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Plant protein hydrolysates support CHO-320 cells proliferation and recombinant IFN-γ production in suspension and inside microcarriers in protein-free media

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Abstract

We have recently developed a protein-free medium (PFS) able to support the growth of Chinese hamster ovary (CHO) cells in suspension. Upon further supplementation with some plant protein hydrolysates, medium performances reached what could be observed in serum-containing media [Burteau et al. *In Vitro Cell. Dev. Biol.-Anim.* 39 (2003) 291]. Now, we describe the use of rice and wheat protein hydrolysates, as non-nutritional additives to the culture medium to support productivity and cell growth in suspension or in microcarriers. When CHO-320 cells secreting recombinant interferon-gamma (IFN-γ) were cultivated in suspension in a bioreactor with our PFS supplemented with wheat hydrolysates, the maximum cell density increased by 25% and the IFN-γ secretion by 60% compared to the control PFS. A small-scale perfusion system consisting of CHO-320 cells growing on and inside fibrous microcarriers under discontinuous operation was first developed. Under these conditions, rice protein hydrolysates stimulated recombinant IFN-γ secretion by 30% compared to the control PFS. At the bioreactorscale, similar results were obtained but when compared to shake-flasks studies, nutrients, oxygen or toxic by-products gradients inside the microcarriers seemed to be the main limitation of the system. An increase of the perfusion rate to maintain glucose concentration over 5.5 mM and dissolved oxygen (DO) at 60% was able to stimulate the production of IFN-γ to a level of 6.6 μg h^{-1} g^{-1} of microcarriers after 160 h when a cellular density of about 4×10^8 cell g^{-1} of carriers was reached.

Abbreviations: AU – arbitrary units; CHO – Chinese hamster ovary; DO – dissolved oxygen; LDH – lactate deshydrogenase; IFN-γ – interferon-gamma; PFA – protein-free medium for adhesion; PFS – protein-free medium for suspension; RP – rice protein hydrolysates; WP – wheat protein hydrolysates.

Introduction

In recent years, the industrial production of recombinant proteins for therapeutic or diagnostic purposes has generated a huge market, which is growing further each year. More and more of these biotherapeutics are produced in recombinant mammalian cell lines (like Chinese hamster ovary (CHO), SP2/0 or NS0 cells) instead of bacteria, yeasts or insect cells, the latter being more convenient as rough expression systems, but unable to perform extensive post-translational modifications

that are required for many biological activities (Chu and Robinson 2001). CHO cells are capable of glycosylation patterns very close to human glycanic structures. Nevertheless, sialic acids are bound to the glycanic structures of human proteins in both the α-2, 3 and α-2, 6 conformation whereas proteins expressed in CHO cells display only sialic acids linked in the α-2, 3 conformation (Bragonzi et al. 2000).

Progressively, serum and animal compounds are banned from the formulation of nutritive media to fulfil biosafety requirements and to ease recombinant protein purification procedures. However, the development of such chemically defined protein-free media requires complex, cell line-specific studies and their optimisation sometimes turns out to be very expensive. Nevertheless, since CHO cells can be adapted to serum-free or to protein-free media, according to some authors, the switch to serum-free media can be easier while using protein hydrolysates (or peptones) as supplements (Jan et al. 1994; Keen and Rapson 1995; Heidemann et al. 2000). Plant peptones, in particular, can be used as a cheap partial substitute to serum to respond to biosafety concerns.

In a recent study, we identified several plant peptones showing a positive effect on CHO-320 cell growth and recombinant interferon-gamma (IFN-γ) productivity in protein-free suspension culture (Burteau et al. 2003). An additional advantage of using CHO cells is their ability to grow as adherent cells, as immobilised aggregates or as free-cells in suspension depending on the formulation of the culture medium utilised. Therefore, different strategies can be used for the production of recombinant proteins by such cell lines. The simple batch suspension culture could be replaced by more complex feeding strategies, such as fed-batch or even continuous perfusion aimed to maintain stable nutrient concentrations and to limit toxic metabolic by-products accumulation.

For perfusion cultures in suspension, the critical point resides in the separation of the cells from the outgoing medium. For that purpose, settling, filtration or centrifugation techniques are usually used, but each suffers from several drawbacks such as filter clogging, detrimental impact on cell viability or contamination hazards (Lee and Palsson 1990; Lee et al. 1993; Nishijima et al. 2000). The utilization of solid supports packed in fixed-bed devices, which allows cell adhesion and/or entrapment is also another way to separate cells from the culture medium. Unfortunately, cell sampling is almost impossible in such systems and the dense packing of the matrix inside the fixed-bed device can be a source of important gradients for some toxic by-products and/or basal metabolites. In particular, non-woven polyester fabrics, (NWPF), have been used successfully in fixed-bed reactor for the cultivation of some cell lines because their high surface-to-volume ratio allows the development of high cell densities. Fibra-cel® carriers, which are used in this paper, are commercially available NWPF products that have been used successfully with insect cell lines (Kompier et al. 1991; Agathos 1996; Ikonomou et al. 2002) and several mammalian cell lines, either anchorage-dependent (Racher et al. 1995; Hu et al. 2000; Kaufman et al. 2000; Mertens et al. 2001) or anchorage-independent (Wang et al. 1992). In most of these studies serum-supplemented media were used, at least during the initial cell propagation phase.

