Cytotechnology **23:** 103–111, 1997.

Special Issue

Monitoring proteolysis of recombinant human interferon-γ during batch culture of Chinese hamster ovary cells *

.

M. H. Goldman[1,2], D. C. James[1,2], A. P. Ison[2] & A. T. Bull[1]
[1] *Research School of Biosciences, University of Kent, Canterbury, Kent CT2 7NJ, United Kingdom;* [2] *Advanced Centre for Biochemical Engineering, University College, London University, Torrington Place, London WC1E 7JE, United Kingdom*

Received and accepted 17 June 1996

Key words: batch culture, Chinese hamster ovary, interferon-γ, mass spectrometry, proteolytic processing, serum-free cultivation

Abstract

Proteolytic cleavage of recombinant human interferon-γ (IFN-γ) expressed in Chinese hamster ovary (CHO) cells during batch fermentation has been monitored by mass spectrometric peptide mapping. IFN-γ was purified from cell-free culture supernatant by immunoaffinity chromatography and cleaved by endoprotease Asp-N. Peptide fragments were resolved by reverse-phase HPLC and identified by a combination of matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) and automated N-terminal peptide sequencing. Using this approach, a peptide was identified as the C-terminal fragment of the IFN-γ polypeptide. Analysis of this peptide by MS indicated that the recombinant IFN-γ polypeptide secreted by CHO cells was truncated by at least ten amino acids, initially at Gln^{133}-Met^{134}. No full length (143 amino acids) polypeptide molecules were observed at any stages of the fermentation. Additional proteolytic cleavages at basic amino acids N-terminal of Gln^{133} occurred during the later stages of the culture resulting in a heterogeneous IFN-γ polypeptide population with 'ragged' C-termini.

Introduction

Mass spectrometry (MS) is an increasingly attractive choice for analysis of recombinant proteins produced by mammalian cell systems. Two methods of ionization, developed relatively recently, are particularly suitable for analysis of peptides and proteins: matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) MS (Siuzdak, 1994). These techniques have proved to be a relatively rapid means of confirming the primary structure of recombinant proteins and analysis of post-translational modifications such as glycosylation which contribute to recombinant protein heterogeneity (Aebersold, 1993; Wang and Chait, 1994; Burlingame, 1996).

In this laboratory we have previously demonstrated the use of MALDI-MS in combination with exoglycosidase array sequencing to examine site-specific N-glycosylation of recombinant human interferon-γ (IFN-γ) produced by eukaryotic expression systems (James *et al*., 1995). This technique was subsequently employed by us to monitor changes in the N-glycosylation of IFN-γ during batch culture of CHO cells (Hooker *et al*., 1995). Further analyses of intact IFN-γ proteins by ESI-MS described the heterogeneity of recombinant IFN-γ populations derived from different eukaryotic host cells in detail (James *et al*., 1996). In this paper we describe the use of MALDI-MS to describe proteolysis of IFN-γ during batch culture.

Proteolysis is an unpredictable and poorly understood process in mammalian cell culture systems. This modification may occur intra- or extracellularly, and its

* This paper is dedicated to the memory of the late Professor Hiroki Murakami

extent will depend on the protein substrate, host cell and process conditions. Proteolysis may itself be desirable; for example, cleavage of inactive recombinant protein precursors by pro-protein endoproteases may be necessary to generate bioactive products. To this end, a soluble, recombinant form of the mammalian endoprotease furin can now be synthesized in CHO cells, and may facilitate the production of mature proteins which have been incompletely processed by the host cell (Nakayama, 1994). In other cases, unwanted proteolysis may be deleterious to recombinant protein function, and has prompted the construction of protease-negative strains of *E. coli*. In eukaryotic production systems, proteolytic activity can occur extracellularly due to the release of proteases from lysed cells, although the enzymes responsible remain largely uncharacterized. Environmental factors may affect proteolysis – Teige *et al*. (1994) demonstrated that higher proteolytic activity was associated both with a switch from serum to serum-free production and the culture lifetime. By the end of fermentation the product, antithrombin III, suffered considerable product degradation probably due to the loss of protease inhibitors present in serum. In addition, recombinant human interleukin–2 was shown to undergo N-terminal truncation under glucose limiting conditions during perfusion culture (Gawlitzek *et al*., 1995)

For production of any recombinant protein product using an eukaryotic production system, an evaluation of product quality is crucial. Proteolytic events may be a key factor in defining product quality. Here we demonstrate that proteolysis of a recombinant protein may vary during a production process, and describe an analytical approach to monitor these changes in detail.

