5	0.64	1.32
20	161	6.29	3.55	6.7	13.0	0.56	1.94

(Table 1). This indicated that the EPO production was not correlated with cell yield.

From this experiment the high glutamine in the culture resulted in a decrease of cell growth and an increase of EPO specific production. This is similar to previously reported results obtained for monoclonal antibody production in high glutamine condition (Flickinger et al., 1992).

2. The effect of glutamine on cell metabolism

Substrate consumption and by-product formation was measured in each of the cultures over 7 days (Figure 2). Glutamine was almost completely depleted in the low gln culture after 4 days and in the standard culture after 5 days. The rate of ammonia production is reflected by the initial glutamine level in the cultures, with the concentration reaching 6.2 mM in the high gln culture and 2.15 mM in the standard culture after 7 days. Specific utilization and production rates were calculated as detailed in the section Materials and methods. Table 1 shows the increased specific rates of glutamine utilization and ammonia production with increasing glutamine level in culture medium. The rate of glutamine consumption and ammonia production in the high glutamine culture (6.29 μmole/10^6 cells per day) was $\times 3$ that of the standard culture. The rate of glucose consumption and lactate production decreased with increased glutamine concentrations in the culture. The metabolic coefficient, Qammonia/Qgln, progressively decreased whereas the coefficient Qlactate/Qglucose increased with higher initial glutamine levels. This metabolic data suggests an reciprocal relationship between the use of glucose and glutamine for energy metabolism as has been reported previously (Zielke et al., 1978).

3. EPO heterogeneity during culture progression

The heterogeneity of EPO during the course of a standard batch culture was examined from culture samples taken at regular intervals over a period of 10 days. The samples were concentrated and analysed by SDS-PAGE and Western blotting detection with an anti-EPO Mab. This analysis showed a gradual increase in the width of the EPO band from day 3 to day 7. By reference to marker proteins, the molecular weight range of EPO at day 3 was 33–39 kDa, whereas on day 7 and day 10, the range extended from 31 to 39 kDa. This suggested a greater heterogeneity of the recombinant EPO at the end of the batch culture (Figure 3), possibly resulting from molecular degradation.

The altered pattern of EPO heterogeneity was confirmed by 2D-electrophoresis combined with Western blot analysis (Figure 4). Protein separation was based on isoelectric focusing in the first direction (horizontal) and SDS-PAGE in the second direction (vertical). Protein patterns are shown at day 4 and day 7 of a standard culture (Figures 4a and b) as well as for day 7 samples taken from a high glutamine (Figure 4c) or low glutamine culture (Figure 4d). Seven protein spots were observed from the 4 day culture supernatant extending over a pI range of 4.06–4.67 and a molecular weight range of 33–39 kDa. The pattern of spots from the day 7 culture supernatant under standard conditions was significantly different. The last three protein bands (with high pI) disappeared and some new spots with lower molecular weight and lower pI were observed (Figure 4b). The patterns observed in Figures 4c and d were taken from day 7 samples of cultures grown under different glutamine concentrations. At low glutamine (2.5 mM; Figure 4d) the pattern appeared to be the same as under standard conditions. However, at high glutamine (16 mM; Figure 4c), the pattern was identical to that at day 4 for a standard culture. In particular, the low molecular weight bands characteristic of a degraded product observed at day 7 in standard and low glutamine cultures (Figures 4b and d) were absent in the sample from the high glutamine culture (Figure 4c). This appeared to indicate less breakdown of EPO under high glutamine conditions.