In this work we used CHO-320 cells constitutively expressing human IFN-γ and previously adapted to cultivation in a protein-free medium supplemented with plant protein hydrolysates (Burteau et al. 2003). We describe the cultivation of CHO-320 cells, either in suspension during batch experiments or immobilised as aggregates inside fibrous microcarriers during perfusion cultures. Cell growth, viability, metabolic analysis, as well as recombinant IFN-γ production and glycosylation patterns are presented.

Materials and methods

Cells, media and cultivation procedures

The CHO-320 cell line was a gift from Dr A. Marc (CNRS and Institut Polytechnique de Lorraine, Nancy, France) and was selected by Wellcome Biotechnology with the DHFR-methotrexate method to synthesize and secrete human IFN-γ (Castro et al. 1995). These cells were used as previously described (Burteau et al. 2003). In brief, after complete adaptation, they were cultivated in a protein-free medium (PFS) optimized from the *basal defined medium* (BDM) previously described (Schneider 1989), which is a 5:5:1 (v:v:v) mixture of Iscove's MDM, Ham's F12 and NCTC 135

media, further supplemented with glutamine to reach a 6 mM final concentration, 32.5 mM $NaHCO_3$ and 60 μM ethanolamine. To grow CHO cells in suspension, BDM was supplemented with 0.1% pluronic F68, 0.04 pM sodium selenite and 500 μM ferric citrate with a 1:1 Fe:citrate ratio and named PFS for suspension (PFS). When cells were grown inside Fibra-cel® microcarriers, ferric citrate concentration was reduced to 50 μM and named PFA for Protein-Free and Adhesion. Both media were further supplemented with plant protein hydrolysates (rice : Hypep 5115, RP; and wheat : Hypep 4605, WP) to promote cell growth and IFN-γ production. Both hydrolysates were kindly provided by Quest International (Naarden, The Netherlands) and added at a concentration of 2 g l^{-1} to grow cells in suspension (PFS-WP) and 1 g l^{-1} in adhesion (PFA-RP).

CHO-320 cells have been previously directly adapted from adhesion in serum-containing medium to suspension in PFS (Burteau et al. 2003). They were routinely cultivated in 125 ml shake-flasks, inoculated with 3×10^5 cells ml^{-1} at 37 °C under a water-saturated atmosphere and 5% (v/v) CO_2 on an orbital shaking platform (New Brunswick Scientific, Edison, NJ) at 100 rpm. Batch cultures, performed in a 1.5 l (working volume) bioreactor (Celligen Plus™, New Brunswick Scientific) were also inoculated with 3×10^5 cells ml^{-1} at 37 °C and under agitation at 60 rpm. Dissolved oxygen (DO) and pH were kept respectively at 50% and 7.2, by surface aeration and sparging using a four-gas mixing system (CO_2, N_2, O_2, air). Agitation was performed by a pitched blade impeller.

Fibra-cel® microcarriers (Bibby Sterilin, Stone, UK) that are 6 mm discs made of NWPF were utilised at 0.34 g per 25 ml of culture medium. Flasks were previously siliconised with Sigmacote® (Sigma, St Louis, MO). Microcarriers were first equilibrated with BDM overnight. The equilibration medium was discarded and the flasks containing the carriers were inoculated with 3×10^6 cells at 37 °C and 5% CO_2 (v:v) with occasional agitation for 4 h in 10 ml of PFA supplemented or not with plant protein hydrolysates. CHO-320 cells used to inoculate microcarriers came from suspension cultures in PFS. Afterwards, the medium was aspirated and replaced by 25 ml of fresh medium and flasks were agitated at 10 rpm on a rocking table. The medium was changed for the first time 72 h after inoculation, then once a day for the next two days and finally twice a day. A sample of the medium was centrifuged to remove any suspended cells and stored either at 4 or at − 20 °C for lactate deshydrogenase (LDH) assay, IFN-γ concentration and metabolites analyses. The free cell density (cells not trapped inside carriers) and cell viability were determined by the Trypan blue dye exclusion method. As cells trapped within the carriers were not accessible for direct enumeration, three to five carriers were taken and immersed in a crystal violet (0.1 %; w/v) and citric acid (0.1 M) solution and, after one day at 27 °C, nuclei were counted in a hemocytometer.

Fixed-bed reactor cultures were performed in a 1.5 l (working volume) Celligen Plus™ bioreactor containing an internal retention device packed with 25 g Fibra-cel® and a vertical mixing system. The medium volume was held constant and DO was kept either at 50% (v:v) or at 60% air saturation and the pH was controlled at 7.2 by surface aeration and sparging. Temperature was set at 37 °C. The bioreactor was inoculated with 225×10^6 cells from a shake-flask suspension culture. After 4 h under gentle stirring (50 rpm), the agitation was set to 80 rpm and was increased gradually along the course of the culture until the maximum value of 150 rpm was reached. After the inoculation, a batch mode was kept until glucose concentration approached 6 mM, at which point perfusion started. The perfusion rates of two reactor were set to maintain glucose concentrations around 4 mM and 50% DO or 5.5 mM and 60% DO. This resulted in perfusion rates ranging from 0.1 to 0.2 h^{-1}. Regular medium samplings allowed the determination of the glucose consumption rate, which was extrapolated for the next hours to readjust the medium perfusion rate. The bioreactor experiments ended when medium was spent.

Analytical methods

IFN-γ quantification assay was performed with the human IFN-γ Duoset® kit (R & D Systems Inc, Abingdon, UK) according to the manufacturer's instructions.