Materials and methods

Chemicals

Analytical grade reagents were purchased from Sigma Chemical Company (Poole, United Kingdom) unless otherwise stated. Monoclonal antibody 20B8 coupled to Sepharose was provided by Celltech Ltd. (Slough, United Kingdom). Endoprotease Asp-N (mutant *Pseudomonas fragi*) was obtained from Boheringer Manheim (Lewes, United Kingdom).

Cell line and culture

Recombinant human IFN-γ was produced by the Duk cell line derived from mutant CHO-K1 cells lacking dihydrofolate reductase (DHFR$^-$) and co-transfected with the genes for IFN-γ and DHFR at Wellcome Research Laboratories (Beckenham, Kent, United Kingdom). This cell line (clone 320) was adapted for growth in serum-free medium in this laboratory (Jenkins *et al*., 1991) and IFN-γ and DHFR were co-amplified by methotrexate selection (1 mM).

Fermentation cultures were inoculated with cells harvested from two 2.5 l Bellco spinner cultures and seeded at 1.5×10^5 cells ml^{-1} into a 15 l fermentor (12 l working volume, InCeltech, Reading, United Kingdom); there was no carry over of spent medium. Conditions of constant dissolved oxygen tension (40% of air saturation by sparging with air), pH 7.2 (maintained by CO_2 and 0.1 M NaOH addition) and a temperature of 37 °C were maintained. Because large sample volumes (0.5–1.5l) were required for IFN-γ analysis the stirrer speed was stepped down with decreasing culture volume to maintain a constant power-to-volume ratio of 5×10^3 W l^{-1} (Hooker *et al*., 1995).

Cell and metabolite analysis

Samples were removed twice daily for analysis and culture supernatants were stored at –80 °C after removal of cells by centrifugation (1000 rpm for 10 min). Cell counts were made in a Neubauer counting chamber and cell viability determined by erythrosin dye exclusion. The cell pellets were fixed in ethanol (70% v/v) for flow cytometric analysis and the nuclei stained with propidium iodide for subsequent flow cytometric analysis on a Coulter Epics II flow cytometer (Jenkins *et al*., 1993). Glucose, lactate and ammonia determinations have been described in an earlier publication (Hayter *et al*., 1993).

Purification of IFN-γ

Supernatant from fermentations was harvested by cell centrifugation and adjusted to pH 6.6 with 50 mM 2-[N-Morpholino]ethanesulfonic acid (MES) and 0.02% (w/v) NaN_3 (buffer 1). Samples of equal volume from days 3 and 4 were combined as were days 5 and 6 and 7 and 8 to provide sufficient analytical material (Hooker *et al*., 1995). Samples were loaded onto a 1×10 cm immunoaffinity column containing the monoclonal antibody anti-IFN-γ 20B8, cyanogen bro-

mide coupled to Sepharose beads attached to an FPLC system (Pharmacia Biotech Ltd., Milton Keynes United Kingdom) and at a flow rate of 1.0 ml min^{-1}. Unbound material was washed off the matrix with 100 ml of 250 mM MES + 1 M NaCl, pH 6.5, and then 100 ml of buffer 1. Epitope mapping of IFN-γ in our laboratory has shown the monoclonal antibody anti-IFN-γ 20B8 to be specific for protein epitopes not associated with the C-terminal region (data not shown). Therefore, purification of IFN-γ was not influenced by changes in peptide processing. Bound IFN-γ was eluted in 13.5 mL of 0.1 M glycine-HCl, pH 2.5 and neutralized with 1.5 mL of 1 M Tris-HCl (pH 8.0). Purified IFN-γ was concentrated and desalted by ultrafiltration with Centriprep® concentrators (Amicon LTD, Stonehouse, United Kingdom) and then buffer-exchanged with 50 mM sodium phosphate buffer, containing 0.02% (w/v) NaN_3 (pH 8.0) and stored at 4 °C.

Protein determinations

Protein assays were performed with an assay kit supplied by Bio-Rad Laboratories Ltd., Hemel Hempstead, United Kingdom. BSA was used as standard.