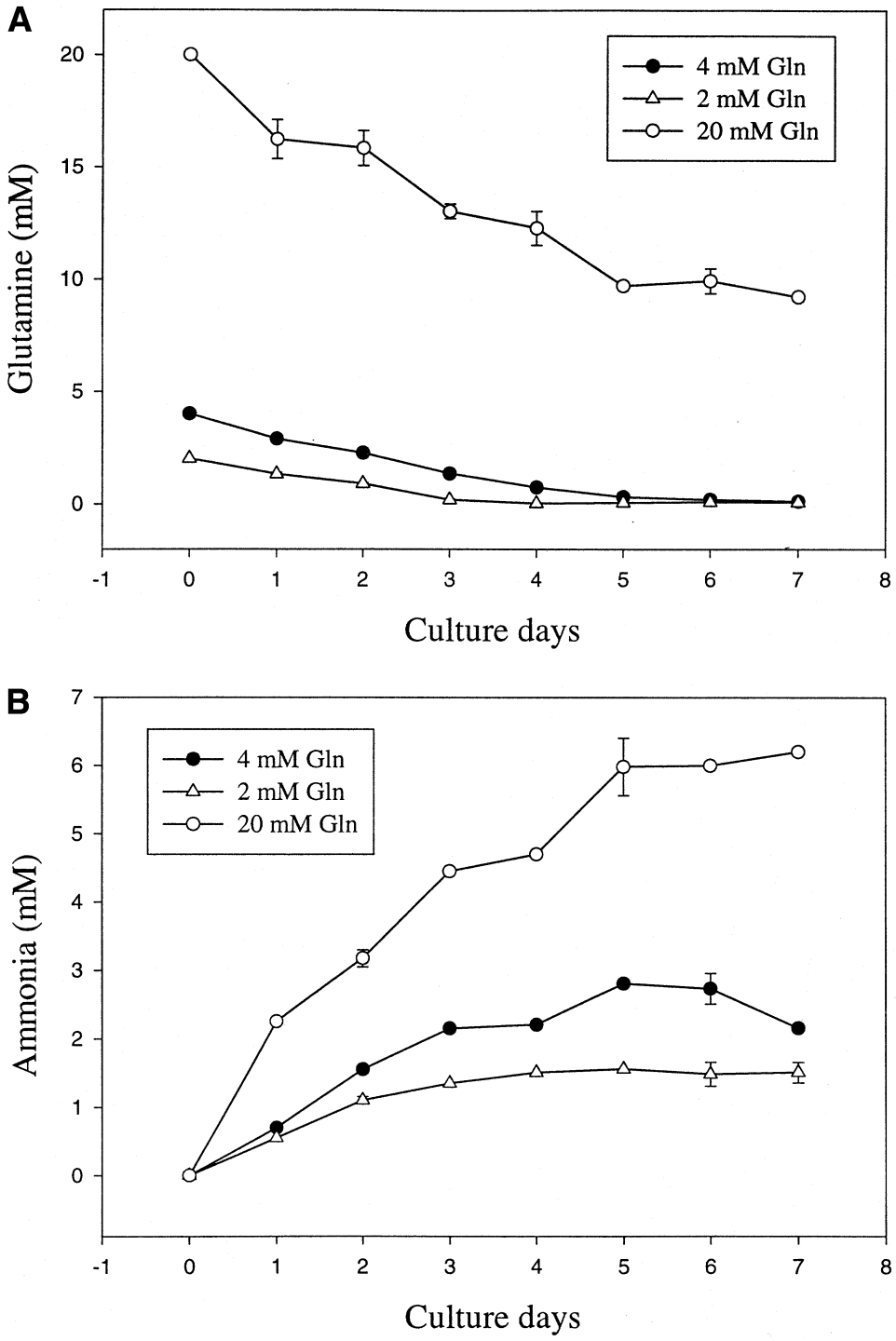
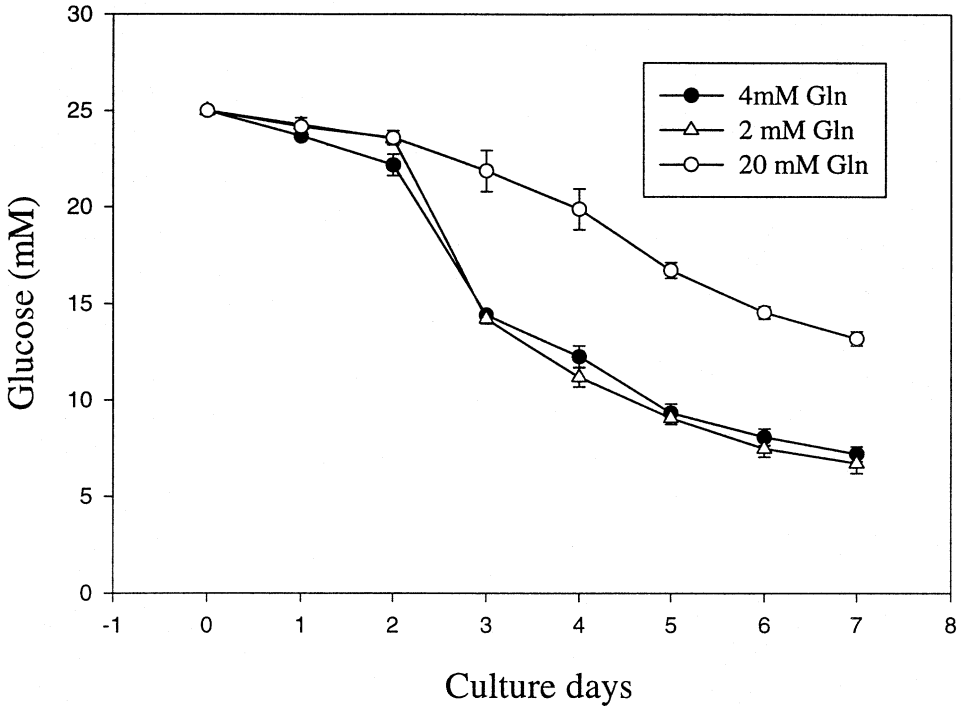


Figure 2A-B. Substrate utilization and by-product formation. CHO cells were inoculated at 10^5 cells ml^{-1} into 7 ml media containing different concentrations of glutamine in 25 cm^2 T-flask and cultured for 7 days. Supernatants were harvested from day 1 to day 7. Glutamine (A), ammonia (B), glucose (C) and lactate (D) concentrations were measured. Values are means ± SE of duplicate cultures.