For western-blots, proteins were separated by SDS-PAGE (Laemmli 1970) and then transferred to a polyvinylidene fluoride membrane (Amersham Biosciences, Little Chalfont, UK) which was first saturated over one h in PBS containing 5%

(w/v) defatted milk powder, 0.05% Tween-20, 0.02% NaN_3. The membrane was then incubated for 2 h with the primary antibody (Endogen, Woburn, MA) prior to a 1 h incubation with a peroxidase-conjugated secondary antibody (Dako, Glostrup, Denmark). Between each incubation with the antibodies and before the peroxidase reaction, the membrane was washed 4 times for 5 min in PBS containing 0.05% Tween-20, 0.5% defatted milk powder. A light-sensitive film (Amersham Biosciences) was utilised to visualise the peroxidase-induced chemiluminescent reaction after incubation of the membrane with the ECL + kit (Amersham Biosciences).

Extracellular LDH activity was quantified with Sigma LD-L reagent. Measurement of glucose and lactate concentrations in the culture medium were carried out using a Bioprofile-100 automated analyser (Nova Biomedical, Waltham, USA). Alanine concentration was measured enzymatically in a multiwell assay plate. 25 μl of diluted samples were mixed with 12.5 μl NAD^+ (240 mM), 150 μl water and 63 μl Tris/hydrazine buffer (Tris 0.04M, 5% (v/v) hydrazine, 1.7 mM EDTA, adjusted at pH 9.0 with 1M HCl). The optical density was read in a microplate reader (Spectracount, Packard, Warrenville, USA) set to 340 nm; after homogenisation, 2.5 μl of alanine dehydrogenase (Sigma) were added and the plate incubated at room temperature for 30 min before reading again the optical density. Other amino acids were quantified by reverse-phase HPLC (Thermo Separation Products, San José, CA) using a pre-column derivatization with o-phthaldehyde, a Spherisorb ODS2, 5 μm, 125 × 3 mm ID column (Waters, Milford, MA) and a EX337 EM452 fluorescence detector (Jasco, Tokyo, Japan) according to Fekkes et al. (1995).

Results

Effects of wheat protein (WP) hydrolysates on bioreactor cultivation of CHO-320 cells in suspension.

Previous results have indicated that WP hydrolysates were the best peptidic supplement tested to promote CHO-320 cell growth and IFN-γ secretion upon cultivation of the cells in suspension (Burteau et al. 2003). CHO-320 cells were cultivated in a 1.5 l (working volume) bioreactor in PFS for cells in suspension supplemented (PFS-WP) or not (PFS) with 2 g l^{-1} of (WP) hydrolysates (Figure 1). Parallel cultures in 25 ml shake flasks (PFS-WP and PFS) were used as controls. Cell growth was similar in shake-flasks and bioreactor, except that the maximal cell density was reached 1 or 2 days sooner in the bioreactor than in the shake-flasks (Figure 1a). WP very significantly stimulated CHO-320 cell proliferation in both bioreactor and shake-flasks, as an increase of about 25% of living cells was observed. CHO-320 cells grew faster but they also died faster in the bioreactor than in shake-flasks, as seen on Figure 1a with an abrupt drop of the viability after 100 h in the unsupplemented bioreactor culture and after 120 h in the culture supplemented with WP Figure 1b).

The IFN-γ production reached a concentration of 4 μg ml^{-1}(Figure 1c) when cells were cultivated with WP, while only 2.5 μg ml^{-1} were attained in PFS alone. That resulted in an overall increase of about 60% in IFN-γ produced. Although, the maximum IFN-γ concentration was reached one day before when cells were cultivated in the bioreactor compared to shake-flask culture, final levels were similar in both conditions.

As previously reported (Burteau et al. 2003), the rate of glucose consumption was largely similar in all conditions, although some glucose remained in the medium of shake-flasks experiments Figure 1d). During the decline phase, CHO-320 cells cultivated with WP consumed about 40% of the lactate produced during the growth phase, while those cultivated in PFS consumed only approximately 10% Figure 1d). Figure 1e displays the free amino acids concentrations in batch cultures of CHO-320 cells in bioreactor cultures, supplemented or not with WP at days 0, 2, 5 and 8. At day 0, no significant differences were observed between peptone-fortified and unsupplemented cultures due to the low content of this wheat hydrolysate in free-amino acids (% of the total amino acids). In contrast, by the end of the batch (day 8), the concentrations of Gly, Thr, Arg, Phe, Leu and Ile increased systematically in WP-supplemented media.

Figure 1F displays IFN-γ glycosylation patterns generated during batches in PFS in bioreactor and shake-flask cultures and in PFS-WP in shake-flask. Most of the IFN-γ was bi-glycosylated (2N) and the relative amount of each glycoform did not vary