Digestion by endoprotease Asp-N and separation of peptides by reverse-phase HPLC

Fifty μg of purified IFN-γ were incubated with 8 M guanidine hydrochloride for 2 h at 50 °C. The denatured samples were adjusted to 1 M guanidine hydrochloride and mixed with 2.5 μg of Asp-N for 18 h at 37 °C. Samples were applied in 0.06% (v/v) trifluoroacetic acid (TFA) to a Vydac 218TP52 reverse-phase column (C18, 2.1 × 250 mm) connected to a Waters 626 Millenium system (Millipore Ltd., Watford, United Kingdom). Peptides were separated with a linear gradient (0–70%) of 80% (v/v) aqueous CH_3CN with 0.052% TFA in 100 min at a flow rate of 0.1 ml min^{-1}. Individual peptide peaks were detected at 210 nm and collected manually. All peaks were collected and centrifuged at room temperature under vacuum to remove CH_3CN and lyophilized overnight, then stored at –20 °C. Each peak was measured by MALDI-MS and molecular masses associated with predicted peptide fragment masses. Those peptides that could not be correlated by mass alone were subjected to N-terminal sequencing. Only small fragments (1–4 amino acids) were not amenable to analysis.

Table 1. Growth, productivity and metabolic quotients

Parameter		Value
μ_{max}	(h^{-1})	0.028
q_{IFN}	(IU 10^6 $cells^{-1}$ h^{-1})	302.65
$q_{Glucose}$	(mM 10^6 $cells^{-1}$ h^{-1})	0.269
$q_{Lactate}$	(mM 10^6 $cells^{-1}$ h^{-1})	0.387
$q_{Ammonia}$	(mM 10^6 $cells^{-1}$ h^{-1})	0.049

Matrix assisted laser desorption/ionization mass spectrometry

All MALDI-MS spectra were obtained with a VG Tof-Spec spectrometer (VG Organic, Manchester, United Kingdom). Lyophilized peptides were dissolved in 5 μl distilled water. Samples (0.5 μl) were mixed with 0.5 μl of a saturated solution of α-cyano–4-hydroxy cinnaminic acid (Aldrich Chemical Co., Gillingham, United Kingdom). Spectra were externally calibrated using the peptide fragment 1–12 of vasoactive intestinal peptide with an average molecular mass of 1425.5. Ions were desorbed by pulses (1 Hz repetition rate) of light from a N_2 laser at 337 nm and accelerated at 20 000 V potential in positive ion mode. After a linear flight path of 0.65 m, ions were detected by a microchannel plate detector (2000 V applied voltage) and digitized at 250 MHz. Observed signals were adjusted so as to obtain maximum peak resolution (optimum signal to noise ratio at minimum laser energy) and the spectra from twenty laser pulses were averaged.

Results and discussion

Batch fermentation

The cells demonstrated typical growth characteristics (Hayter *et al.*, 1991) and reached a maximum viable cell density of 0.54×10^6 cells ml^{-1} and a total cell density of 0.7×10^6 cells ml^{-1} after 95 h of culture (Figure 1a). Cell viability dropped sharply after peak cell densities had been reached (Figure 1a). IFN-γ accumulated during the growth phase to concentrations of 11×10^3 IU mL^{-1} (Figure 1b). The maximum specific growth rate of the CHO cells under these conditions was 0.028 h^{-1} (μ_{max}) (Table 1). Maximum specific IFN-γ production rates (q_{IFN}) also were associated with this period of growth and the culture produced approximately 300 IU 10^6 $cells^{-1}$ h^{-1}.