(C)



(D)

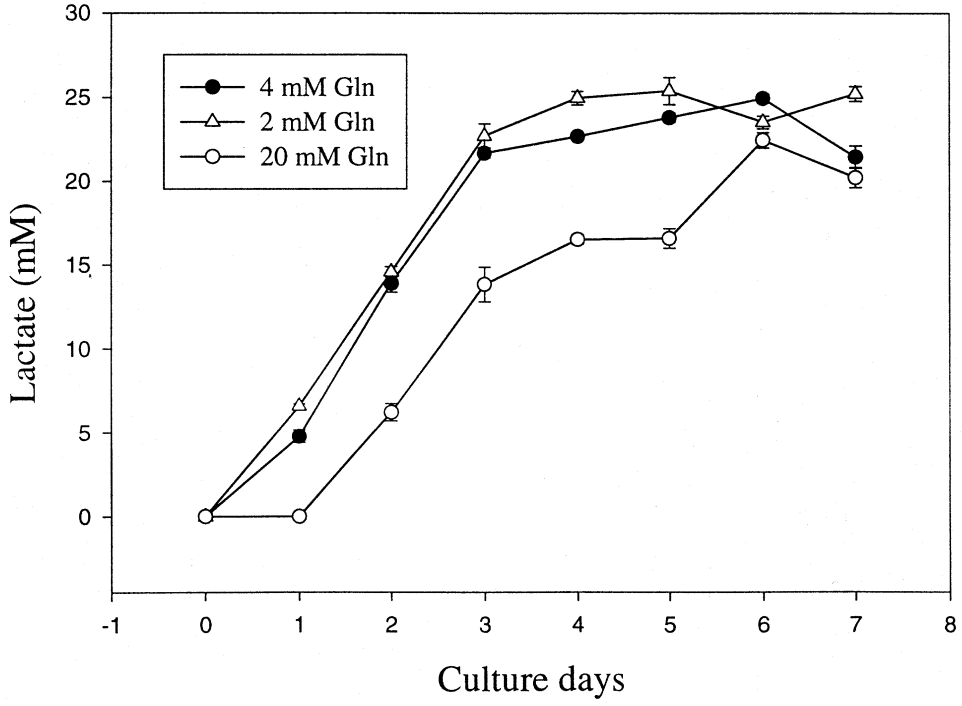


Figure 2C-D.

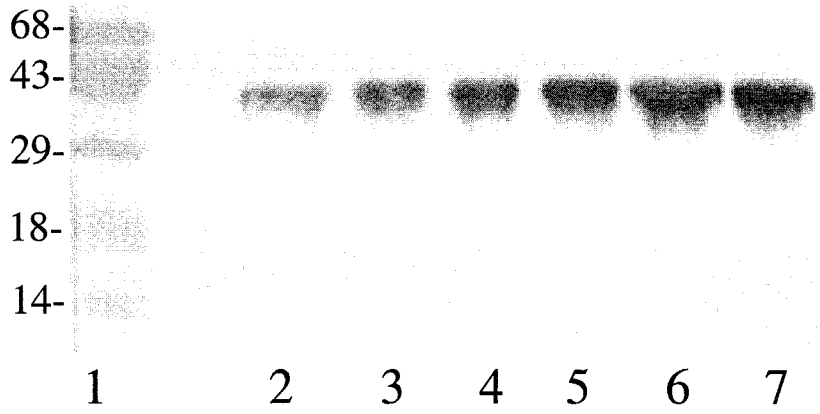


Figure 3. Gel electrophoresis of EPO during the course of a culture. Supernatants were harvested at time intervals from standard cultures, concentrated and separated by 14% SDS-PAGE. The proteins were transferred to a NC membrane and detected by mouse monoclonal anti-EPO antibody. Lane 1 shows the separation of protein molecular weight marker (kDa). Lane 2–7 are culture supernatants harvested at days 3, 4, 5, 6, 7, and 10.

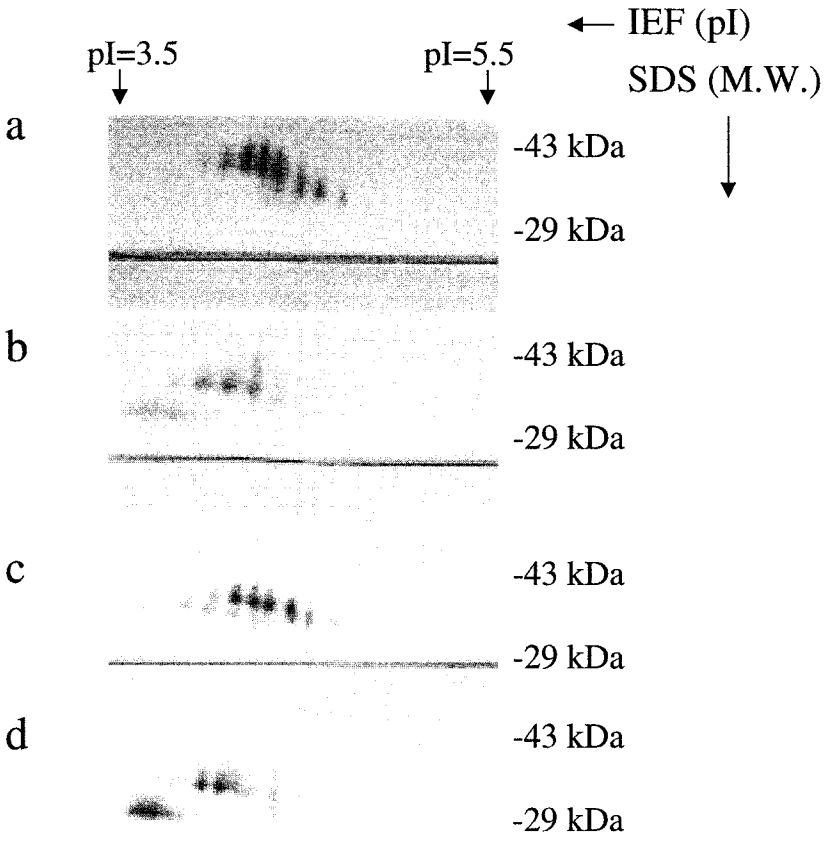


Figure 4. Two-dimensional electrophoresis and Western blot analysis of EPO. Supernatants from CHO cell cultures were desalted and concentrated. EPO were separated by pI in the first dimension followed by SDS-PAGE in the second dimension and analysed by a Western blot. (a) Standard culture at day 4. (b) Standard culture at day 7. (c) High gln (16 mM) culture at day 7 and (d) low gln (2.5 mM) culture at day 7.