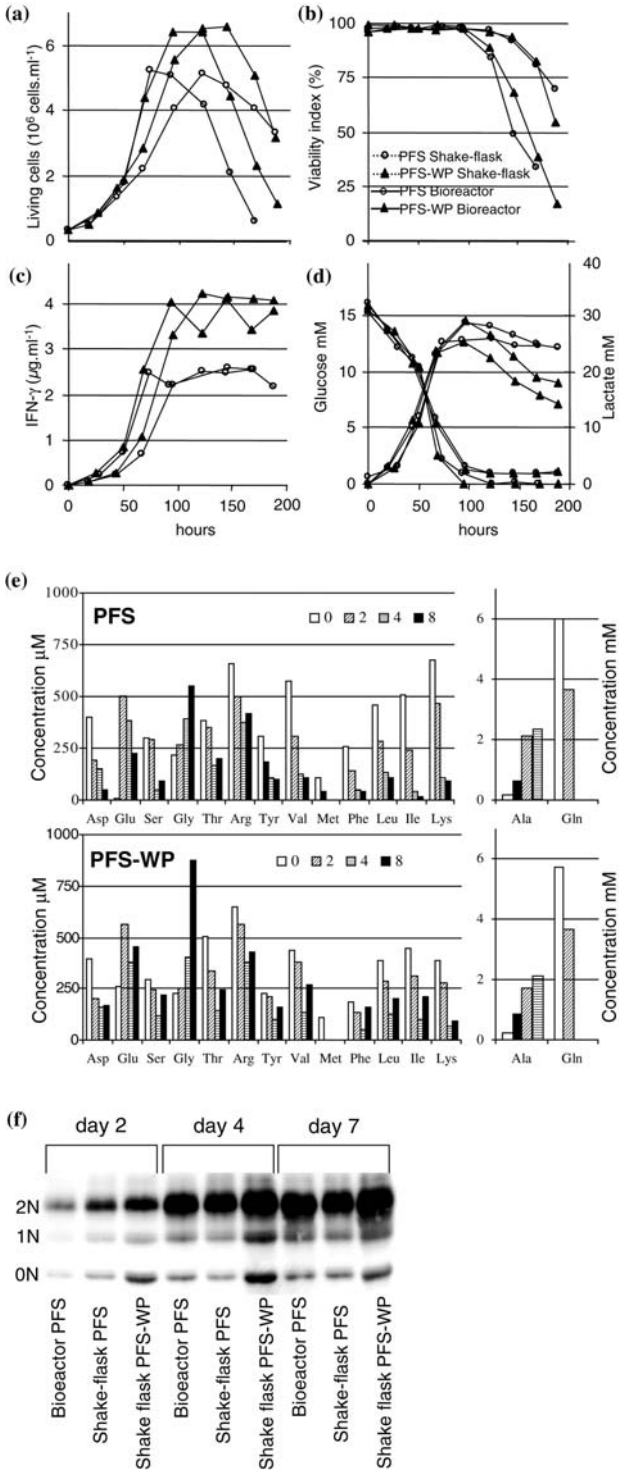

Figure 1. CHO-320 cells were inoculated at 3×10^5 cell ml^{-1} in batch mode in PFS supplemented (▲) or not (O) with wheat protein hydrolysates (WP) at 2 g l^{-1} in shake-flasks (SF : - - -) or in bioreactor (BR : —). (a) Trypan blue evaluation of living cells density (10^6 cell ml^{-1}), (b) Viability index (%), (c) ELISA quantification of secreted recombinant IFN-γ (μg ml^{-1}), (d) Glucose consumption and lactate production analyses (mM), (e) HPLC analysis of several amino acids concentration at day 0, 2, 5 and 8 and (f) Western-blot analysis of recombinant IFN-γ glycoforms at days 2, 4 and 7.

from day 2 to day 7. Western-blot analyses further confirmed that the total concentration of IFN-γ (non- (0N), mono- (1N) and bi-glycosylated (2N)) was clearly higher when the cells were cultivated with WP.

Effects of rice protein (RP) hydrolysates on CHO-320 cells cultivated in shake-flasks inside fibrous microcarriers

Although WP were the best plant hydrolysates to support CHO-320 cell growth and IFN-γ production, in suspension, preliminary results indicated that RP hydrolysates were also strong enhancers of IFN-γ production and, to a lesser extent, of cell growth during cultivation in PFA inside micro-carriers (results not shown). Under such cell culture conditions, cells were trapped inside Fibra-cel® carriers and grew as small aggregates (results not shown).

Results presented in Figure 2 are expressed as cumulative data reported per gram of microcarriers to better compare culture parameters. Figure 2a indicates a slight increase in the number of cells (detected as nuclei) inside microcarriers during cultivation in PFA-RP compared to PFA but less than what was observed in suspension in PFS-WP as compared to PFS (Figure 1). Confocal microscopy analyses helped to visualize the state and morphology of the cells trapped inside the carriers. CHO-320 cells grew as loose aggregates but not as spreading cells (results not shown). This is consistent with our previous observations in