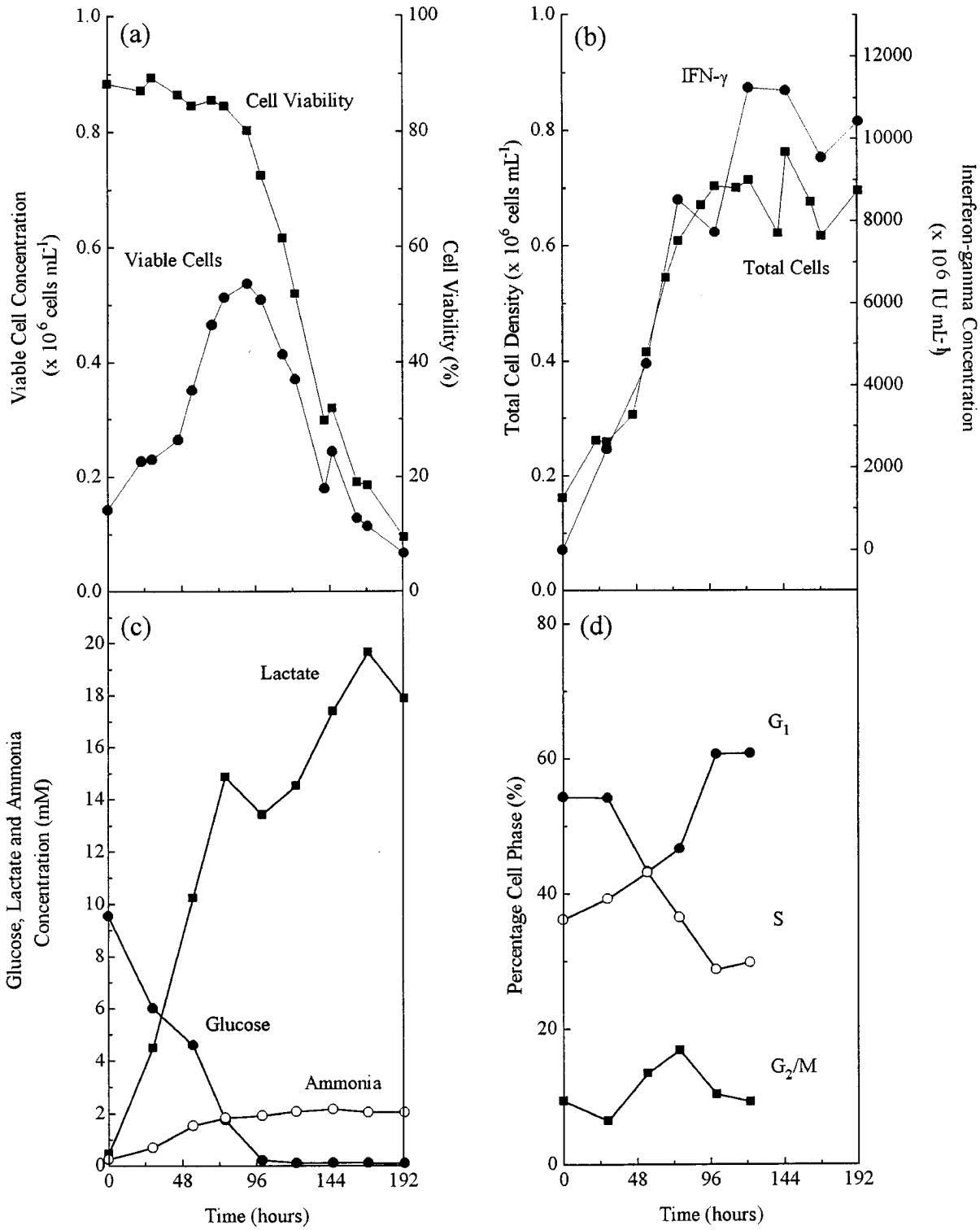

Figure 1. Fermentation profiles of CHO cells. (a) viable cell density and viability, (b) total cell density and IFN-γ production; (c) glucose consumption, lactate and ammonia production curves; (d) cell cycle distribution.

Glucose was utilized rapidly and was depleted after 100 h (Figure 1c). The maximum specific glucose utilization rate was 0.269 mM 10^6 cells^{-1} h^{-1} (q_{Glucose}). Lactate and ammonia both accumulated during the culture (Figure 1c). Lactate showed a rapid initial increase reaching a final concentration of 18 mM; accordingly, the specific lactate production rate was 0.387 mM 10^6 cells^{-1} h^{-1} (q_{Lactate}). Ammonia levels increased slowly and reached 2 mM; the rate of specific ammonia production was 0.049 mM 10^6 cells^{-1} h^{-1} (q_{Ammonia}). Thus a complete depletion of glucose is related to the end of the growth phase and lactate and ammonia production is related to the profile of batch growth.

Cell cycle distributions in the CHO cell population are shown in Figure 1d. Results are not displayed for the whole culture lifetime due to the build up of cell debris which prevent accurate determinations of cell cycle distributions. When the late exponential period had been reached an increase in the percentage of cells in the G_1-phase occurred. G_1-phase cells formed about 60% of the total cells after 100 h of growth. The proportion of cells in S-phase decreased after an initial increase, changes that were the reverse of the proportion of cells in G_1. The quantities of cells in G_2/M-phase increased during the growth phase reaching 17%.