In order to determine if the degradation of EPO from day 4 to day 7 occurred as a result of extracellular components, a cell-free supernatant taken from the 4 day culture was incubated for 3 days at 37 °C. Further analysis by 2-D electrophoresis showed an identical pattern to the original 4 day sample. This indicated that the observed altered pattern of EPO from day 4 to day 7 required the presence of cells. This suggests that any degradative enzymes causing these changes would be produced by the cells over this time period.

4. Analysis of the N-linked glycan profile of EPO by FACE

The N-linked oligosaccharide profile of EPO was analyzed by FACE from samples taken from a batch culture. EPO was purified from culture samples taken at days 4 and 7. The N-linked glycans were removed by incubation with PNGase F with or without the presence of sialidase. The resulting glycans were labeled with ANTS which provides a negative charge as well as the necessary fluorescence for detection. Separation of these samples by electrophoresis (Figure 5) indicated that there was no difference in the resulting patterns between day 4 or day 7 samples for either the PNGase F treated (lanes 3 and 4) or the PNGase F/sialidase treated (lanes 5 and 6) samples. The sialylated glycans (lanes 3 and 4) from purified EPO contained at least seven different structures with one prominent band at a position corresponding to 8 GU (glucose units) as related the glucose ladder in lane 1.

The desialylated glycans were separated into 5 bands (lanes 5 and 6) and compared with the standards in lane 2. Following desialylation of EPO the major band shifted to a position >11 GU which had a similar migration distance to asialo tetraantennary structure. Two bands lower than the major band at approximately 8.5 and 10 GU were close to the asialo bi- and triantennary standards. Two bands at a higher molecular size than the major band were identified as tetraantennary containing one or two lactosamine repeats (Gal-GlcNAc) by comparison with previously published structural data on glycans from rHuEPO (Morimoto et al., 1999; Takeuchi et al., 1988). The proportion of the di-, tri- and tetra-antennary (with zero, one or two lactosamine repeats) moieties was 11/21/68, respectively, as analyzed by densitometry from four independent samples.

The result of the analysis by FACE indicated that the profiles of sialylated and asialylated N-linked glycans structures of EPO did not change over the culture period between days 4 and 7. This indicated that any EPO heterogeneity would have to be explained by changes in the polypeptide structure.

5. Analysis of the N-glycan profile of EPO by HPLC

In order to confirm the FACE analysis by an independent technique, the sialylated N-glycans released from purified EPO were also analyzed by HPLC using the anion exchange column, GlycoSep C. The oligosaccharides were detected by a 2-AB label. A typical chromatogram of EPO oligosaccharides is shown in Figure 6. There are 7 peaks which corresponds to the number of bands identified by electrophoresis.

EPO oligosaccharide peaks were identified by comparison with the retention times of oligosaccharide standards containing variable sialic acids. The relative peak areas of the mono- (peak 1), di- (peak 2), tri- (peak 3–4) and tetra- (peak 5–7) sialylated structures were calculated as 12, 15, 28 and 45%, respectively (Figure 6a). The data showed that the major portion of the EPO glycans were tetra-sialylated with the most prominent peak occurring at a retention time of 24 min. The multiple peaks corresponding to each state of sialylation probably reflect different antennarity or variable lactosamine repeats.

Analysis of samples for days 4 and 7 by HPLC indicated identical glycan patterns. This result confirmed that the sialylation of EPO at the end of the batch culture was not reduced which is consistent with the results obtained by FACE.

6. Protein degradation during culture

We attempted to determine whether the observed time-dependent increase in EPO heterogeneity during batch culture was due to changes in polypeptide structure. The N-linked and O-linked oligosaccharides were removed from EPO by enzymatic deglycosylation and the resulting peptides were analyzed by SDS-PAGE and Western blotting (Figure 7). A 4-day sample from a culture in standard medium (containing 4 mM glutamine) resulted in a protein band with a molecular weight of 16.5–19.1 kDa (lane 1). This corresponded to the position of the band from non-glycosylated EPO from *Streptomyces*. (not shown). However, the 7-day sample of the same CHO culture resulted in a significantly broader band with molecular weight range of 15.8–19.9 kDa (lane 2). This suggested that a proportion of the EPO peptide may have been degraded toward the later stages of the batch culture. This evidence combined with the observed consistent

