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Monoclonal antibody production with on-line harvesting and process monitoring

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Abstract

A semi-automated system has been designed for on-line harvesting and monitoring of monoclonal antibody (mAb) production. [The antibody was directed against the peptide AGPAGTGKTTKDL.] Analytical and purification units were interfaced to the fermenter via a hollow fiber cartridge in which fermentation broth was continuously circulated through the lumen of the hollow fiber system. Permeate from the hollow fiber cartridge was pumped through either an analytical sampling loop or a preparative Protein G column where antibody species were captured. Switching between monitoring and harvesting was achieved by two 3-way toggle valves. Samples from the analytical sampling loop were transported to an analytical Protein G chromatography column for quantitation of all immunoglobulin G species in the fermenter. Data acquisition and processing was performed by the data system of the liquid chromatograph. All valves in the system except the two toggle valves were controlled by the liquid chromatograph. Antibody biosynthesis was monitored for the first 60 h of fermentation. Harvesting was initiated when mAb accumulated in the fermenter. Complete harvesting took approximately 90 h. © 1998 Elsevier Science B.V.

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1. Introduction

Production of monoclonal antibodies (mAb) is of broad interest in biotechnology because of their utility as analytical and therapeutic agents. The quality of antibody produced is subject to several variables. One is variation in the growth medium during the several weeks of a fermentation campaign. Glycosylation variants in particular are formed as rapidly consumed carbohydrate species are exhausted. Another is proteolysis by enzymes released from dead cells [1] or degradation from other cell culture components [2]. This means that structure of accumulating antibody can be altered both during the production phase and at the end of cellular productivity when cells begin to lyse. The potential for proteolysis is diminished by harvesting immediately after cessation of production. For this reason, frequent monitoring and harvesting would be an even better solution to the problem of trying to maintain mAb quality.

Monoclonal antibody is excreted into the growth medium of the cell cultures at concentrations in the range 1–200 μg/ml [3] depending on the cell line and the stage of fermentation. Antibody concentration is almost always determined off-line with a binding protein assay, generally an immunological assay. The problem with off-line immunological

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assays and the several on-line methods described to date is that they are (i) slow, (ii) labor intensive, (iii) difficult to automate, (iv) high maintenance, and (v) not easy to integrate into the purification process [4–6]. The single on-line method which has been described [7] addressed several of these problems but was not integrated into the processing system.

The objective of the work presented here was to develop an integrated system for producing, monitoring and harvesting monoclonal antibody from hybridoma cell cultures. Antibody was extracted from the fermenter by using hollow fiber membranes and continuously circulating culture broth through the lumen of the fiber bundle. Valving allowed permeate to be directed either to a chromatographic harvesting column or to an analytical chromatography column. Analytical data derived from the Protein G column was used to direct harvesting.

2. Experimental

2.1. Reagents and buffers

Hydrochloric acid was obtained from J.T. Baker (Phillipsburg, NJ, USA); sodium chloride, tris-(hydroxymethyl)aminomethane free base (tris base) and tris(hydroxymethyl)aminomethane hydrochloride (tris-HCl) were purchased from Mallinckrodt (Paris, KY, USA). Anti-bovine serum albumin (BSA) was bought from Sigma (St. Louis, MO, USA). Fetal bovine serum (low IgG concentration) and Dulbecco's Modified Eagle medium (DMEM) (high glucose, with L-glutamine, 25 m*M* HEPES buffer, without sodium pyruvate) were purchased from Life Technologies (Gaithersburg, MD, USA). Gelman Science 0.2 μm air filters were obtained from Fisher Scientific (Pittsburgh, PA, USA). All buffers were filtered with a 0.2 μm membrane.

2.2. Hybridoma cells

The hybridoma cell line, which produced antibody against the peptide CAGPAGTGKTETTKDL, was obtained from the antibody production facility in the Department of Biological Science at Purdue University.

2.3. Instrumentation

A BioCAD chromatography workstation from PerSeptive Biosystems (Framingham, MA, USA) was employed to perform all chromatographic separations. The system computer was used to control column switching, sampling, gradient elution and flow-rates in the range 0.2–20 ml/min. Fermentation was achieved in a 1 l stirred vessel with asceptic inlets and outlets for air and the hollow fiber interface. When the injection valve 2 is in the loading position shown in Fig. 2, the permeate from the bioreactor is pushed by the peristaltic pump 1 into the preparative column 4 or the 0.25 ml loop 6 determined by the two toggle valves 3; the permeate is then circulated back into the lumen of the hollow fiber. When the injection valve 2 is in the injecting position, the mobile phase from the BioCAD system enters either the preparative column 4 or the 0.25 ml loop 6. For antibody monitoring, the 0.25 ml loop is put in-line and therefore the permeate is pushed into the 0.20 ml loop 7, after which the injection valve 2 is immediately set to the loading position. The permeate inside the 0.2 ml loop is then loaded into the analytical Protein G column 5 for analysis. For antibody harvesting, the preparative Protein G column 4 is in-line, washed by the mobile phase and the bound antibodies are eluted by the acidic mobile phase. During that process, the switching valves 8 are bypassed and the flow goes directly through the UV–Vis detector.