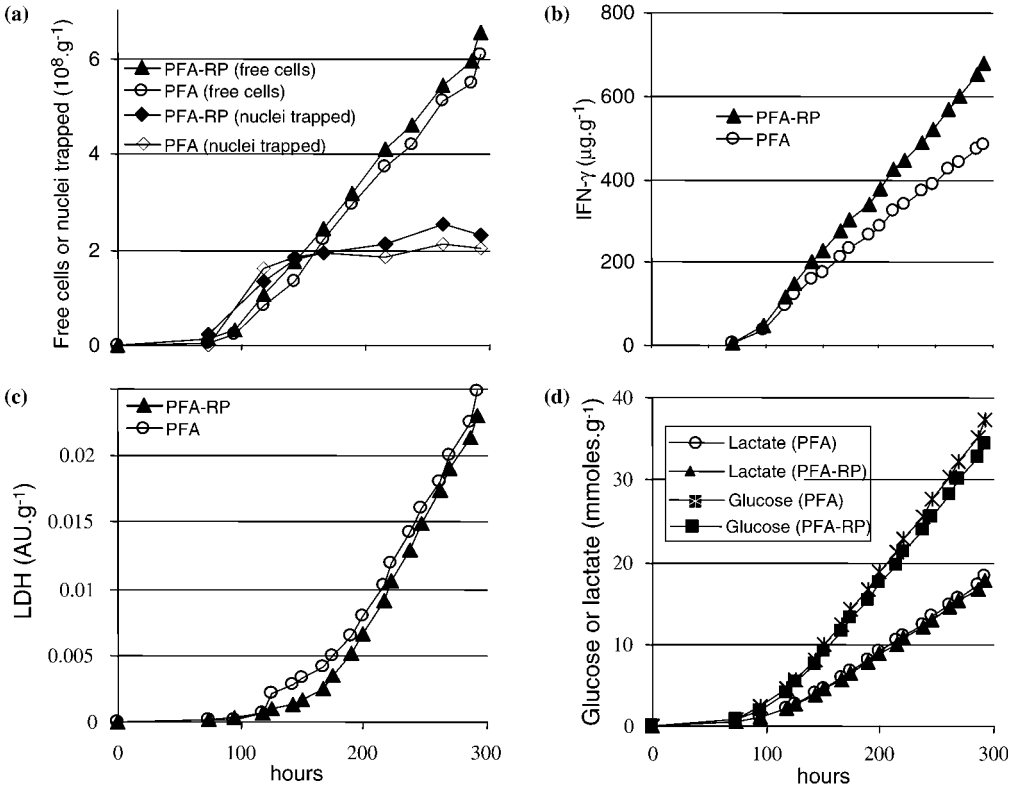

Figure 2. CHO-320 cells, previously cultivated in suspension, were utilised to inoculate Fibra-cel® micorcarriers in PFA supplemented or not with RP hydrolysates at 1 g l^{-1}. After 48 h, medium was changed twice a day. (a) Trypan blue evaluation of living cell density (— : cell g^{-1} of dry Fibra-cel®) in the supernatant of the cultures in PFA (O) and PFA-RP (▲) and Crystal Violet counting of nuclei entrapped inside Fibra-cel® microcarriers (- - - : nuclei g^{-1} of dry Fibra-cel®) of the cultures in PFA (◇ ■ Please check the insertion of symbols■) and PFA-RP (◆). (b) ELISA quantification of secreted recombinant IFN-γ (μg g^{-1} of dry Fibra-cel®) produced in PFA (O) and PFA-RP (◆). (c) lactate deshydrogenase release (arbitrary units : AU g^{-1} of dry Fibra-cel®) in PFA (O) and PFA-RP (◆) supernatants. (d) Analysis of glucose consumption in PFA (O) and PFA-RP (◆) and lactate production in PFA (■) and PFA-RP (×) (mmole g^{-1} of dry Fibra-cel®).

static conditions as only serum and extracellular matrix proteins like collagen were able to promote cells spreading. Otherwise, in PFS, cells grew as immobilised aggregates (results not shown).

After 100 h cultivation, cells began to detach from the aggregates inside the carriers (Figure 2a). For practical reasons, the resulting free cells were only counted once a day and consequently the curve may have underestimated the cell lost. As shown in Figure 2a, after 100 h, the number of free cells released from the carriers and the number of cells trapped within the carriers started to increase.

Figure 2b clearly illustrates that the total secretion of recombinant IFN-γ increased by about 30% when CHO-320 cells were cultivated in PFA-RP instead of PFA. This could simply be the result of the total number of living cells in the flasks (free + trapped), which is always slightly higher with respect to the unsupplemented culture. However, IFN-γ specific production rate was also better in PFA-RP compared to PFA (Table 1). At the early beginning of the growth, the ratio between the specific production rate in PFA-RP and in PFA was 1.58, while after 166 h of cultivation, it decreased slightly but remained in favour of PFA-RP. After 166 h, during short intervals of medium renewal, it was higher (1.49) than what observed when glucose was depleted (1.33) as during long intervals of medium replenishment glucose was totally consumed. Figure 2c presents the total cumulative LDH activity released in the conditioned medium per gram of microcarriers. We can observe that when LDH accumulated in the conditioned medium, the number of cells trapped inside microcarriers increased. Furthermore, LDH was released earlier in PFA and then after 200 h, the LDH release rates were the same in PFA-RP and PFA, as both curves had the same slope. Based on correlations between the number of cells and LDH activity generated through cell lysis, the viability was expected to be over 95% as the LDH activity measured corresponded to about 1–5% of the total number of cells.

Finally, Figure 2d shows the total amount of glucose consumed and of lactate produced during both cultivation processes. CHO-320 cells grown in PFA-RP and in PFA were consuming glucose and producing lactate apparently at the same rate, but it should be kept in mind that during long intervals of medium renewal, glucose was depleted. This raised the possibility that under such conditions lactate could be consumed as it was seen in suspension Figure 1d).

Table 1. IFN-γspecific production rates ($10^{-8}\mu$g cell^{-1} h^{-1}) during cultivation in PFA oer in PFA-RP inside Fibra-cel® microcarriers as described in Figure 2

Time	From 95 to 125 h	From 166 h	
		Long intervals*	Short intervals
PFA-RP	2.8	1.2	1.5
PFA	1.8	0.9	1.0
Ratio PFA-RP/ PFA	1.58	1.33	1.49

*glucose depletion

Effects of RP hydrolysates on CHO-320 cells cultivated inside fibrous microcarriers packed in a fixed-bed bioreactor