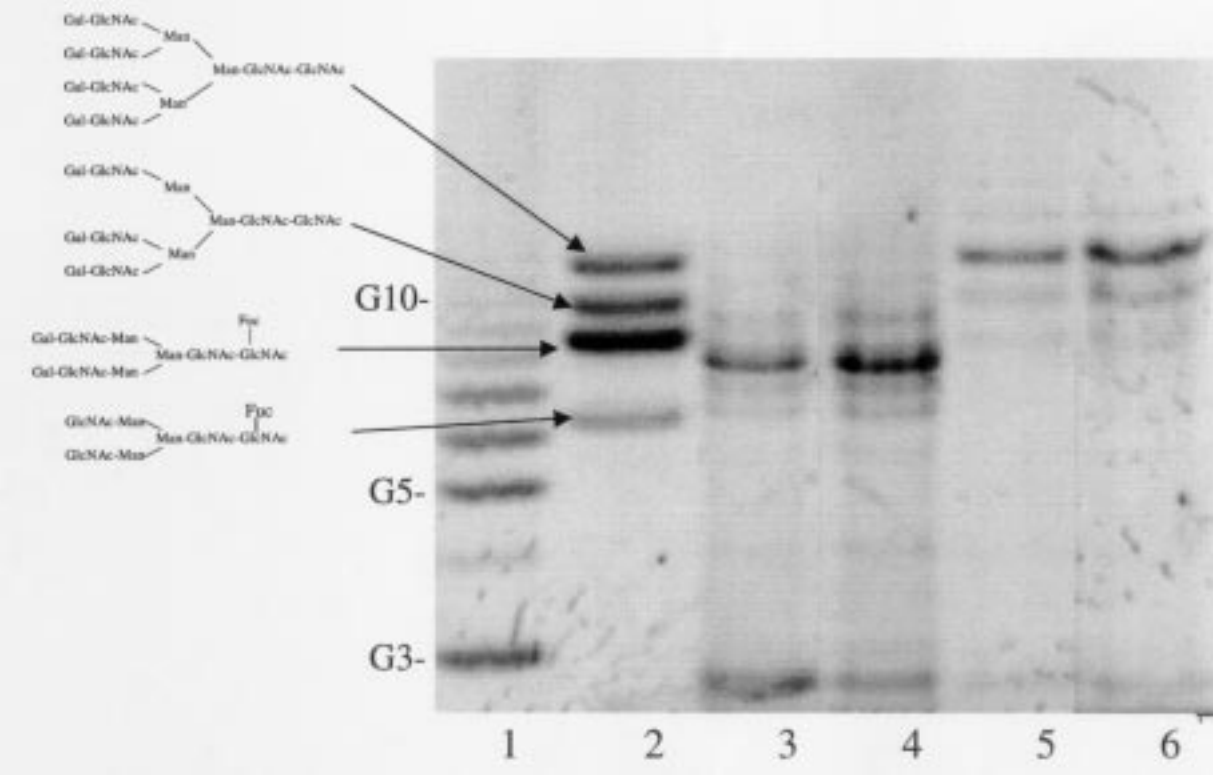


Figure 5. EPO N-linked oligosaccharide profiles analyzed by FACE. Culture supernatants harvested on day 4 and day 7 were purified by an immunoaffinity column. The N-linked oligosaccharides were released by PNGase F or plus sialidase at 37 °C for 16 h. The ANTS labeled carbohydrates were separated by polyacrylamide gel and visualized by detection of the fluorescence under UV illumination. Lane 1: glucose ladder; lane 2: carbohydrate standards: asialo-agalacto-biantennary with core fucose; asialo biantennary with core fucose; asialo triantennary and asialo tetraantennary. Lane 3 and 4 are sialylated N-linked glycans from culture supernatants collected on day 4 and day 7. Lane 5 and 6 are asialo N-linked glycans from culture supernatants collected on day 4 and day 7.

glycosylation pattern suggests that the enhanced EPO heterogeneity during batch culture was likely to be due to proteolytic break down.

7. Effect of glutamine on EPO heterogeneity

Evidence from the data presented from the 2-dimensional electrophoresis in Figure 4 suggested that glutamine levels in the culture had an effect on EPO heterogeneity, with an apparent decrease in degradation at high glutamine. This finding was confirmed by the appearance of the deglycosylated EPO bands in lanes 3 and 4 of Figure 7. These were taken from samples of culture at day 7 at low and high glutamine, respectively. The narrow band width for the sample from the high glutamine culture is similar in appearance to the day 4 band under standard conditions (lane 1), whereas the low and standard glutamine culture samples at day 7 (lanes 2 and 3) are wider and are consistent with the occurrence of degradation.

The effect of excess glutamine on EPO degradation was confirmed by analysis of these samples by simple one dimensional SDS-PAGE (Figure 8). The results showed that the increased breadth of the protein band from day 4 to day 7 observed under low or standard glutamine conditions was not observed at high glutamine. Thus, considering that there was no observed change in the glycosylation pattern, glutamine appeared to have an effect in reducing EPO peptide degradation during the course of the batch culture.

The effect of glutamine was also determined in a fed-batch culture in which it was attempted to maintain a standard concentration of glutamine throughout the growth period. Cells were inoculated into standard medium (4 mM glutamine) and fed with glutamine (4 mM) on day 3 and day 5. Thus, a total of 12 mM glutamine was added to the culture. However, the characteristics of the isolated EPO from this culture were identical to those from a batch culture in standard medium. This showed that under these conditions of