2.4. Fermentation conditions

The fermentation setup is shown in Fig. 1. Hybridoma were grown in a medium composed of 475 ml of DMEM and 25 ml of fetal bovine serum. Cell density was approximately 100 000 cells/ml. The bioreactor was maintained at room temperature while the medium was continuously purged with air containing 10% CO_2.

2.5. Chromatography columns

Analytical separations were performed on a 30× 2.1 mm (20 μm particles) POROS G/M Protein G column from PerSeptive Biosystems. Harvesting and preparative separations were conducted with a 50× 4.6 mm (50 μm particles) POROS G/P Protein G

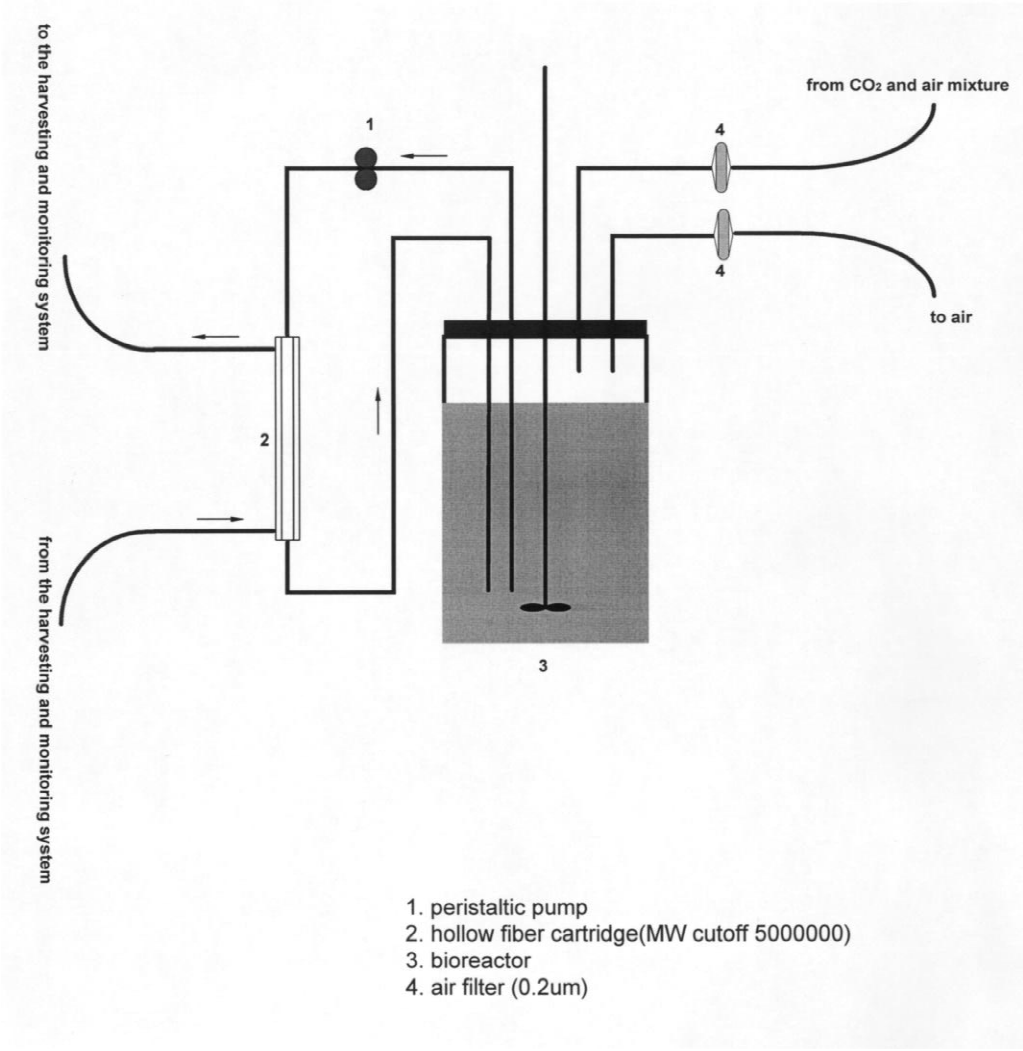

Fig. 1. Fermentation setup for antibody production. Designated components are the peristaltic pump (1), hollow fiber cartridge (MW cut-off=500 000) (2), bioreactor (3) and air filter (4).

column from PerSeptive Biosystems. Both columns were kindly donated by PerSeptive Biosystems. The loading buffer for the Grotein G columns was 100 m*M* NaCl in 10 m*M* phosphate buffer (pH 7.0). Elution was achieved with 100 m*M* NaCl in 12 m*M* HCl. Mobile phases were filtered with 0.2 μm membrane filters.

2.6. Simulations

The purpose of this experiment was to determine the clearance rate of antibody from the fermenter through the use of a hollow fiber interface and a Protein G adsorbent column. A 30.0 mg aliquot of anti-BSA was dissolved in 500 ml of 100 m*M* NaCl with 10 m*M* phosphate buffer (pH 7.0) and used in a simulated fermentation campaign.