IFN-γ peptide mapping by reverse-phase HPLC and mass spectrometry

The elution profile of Asp-N peptides by rp-HPLC is shown in Figure 2. All the collected peaks were subjected to MALDI-MS and masses matched to predicted peptide fragments (Table 2). It was evident that cleavage events were occurring at Glu residues in addition to Asp residues. This is to be expected with the digestion conditions employed: low protein:protease ratio, extended digestion time and denatured substrate. Protein sequence analysis predicts 20 peptides or amino acids ranging in size (Figure 3). The majority of the IFN-γ polypeptide was mapped in this manner (92%), with only small peptide fragments ($<$500 Da) not amenable to analysis, due to interference by matrix ions. Those peptide fragments that could not be assigned on mass criteria alone were subjected to N-terminal sequencing to confirm their identity. The C-terminal peptide fraction eluted early (peak 1) and its identity was confirmed by N-terminal sequencing. Using endoprotease Asp-N, it was not possible to assign an N-terminal amino acid, however, previous studies have shown unambiguously that the N-terminal amino acid of IFN-γ produced by CHO cells is pyroglutamic acid (James *et al*., 1996).

Changes in the C-terminus

Mass spectra of collected C-terminal peptide fragments are shown collectively from various stages of the culture in Figure 3. A polypeptide truncation was revealed of at least 10 amino acids beginning at Gln133, corresponding to a maximum molecular weight of 1658 Da. This truncation was apparent in all samples; no full length IFN-γ molecules (143 amino acids) were found at any stage of the fermentation. In early culture there was only one major IFN-γ polypeptide species of 133 amino acids. This was also visible in the late and death phase samples. There was evidence of further truncation later in culture by the increase in peptide heterogeneity. These new peptide species were present at lower intensities during early culture and at greater intensities at late and death stages of culture. The death phase sample showed the greatest heterogeneity due to the addition of a species of molecular mass 71 Da to several of the peptide fragments. A peak of molecular mass 1065 Da in the late and death phase samples was not conclusively identified.

The major cleavage event occurs at Gln133, the enzyme responsible for this cut is not known. James *et al*. (1996) demonstrated by EI MS that CHO cell derived IFN-γ polypeptides from late culture terminated between Gly127 and Gln133. Since this truncation occurs at all stages of the fermentation and at a point of relatively low cell death the event may be intracellular in origin. Protease levels in the medium during early culture are likely to be low due to the relatively high culture viability. Curling *et al*. (1990) have demonstrated that proteolysis of IFN-γ occurs prior to secretion from CHO cells. The truncations seen in later culture may have occurred sequentially by the loss of individual amino acids, as indicated in Figure 3.

It is likely that the late and death phase modifications were caused by the action of cellular carboxypeptidases or serine proteases released into the culture supernatant. The C-terminus of IFN-γ is rich in basic amino acids which are good substrates for these enzymes. These types of enzymes are thought to be released by dead cells (Teige *et al*., 1994). In addition, the sequence Lys-Arg-Lys-Arg is the cleavage site for the endoprotease furin. The additional mass difference of 71 Da to several of the peptides in the late culture sample may be due to the presence of the residue Ala117. The enzyme responsible for this event is not