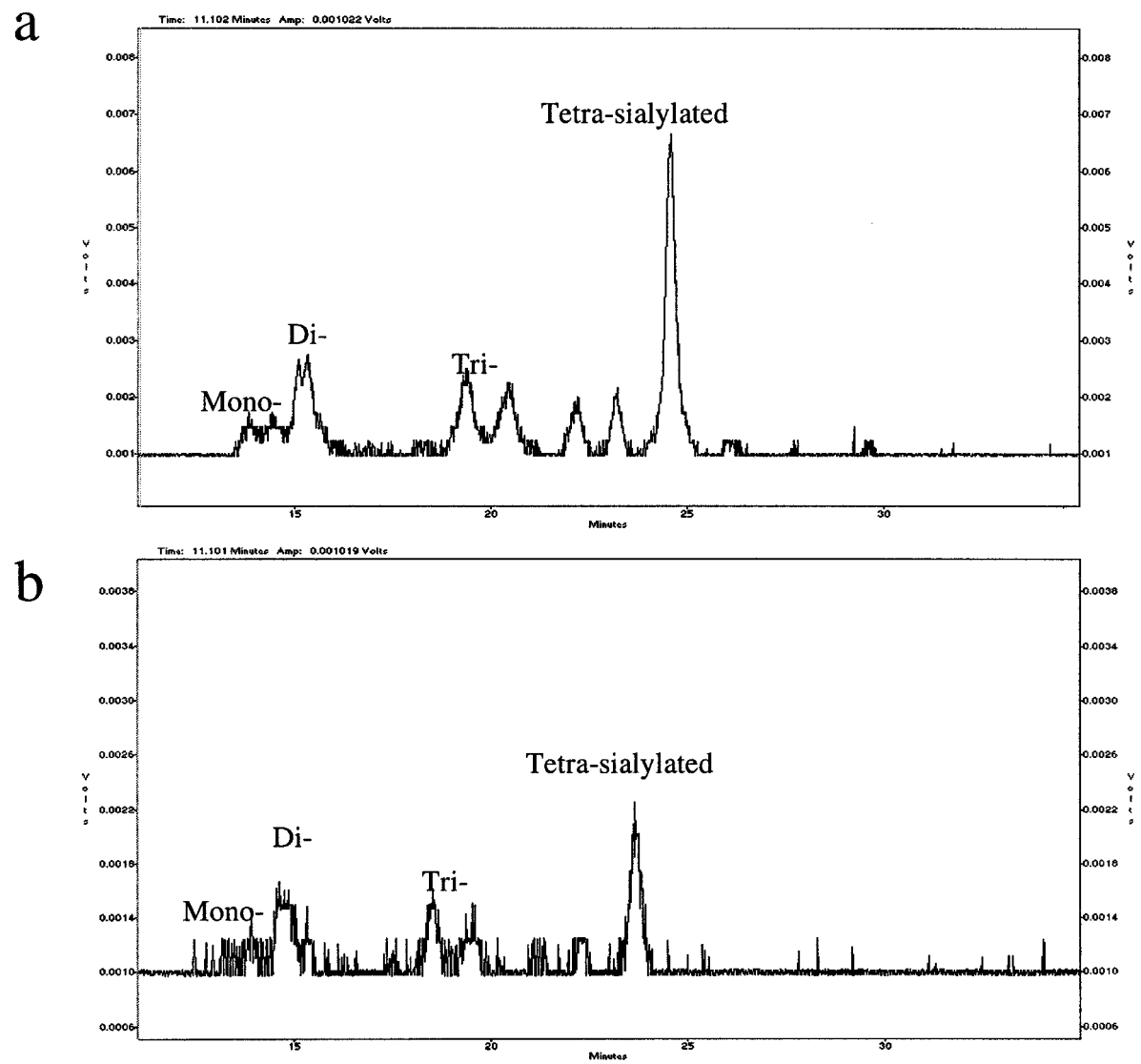


Figure 6. Analysis of sialylated N-glycans of EPO by HPLC. N-linked glycans released from 4- (a) and 7-day (b) culture samples were labeled with 2-AB and analyzed by ion exchange chromatography with a GlycoSep C column. The peaks were identified by comparison with the retention times of oligosaccharide standards.

glutamine feeding the extent of EPO degradation was not reduced.

Discussion

The structural heterogeneity of glycoproteins produced from cells in culture is due to a number of factors including variable intracellular glycosylation, variable extracellular glucosidase and proteolytic degradation. The cell culture parameters known to affect glycosylation include glucose depletion (Gershman and Robbins, 1981; Hooker et al., 1995), ammonia accumulation (Andersen and Goochee, 1995; Borys et al., 1994; Gawlitzek et al., 1998; Jenkins and Curling, 1994; Yang and Butler, 2000), growth rate (Hayter et al., 1993), lipid availability (Jenkins et al., 1994) and dissolved oxygen (Kunkel et al., 1998). These parameters may change during the course of a batch culture and this may well result in an increased heterogeneity over time. Such changes are exacerbated by the release of variable glycosidases and proteases secreted from viable cells or from lysis of dead cells.