A 1.5 l bioreactor containing 25 g Fibra-cel® packed in a volume of 250 ml was inoculated with 225×10^6 CHO-320 cells in PFA–RP (Figure 3). The seeding was efficient since no free cells were found in the medium after 3.5 h under slow stirring. We decided to set the perfusion rate of the reactor on the basis of glucose consumption to stabilize its concentration to a fixed value. A first experiment aimed at maintaining the glucose concentration around 4 mM, with 50% DO and a second around 5.5 mM with 60% DO. Unfortunately, as microcarriers were not accessible during the whole length of the cultivation process, densities of entrapped cells were only measured at the end of both experiments. Therefore, we have no experimental results indicating the behaviour of the cells during the early stages of the growth. A level of 7.3×10^8 nuclei g^{-1} was reached in the reactor set to 50% DO, whereas 4.38×10^8 nuclei g^{-1} were measured in the reactor maintaining 60% DO. Figure 3a shows that the total population of free cells released from the microcarriers increased dramatically when cells were maintained at 60% DO. At 50% DO, cell release occured to considerably lower extent.

Figure 3b presents IFN-γ productions in both reactors. During the first 125 h, IFN-γ was secreted at the same rate but afterwards, the production slowed down in the bioreactor with 50% DO. After 175 h in the reactor set to 60% DO, the production reached about 600 μg g^{-1}, as

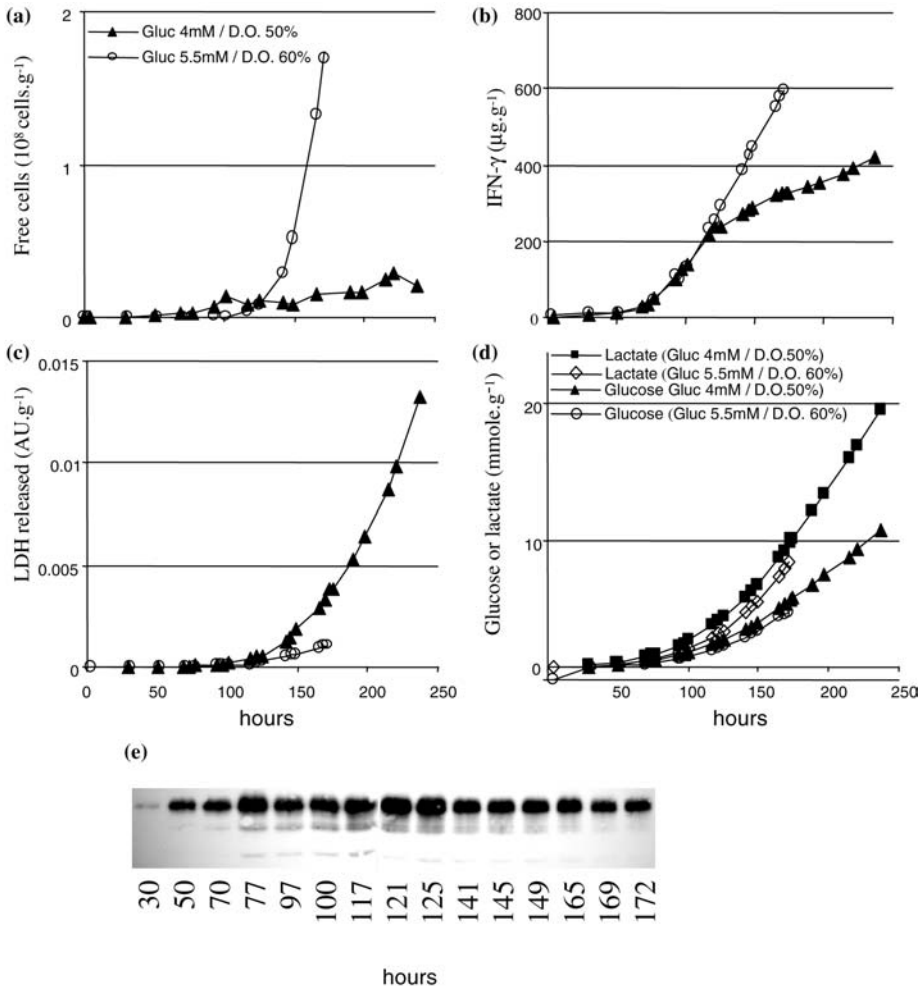

Figure 3. CHO-320 cells, previously cultivated in suspension, were utilised to inoculate Fibra-cel® microcarriers packed in a fixed bed device inside a Celligen™ bioreactor and cultivated in PFA supplemented with 1 g l^{-1} of RP hydrolysates. The first reactor was designed to maintain at least 4 mM glucose and 50% of air saturation (full symbols) and the second 5.5 mM and 60% of air saturation (empty symbols). (a) Trypan blue evaluation of living cell density (cell g^{-1} of dry Fibra-cel®) in the reactors set to maintain 4 mM glucose and 50% of air saturation (▲) and 5.5 mM glucose and 60% of air saturation (O). (b) ELISA quantification of secreted recombinant IFN-γ (μg g^{-1} of dry Fibra-cel®) in the reactors set to maintain 4 mM glucose and 50% of air saturation (▲) and 5.5 mM glucose 60% of air saturation (O). (c) LDH release in the reactors set to maintain 4 mM glucose and 50% of air saturation (▲) and 5.5 mM glucose and 60% of air saturation (O) (arbitrary units : AU g^{-1} of dry Fibra-cel®). (d) Glucose consumption in the reactor maintained at 4 mM glucose and 50% of air saturation (▲) and 5.5 mM glucose and 60% of air saturation (O) and lactate production in the reactors set to maintain 4 mM glucose and 50% of air saturation (■) and 5.5 mM glucose and 60% of air saturation (◆) (mmole g^{-1} of dry Fibra-cel®). (e) Western-blot analysis of recombinant IFN-γ glycoforms during perfusion.