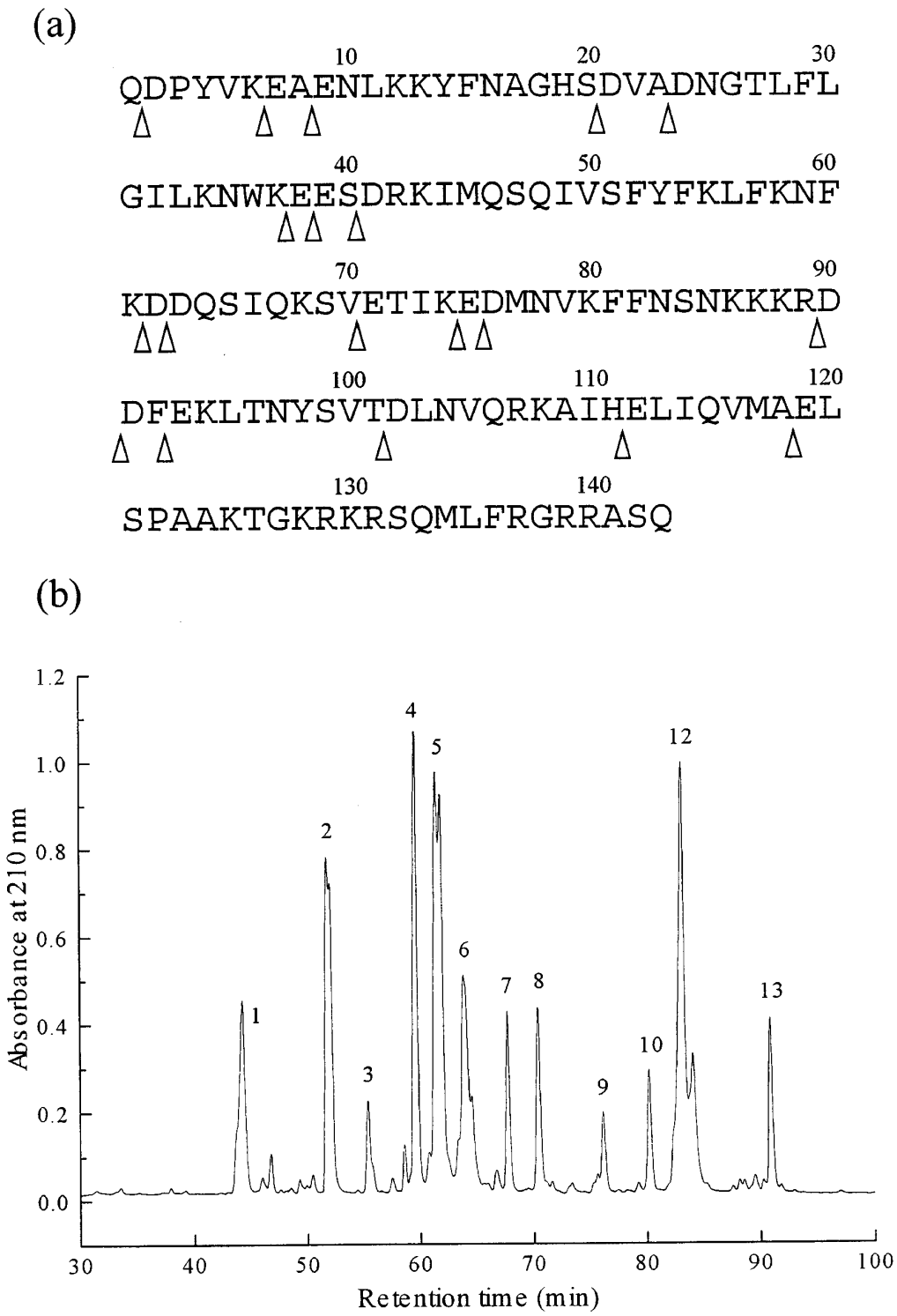

Figure 2. (a) IFN-γ sequence (without leader sequence) with observed endoprotease Asp-N cleavage sites; (b) elution profile of endoprotease Asp-N digested purified IFN-γ samples separated by rp-HPLC. Peak numbers indicate the individually collected peaks analysed by MALDI-MS.

Table 2. Predicted and experimental masses of endoprotease Asp-N peptides of IFN-γ with N-terminal sequencing data

Peak number	Measured mass (Da)	Correlated mass (Da)	Residues	Sequence
1	1658.2	4430.2	119–133	ELSPAAKTGKRKRSQ
2	1194.3	1193.4	102–111	DLNVQRKAIH
3	1777.9*	1756.1	76–89	DMNVKFFNKKKR (N)
4	1407.8	1407.6	9–20	ENLKKYFNAGHS
5	1757.4	1756.1	76–89	DMNVKFFNKKKR
	1607.1	1607.8	7–20	EAENLKKYFNAGHS
6	variable	1054.1	93–101 (G)	EKLTNYSVT
7	1431.3	1431.5	90–101	DDFEKLTNYSVT (N)
8	803.7	803.0	112–118	ELIQVMA (N)
9	903.6	904.0	63–70	DQSIQKSV
	2210.2	2210.4	2–20	DPYVKEAENLKKYFNAGHS
10	2325.1	2228.5	2–20	DPYVKEAENLKKYFNAGHS (N)
11	variable	1748.0	24–37 (G)	DNGTLFLGILKNWKE
12	2670.2	2668.2	41–61	DRKIMQSQIVSFYFKLFKNFK

G = glycosylated; N = N-terminal sequenced; * = possible Na^+ addunct.

known although the endoprotease chymotrypsin acts weakly on the C-terminal of methionine. The species of mass 1065 present in late and death stages of culture could be a sodium-potassium addunct of a species of mass 1005 Da that was seen only in the death phase sample.

This increase in product heterogeneity may have important implications for quality control and biological activity. There is an industrial practice of adding protease inhibitors to serum free medium to prevent product degradation due to the action of released cellular proteases. This practice is unlikely to prevent the earliest truncation event at Gln133. However, the events are particularly cell line and product specific. The biological activity and receptor binding of IFN-γ are both linked to its C-terminal region (Farrar and Schreiber, 1993; Lundell *et al.*, 1991). Death phase samples were found to bind to the IFN-γ receptor at a 20-fold lower rate than commercial grade IFN-γ from *E. coli* (data not shown). IFN-γ from early culture is likely to be the most bioactive; the region Lys-Arg-Ser is of particular importance to the bioactivity of IFN-γ. Work is required to determine the mechanisms behind these proteolytic events and their influence on product activity. The number of cleavage sites in IFN-γ makes it an ideal model system for these studies.