Changes in the glycan structure have been analysed

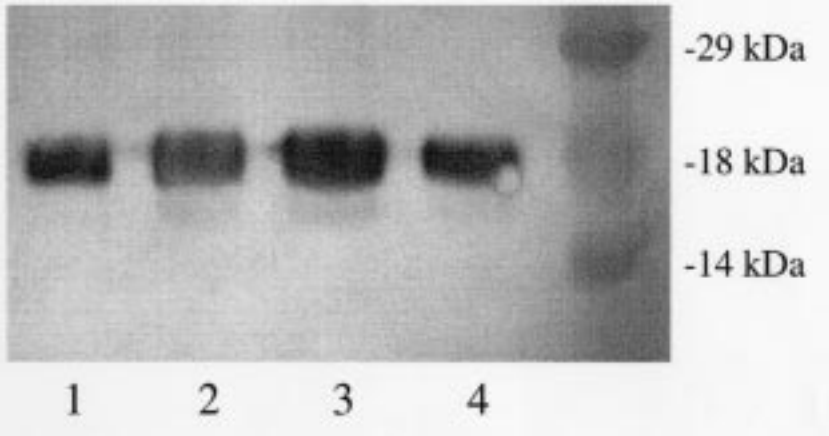


Figure 7. Immunoblot analysis of enzymatically deglycosylated EPO. Culture supernatants were collected on day 4 or day 7. The desalted samples containing EPO were concentrated and treated with PNGase F, O-glycosidase and sialidase at 37 °C for 16 h. The treated samples were separated by 14% SDS-PAGE and transferred to NC membrane. Lane 1 is supernatant containing 4 mM glutamine collected on day 4, Lane 2–4 are supernatants containing 4, 2.5 and 16 mM glutamine, respectively, harvested on day 7. Molecular weight markers are indicated.

during the course of many culture systems (Ferrari et al., 1998; Gawlitzek et al., 1995; Hooker et al., 1995; Munzert et al., 1996). The presence of extracellular sialidase has been reported to be responsible for the loss of terminal sialic acid during the course of anti-thrombin III (Munzert et al., 1996) and DNase (Ferrari et al., 1998) in batch culture of CHO cells. A progressive loss of sialic acid caused by an increased level of sialidase in the culture fluid can also be shown (Gramer et al., 1995). For EPO production from CHO cells we report the presence of at least 7 different glycoforms as analysed by both FACE and HPLC anion exchange, showing that the predominant structure was a tetra-antennary sialylated structure with other structures having lower antennarity and sialylation. However, the glycan patterns were identical throughout the culture, which indicated that extracellular sialidase activity was not evident in our culture system.

The stability of the EPO oligosaccharide pattern in our culture system may be due to a number of reasons. Firstly, glucose was not completely consumed and was maintained at a relatively high level (7 mM) even at the end of the culture period. Secondly, the level of ammonia at the end of culture may not have been high enough to induce the carbohydrate heterogeneity of EPO (Yang and Butler, 2000). Thirdly, the susceptibility of a glycoprotein to glycosidase degradation may vary between proteins and may depend upon the protein structure. It is possible that a fully glycosylated and non-denatured EPO is resistant to any glycosidase that may be present in the cultures.

The enhanced structural heterogeneity of EPO during the course of our CHO batch culture was found to be entirely due to peptide degradation. The evidence for this was the increased width of the electrophoretic band of de-glycosylated EPO and an altered pI range at the end of the culture. The degradation occurred during the latter part of the culture (day 4 to 7) and did not occur as result of incubation in conditioned cell-free medium. This evidence suggests that the effect was due to a protease activity released from the cells. The high viability of the cell population throughout the culture, including the period in which peptide degradation occurred, argues for the secretion of protease from metabolically active cells.

Although proteases may be secreted from many cell lines in culture, the enzymatic activity may be minimised in cultures supplemented with serum. Serum contains a large quantity of protease inhibitors, estimated at up to 15% of the total protein (Travis and Salvesen, 1983). However, despite the many advantages of serum-free media, the potential proteolytic degradation of secreted proteins is enhanced in media not supplemented with serum (Curling et al., 1990; Schlaeger et al., 1987; Sugimoto et al., 1992; Teige et al., 1994). The CHO cultures described here were grown in a serum-free medium formulation developed in our laboratory and did not include components likely to inhibit any protease activity.

The characteristics of the proteases released by various mammalian cell lines have been studied with respect to the type of protein cleavage and susceptibility to inhibition. Proteases of hybridoma cultures have

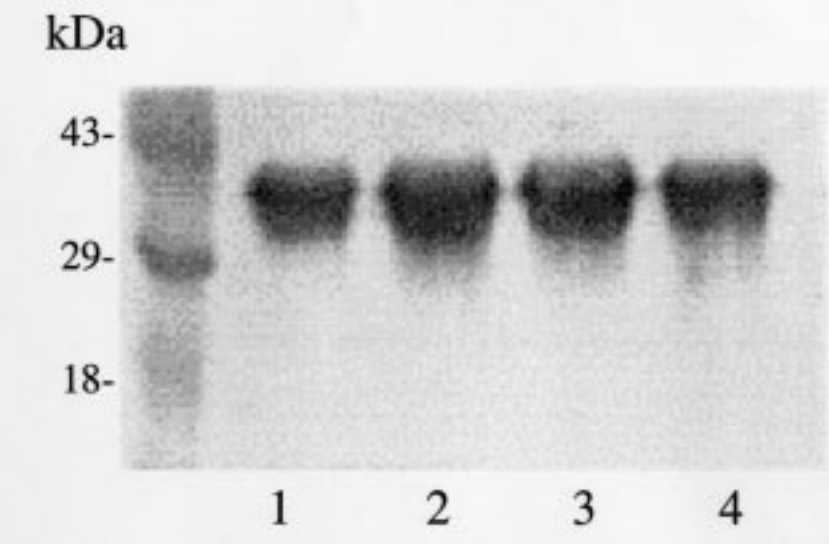


Figure 8. Gel electrophoreis of EPO from cultures with different glutamine levels. Culture supernatants with different concentrations of glutamine were collected on day 4 or day 7. Samples were concentrated and separated by 14% SDS-PAGE. The protein was transferred to NC membrane and detected by Western blot analysis with a monoclonal anti-huEPO antibody. Lane 1 is the supernatant from a standard containing 4 mM glutamine harvested on day 4. Lane 2–4 are supernatants containing 4, 2.5 and 16 mM glutamine, respectively, harvested on day 7. Molecular weight markers are indicated.