compared to 300 μg g^{-1} in the reactor keeping 50% DO and 300 μg g^{-1} in the PFA-RP shake-flasks study presented in Figure 2. These results might be linked to the cell mortality evaluated by the LDH activity released in the culture medium after cell lysis (Figure 3c). After 125 h in the reactor kept at 60% DO, the number of free cells outside the carriers rose while rather low levels of LDH were observed. At that time, in the reactor set to 50% DO, low densities of free cells were observed while high levels of LDH were monitored. We have no information on the state of the cells remaining in the microcarriers as these were inaccessible as these supports were packed in the retention device. However, it is likely that in the 50% DO reactor, cells could undergo necrotic or apoptotic death.

From a metabolic point of view, the consumption of glucose and the production of lactate displayed little difference (Figure 3d). At 60% DO, the total glucose consumption and lactate production were slightly lower than in the 50% DO reactor, but curves remained parallel and consequently glucose consumption and lactate production rates were similar.

Finally, Figure 3e illustrates that IFN-γ, produced in the reactor maintained at 60% DO, is very well glycosylated during the whole cultivation process with most of the protein displaying two glycosylation chains (2N). Similar results were obtained for IFN-g secreted during the experiment at 50% DO (results not shown). The total IFN-γ concentration rose up to the point seen in lane 8 (125 h) and stabilised before decreasing as the perfusion rate was rising to maintain a stable glucose concentration around 5.5 mM.

Discussion

CHO-320 cells, which secrete recombinant human IFN-γ as a model glycoprotein, were used to study growth, metabolic parameters, IFN-γ secretion and glycosylation pattern upon cultivation either as free cells in suspension or as cells entrapped inside microcarriers during perfusion. Nowadays, most bioengineering processes impose biosafety considerations at each step of the culture, which have progressively led to banning animal proteins from culture media formulations. We have recently developed a basal serum- and protein-free medium (PFS) able to replace protein- and serum-containing media designed for CHO cell lines (Burteau et al. 2003). In suspension cultures, PFS supports CHO-320 cell growth and IFN-γ secretion as well as quantitatively similar performances to those observed in serum-containing media provided that it is supplemented with plant protein hydrolysates. Furthermore, upon cultivation in PFS supplemented or not with plant protein hydrolysates, the IFN-γ secreted was mostly bi-glycosylated while in serum-containing media, IFN-γ was non-, mono- and bi-glycosylated in similar proportions (Burteau et al. 2003). Microheterogeneities have also been shown to be tightly dependent on environmental conditions. Indeed, ammonium chloride could affect glycosylation patterns of recombinant mouse placental lactogen-1 (mPL-1) in a pH-dependent manner (Borys et al. 1994). In contrast, Yang and Butler (2000) showed that ammonia reduced the proportion of terminal sialic acid as well as the proportion of *O*-linked glycans found on recombinant erythropoietin produced by CHO cells. Such analyses should also be performed to investigate the effects of plant peptones and cell aggregation on the microheterogeneities of the glycans linked to the IFN-γ. Indeed, the addition of meat hydrolysates has been demonstrated to have a deleterious effect on the sialylation of recombinant IFN-γ (Gu et al. 1997), whereas Watson et al. (1994) described that cell adhesion to microcarrier beads resulted also in a reduced amount of sialylated glycans as compared to suspension processes.

In this work, we used two basal protein-free media to cultivate CHO-320 cells either in suspension or inside Fibra-cel® carriers. The only differences between these media resided in the choice of the plant protein hydolysates and the concentration of ferric citrate. In suspension, WP hydrolysates were chosen while RP hydrolysates had given the best results for the cultivation inside Fibra-cel® carriers. WP significantly increased the growth rate of CHO-320 cells in shake-flasks (Burteau et al. 2003) but also in the reactor (Figure 1), even if in this case, a better control on environmental parameters (pH, DO) allowed a shorter lag phase than in shake-flasks. The IFN-γ volumetric production was also improved in PFS-WP. This effect was related to an increase in the cell density, but mostly to a direct stimulation of the IFN-γ specific production, which reached about 0.65 μg 10^{-6} cells in PFS-WP to be compared to 0.5 μg 10^{-6} cells in PFS. The IFN-γ production by CHO-320 cells has already been described to be at least partly growth-associated (Hayter et al. 1993; Leelavatcharamas et al. 1999), even if this is sometimes unclear (Hayter et al. 1991; Castro et al., 1992; Goldman et al. 1998).

From a metabolic viewpoint, upon supplementation with WP, when glucose concentration became too low in the medium lactate was clearly consumed whereas in PFS it was not happening to the same extent. Moreover, at the end of the batch, the concentrations of several amino acids (Glu, Ser, Gly, Thr, Arg, Tyr, Val, Phe, Leu and Ile) rose. This can be explained by either of two hypotheses. First, proteolytic cleavages of plant protein hydrolysates generating smaller peptides and amino acids could occur either at the cell

surface or in the extracellular medium. If they were internalised through specific transporters (Heidemann et al. 2000; Burteau et al. 2003), once viability dropped, they could accumulate in the extracellular medium. This assumes that the hydrolysis of peptones is slower than the transport of the resulting amino acids, di- or tripeptides inside the cells. Otherwise, amino acids should accumulate in the culture medium even during the exponential growth phase. Alternatively, after cell lysis, intracellular proteases could be released inducing an increase in the proteolytic potential of the extracellular medium and the subsequent proteolytic degradation of the peptides. In contrast, IFN-γ was not undergoing any proteolytic processing as no bands of molecular weight lower than the non-glycosylated form of IFN-γ were observed at any time (not even at day 7).