Conclusions

We have demonstrated the value of MALDI-TOF MS for monitoring changes in the proteolytic processing of IFN-γ during batch culture of Chinese hamster ovary cells. No full length molecules were detected at any stage of cultivation. At an early stage of culture, IFN-γ appears to be proteolytically processed intracellularly by an unidentified endoprotease. This event has implications for the addition of protease inhibitors to improve product quality in mammalian fermentation systems. Further cleavage occurs later in culture probably by the action of carboxypeptidases or serine proteases in the culture supernatant. These events result in a heterogeneous population of mixed length polypeptides lacking residues important for biologically activity.

Acknowledgments

UCL is one of the Biotechnology and Biological Science Research Council's Advanced Centres for Biochemical Engineering and the Council's support is gratefully acknowledged. We thank The Wellcome Foundation for the gifts of recombinant CHO cells containing IFN-γ, Celltech Biologics Ltd. for monoclonal 20B8. We thank Professor Nigel Jenkins of the DeMontfort University for helpful discussions.

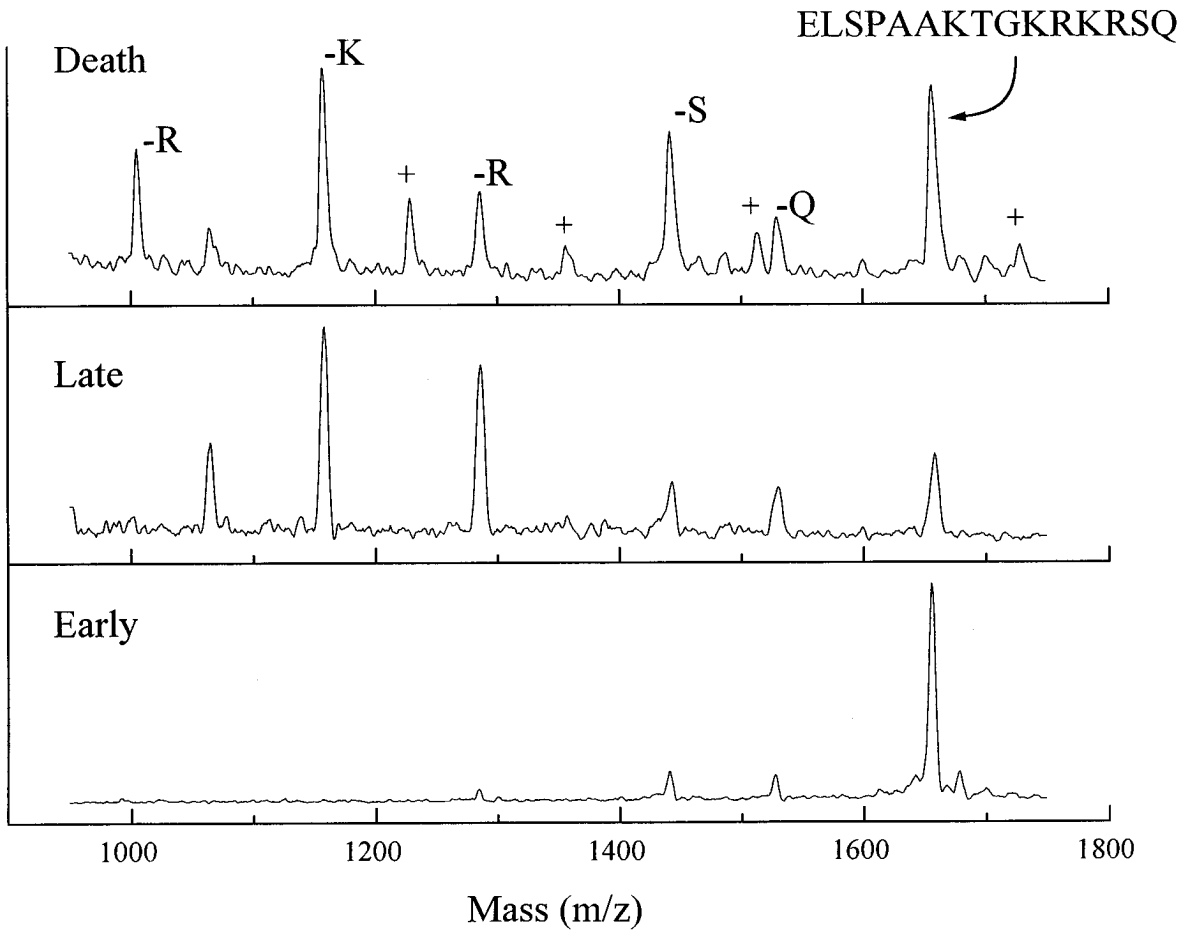

Figure 3. Mass spectra of IFN-γ time course samples. Early = 76 and 100 h, Late = 122 and 146 h and Death = 169 and 193 h; + = addition of Ala^{117}.

References

Aebersold R (1993) Mass spectrometry of proteins and peptides in biotechnology. Current Opinion Biotech 4: 412–419.

Burlingame AL (1996) Characterization of protein glycosylation by mass spectrometry. Current Opinion Biotech 7: 4–10.

Curling EMA, Hayter PM, Baines AJ, Bull AT, Gull K, Strange PG and Jenkins N (1990) Recombinant human interferon-γ. Differences in glycosylation and proteolytic processing lead to heterogeneity in batch culture. Biochem J 272: 333–337.

Farrar, MA and Schreiber MD (1993) The molecular cell biology of interferon-γ and its receptor. Ann Rev Immunol 11: 571–611.

Gawlitzek M, Conradt HS and Wagner R (1995) Effect of different cell culture conditions on the polypeptide integrity and N-g lycosylation of a recombinant model glycoprotein. Biotechnol Bioeng 46: 536–544.

Hayter PM, Curling EMA, Baines AJ, Jenkins N, Salmon I, Strange PG and Bull AT (1991) Chinese hamster ovary cell growth and interferon-γ production kinetics in stirred batch culture. App Microbiol Biotech 34: 559–564.