been characterised as similar to lysosomal cathepsin D (Schlaeger et al., 1987) or as a serine protease (Kratje et al., 1994). For BHK cell cultures, a dipeptidyl aminopeptidase has been described (Gawlitzek et al., 1995). Satoh et al., (1990) described the production of two types of proteases from CHO cultures – exopeptidases and endopeptidases. The exopeptidase had an aminopeptidase activity which increased linearly with the time and correlated with an increase in the non-viable cell count, suggesting release from lysed cells. The endopeptidase was described as a cysteine protease which was secreted continuously by viable cells.

Wang et al. (1985) analysed the susceptibility of human erythropoietin to proteolytic cleavage. They showed that there is a small region of the peptide that is sensitive to digestion by trypsin, chymotrypsin, V-8 protease and Lys C. Limited proteolysis with trypsin leads to the fragmentation of EPO into two domains of 16 kDa. However, the proteolytic cleavage we show for EPO in the CHO culture is clearly quite different with an estimated reduction in size of the native peptide chain of 700 Da which approximates to the cleavage of about 6 amino acid residues. This phenomenon of limited fragmentation has also been shown in other systems. Gawlitzek et al. (1995) reported the removal of up to 8 amino acids following proteolytic degradation of interleukin-2 secreted from BHK cells. A fragmentation of up to 10 amino acids was reported from interferon-γ secreted from CHO cells (Goldman et al., 1997).

The observed molecular size of EPO analyzed from the early phase of the CHO cultures suggests that this consists of an intact peptide structure, corresponding to authentic human EPO (34–38.5 kDa) (Sasakiet al., 1987). Peptide heterogeneity is observed only during the later stages of culture with the appearance of a truncated peptide. This differs from previous work on recombinant IFN-γ produced by CHO cells, in which Curling et al. (1990) and Goldman et al. (1997) reported that no full length peptide was detected at any stage of cultivation.

The effects of changes of carbon substrate levels in culture have been studied for many cell lines. High concentrations of glutamine caused reduced growth rates but enhanced antibody production of hybridomas (Omasa et al., 1992; Flickinger et al., 1992). Similarly our results showed that a high glutamine concentration caused a decreased specific cell growth rate of the CHO cells but an enhanced specific EPO productivity. The high glutamine also showed the characteristic metabolic changes of a decreased Qamm/Qgln coefficient but an increased Qlac/Qglc coefficient as has been noted previously for other mammalian cell lines (Hassell and Butler, 1990; Butler et al., 1991). However, the net ammonia production in cultures is

increased at high glutamine. In a previous publication we showed that the IC-50 of ammonia added to a CHO culture was 33 mM and that cell yield was reduced by 19% at 10 mM NH_4Cl (Yang and Butler, 2000). Although this suggests that CHO cells may be less sensitive to ammonia than many cell lines (Hassell et al., 1991), the generation of ammonia at high glutamine was likely to be the inhibitory factor to cause the observed reduction in the cell growth rate of the CHO cells.

A surprising effect of a high glutamine was that the apparent peptide degradation observed in our standard CHO cultures was eliminated. This result is consistent with several studies which have shown that the induction of protease may be a direct result of amino acid starvation (Cartwright, 1994). Froud et al. (1991) showed that production of a truncated form of the HIV envelope protein, gp120 from CHO cells was due to extracellular protease activity and this coincided with the depletion of several amino acids in the culture medium. A reduction of this proteolytic activity was achieved in a fed-batch culture in which the amino acid concentration was maintained at a critical level. Gawlitzek et al. (1995) showed that several truncated variants of IL-2 lacking up to 8 N-terminal amino acids were observed when cells were grown under limiting serine or aspartate concentrations. The fact that there was significant peptide degradation in our fed-batch culture which maintained glutamine at around the standard concentration (4 mM) suggests that this was below the critical glutamine concentration for reduced protease induction.

There are several possibilities for the mechanism of reduced protease activity in cultures with a high level of glutamine. It is possible that a low level of amino acids in the standard CHO cultures at day 4 cause the induction of a protease to be secreted from viable cells. This relates to previous work from our laboratory in which we showed that cultures supplemented with glutamine dipeptides but with a low level of amino acids induced the secretion of an extracellular protease from hybridoma cells (Christie and Butler, 1994). However, we cannot exclude the possibilities of a specific effect of glutamine in inhibiting protease function or of protecting EPO from protease attack in the medium. Further work is required to determine the precise mechanism behind the influence of glutamine on the proteolytic enzyme activity.

In conclusion, we have shown that in a batch culture of stably transfected CHO cells EPO was produced with a consistent glycosylation pattern throughout but with a truncated peptide in the later stage of the culture. Supplementation of the culture with a high level of glutamine eliminated the observed reduction in peptide size. It is possible that a regime of continuous feeding of nutrients, particularly amino acids may be important to ensure the synthesis of a recombinant glycoprotein with a consistent molecular structure.

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