As in the case of suspension cultures, the addition of plant protein hydrolysates improved by 30% IFN-γ secretion when CHO-320 cells were cultivated inside Fibra-cel® carriers. This could not be explained as a result of mortality, which strongly suggests that, as previously mentioned (Burteau et al. 2003), plant protein hydrolysates could improve biosynthesis either on a nutritional basis or/and due to bioactive peptides acting as cell signal inducers (Rassmussen et al. 1998). Some authors have suggested that most of the effects shown by some protein hydrolysates may be of a strictly nutritional nature (Heidemann et al. 2000). In this hypothesis, peptones are a cheap source of amino acids, possibly with a better uptake potential through peptide transporters. Nevertheless, it seems now established that many hydrolysates do not only act as a source of amino acids, since the use of roughly equivalent amino acid mixture supplements could not match their effects (Jan et al. 1994; Burteau et al. 2003). Franek et al. (2000, 2002) reported that some peptidic fractions purified out of peptones as well as some synthetic peptides had biological effects on growth or protein production by mammalian cells. One of the hypotheses to explain these effects is that some peptides could interact with specific cell surface receptors implicated in growth, anti-apoptotic signalling or protein biosynthesis and that their frequency or concentration could vary from on type of hydrolysate to another.

Although a very efficient initial attachment was observed, when the biomass increased, CHO-320 cells cultivated inside Fibra-cel® detached from the carriers unless insufficient oxygenation and feeding were imposed to the cells (Figure 3). We investigated whether a decrease in the citrate concentration (present in the medium as ferric citrate) could affect this cell loss by complexing Ca^{2+}, which is normally involved in stabilising adhesive proteins like integrins. Although no significant effects could be observed, we chose to reduce ferric citrate concentration in PFA at 50 μM, whereas 500 μM was used in PFS as already described (Landauer et al. 2003), to enhance cell entrappment or aggregation. As suggested by Figure 3, local nutrients, toxic byproducts or O_2 gradients were not likely to be the main reason of this cell detachment as the reactor presenting a lower viability (based on LDH release) did not lose many cells. Furthermore, the free cell population displayed a high viability index. It should also be considered that CHO-320 cells were adapted to suspension and that during this adaptation process, the expression of adhesion proteins could have been reduced. Finally, to favour cell aggregation conditions, it could also be proposed to increase Ca^{2+} concentration (Peshwa et al. 1993), especially since Ca^{2+}-dependent cadherins have been reported to play an important role in CHO-320 cells aggregation (Coppen et al. 1995). Upon cultivation with Fibra-cel® microcarriers, IFN-γ production rate decreased strongly when the feeding rate became insufficient to maintain nutrient concentrations over a certain value or toxic by-products below a certain level. For this, glucose was chosen as an indicator of total medium consumption to adjust the perfusion rate (Figure 3). Presumably, either nutritional shortage or toxic compounds accumulation could be the cause of this IFN-γ production rate drop, since both appeared related to the perfusion rate. In fact, when the IFN-γ production rate decreased, glucose concentrations measured in the reactor maintained at 50% DO were higher than those reported in the literature to affect significantly CHO cell growth or production in suspension (Lao and Toth 1997). These authors also described that lactate concentrations between 20 and 30 mM were usually measured at the beginning of the stationary phase and that lower concentrations were unable to affect cell growth (Lao and Toth 1997). As the values reached in Fibra-cel® cultures were usually lower, lactate accumulation is also unlikely to account for the decrease in IFN-γ

production, unless important concentration gradients developed inside or around the microcarriers. In batch cultures supplementation with vitamins, lipids, amino acids and nucleic acid precursors as well as higher glucose and glutamine concentrations were also tested in an attempt to lengthen the lifetime of the cultures, but so far this approach has not been successful (unpublished results). Nonetheless, glucose concentration was an efficient and convenient tool to monitor overall medium consumption during perfusion.

When cells are cultivated inside fibrous microcarriers like Fibra-cel®, pH, DO or nutrients/toxic by-products concentrations at the direct vicinity of the cells could be totally different from those observed in the medium. Gradients could occur at two scales in this system : inside a single microcarrier and due to the dense packing of the carriers inside the fixed-bed device. As in the shake-flask studies (Figure 2) microcarriers were floating free in the medium, the second hypothesis is not likely but as we can see on Figure 2, compared to Figure 3, the LDH release is nearly the same as in the reactor maintained at 50% DO. This supports the hypothesis of major gradients inside the individual microcarrier. This strengthens the view that glucose, glutamine and lactate concentrations were not individually the main cause of the decline or stagnation of cell growth in the carriers but concentrations gradients of these compounds cannot be ruled out to explain growth retardation, cell death and reduced IFN-γ production.

Plant protein hydrolysates or peptones and particularly rice and wheat peptones are useful supplements for the formulation of protein-free media designed to cultivate CHO cells either trapped inside microcarriers or in suspension. These compounds exert biological stimulation of CHO-320 cell growth and recombinant IFN-γ secretion in a way that could not only be attributed to a simple nutritive effect. Finally, we postulate that peptones like rice and wheat peptones contain some peptides able to interact with cell surface receptors involved in the stimulation of cell growth and/or protein biosynthesis.

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