Hayter PM, Curling EMA, Gould ML, Baines AJ, Jenkins N, Salmon I, Strange PG and Bull AT (1993) Glucose-limited chemostat culture of Chinese hamster ovary cells producing recombinant interferon-γ. Biotechnol Bioeng 39: 327–335.

Hooker AD, Goldman MH, Markham NH, James DC, Ison AP, Bull AT, Strange PG, Salmon I, Baines AJ and Jenkins N (1995) N Glycans of recombinant human interferon-γ change during batch culture of Chinese hamster ovary cells. Biotech Bioeng 48: 639–648.

James DC, Freedman RB, Hoare M, Ogonah O, Rooney OW, Rooney BC, Larinov OA, Dobrovolsky VN, Lagutin OV and Jenkins N (1995) N-glycosylation of recombinant human interferon-γ produced in different animal expression systems. Bio/Tech 13: 592–596.

James DC, Goldman MH, Oliver R, Hoare M, Jenkins N, Oliver RWA, Green BN and Freedman RB (1996) Posttranslational processing of recombinant human interferon-γ in animal expression systems. Protein Sci 5: 331–340.

Jenkins N (1991) Growth factors. In: Butler M (ed.), Mammalian Cell Technology: a practical approach. Vol. 1 (pp. 39–53) University Press, Oxford, United Kingdom.

Jenkins N, Wingrove C, Strange PG, Baines AJ, Curling EMA, Freedman RB and Pucci P (1993) Changes in the glycosylation pattern of interferon-γ during batch culture. In: Kaminogawa S, Ametani A and Hachimura S (eds.), Animal Cell Technology: Basic and Applied Aspects, Vol. 5 (pp. 231–235) Kluwer Academic Publishers, Dordrecht, The Netherlands.

Lundell D, Lunn C, Dalgano D, Fossetta J, Greenburg R, Reim R, Grace M and Nakayama K (1994) Purification of recombinant soluble forms of furin produced in Chinese hamster ovary cells. Methods Enzymol 224: 167–175.

Teige M, Weidemann R and Kretzmer G (1994) Problems with serum-free production of antithrombin III regarding proteolytic activity and product quality. J Biotech 34: 101–105.

Siuzdak G (1994) The emergence of mass spectrometry in biochemical research. Proc Natl Sci 91: 11290–11297.

Wang R and Chait BT (1994) High-accuracy mass measurement as a tool for studying proteins. Current Opinion Biotech 5: 77–84.

Address for Correspondence: M. H. Goldman, Research School of Biosciences, University of Kent, Canterbury, Kent CT2 7NJ, United Kingdom.