2.7. Monitoring and harvesting

The setup of the monitoring and harvesting is illustrated in Fig. 2. A hollow fiber cartridge with a 500 000 MW cut-off from A/G Technology (Needham, MA, USA) served as the interface be-

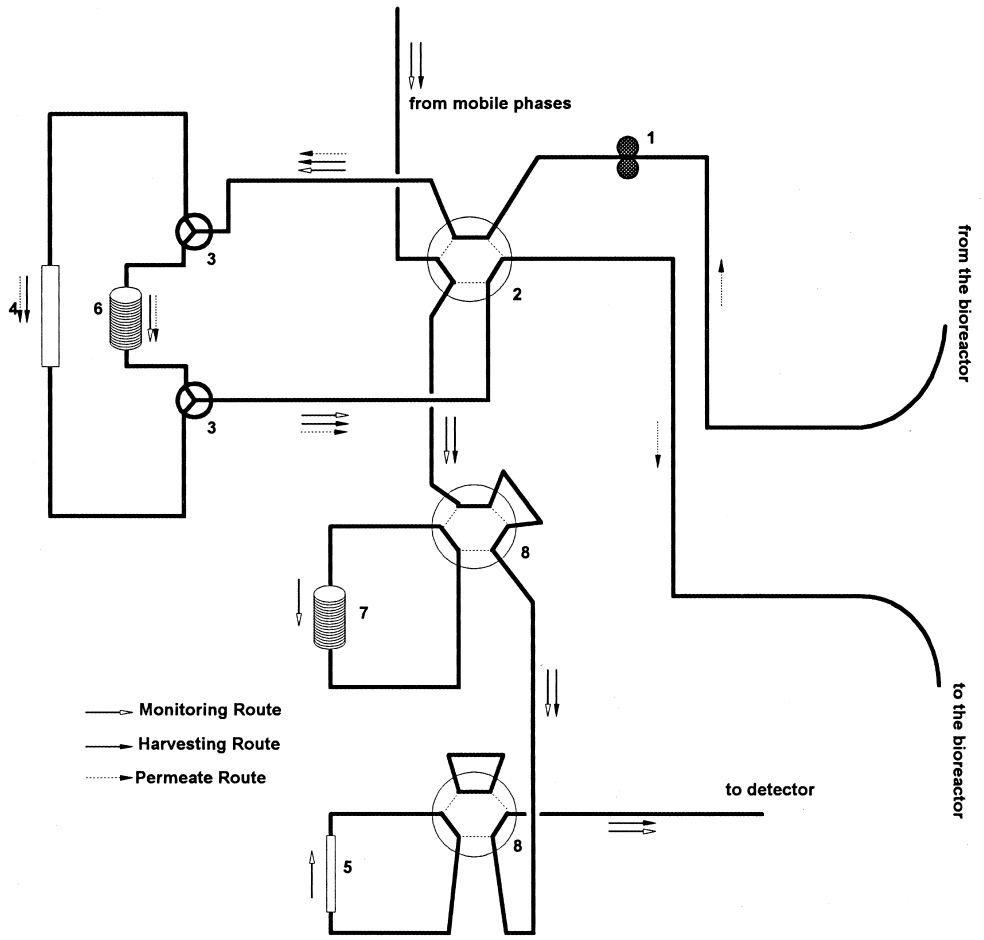

Fig. 2. System setup for antibody harvesting and monitoring. Components designated are the peristaltic pump (1), injection valve (2), 2-way toggle valve (3), preparative Protein G column (4), analytical Protein G column (5), 0.25 ml loop (6), 0.2 ml loop (7) and a switching valve (8). '———' indicates for loading position and '- - -' for the injecting position for the injection valve (2). For the switching valves (8) '———' indicates the bypass position and '- - -' for the in-line position.

tween the BioCAD system and the bioreactor. The fermentation medium was pumped through the lumen inside the cartridge by a peristaltic pump (Cole-Parmer Instrument, Chicago, IL, USA). A second peristaltic pump (Gilson, France) was used to transport permeate for either monitoring or harvesting. Switching between monitoring and harvesting was achieved by two manual three-way toggle valves from Alltech Associates (Deerfield, IL, USA).

2.8. Analytical chromatography

Permeate from the 200 μl loop was injected into the analytical Protein G column and the analytes eluted at 2 ml/min by means of a gradient. The column was washed with loading buffer for 1.2 min and the bound antibodies subsequently eluted with acidic buffer. Columns were regenerated with the loading buffer at 4 ml/min for 3.2 min.

2.9. Monoclonal antibody assay

Harvested antibody concentration was estimated by the Warburg–Christian method [8]. The harvested monoclonal antibody fraction was immediately stored in the freezer and used without further treatment to obtain an adsorption spectrum on a diode-

array spectrophotometry (Hewlett Packard, Palo Alto, CA, USA). Based on the ratio of the adsorption readings at 260 and 280 nm, monoclonal antibody concentration was estimated from a nomograph [8]. Purity of the harvested antibody was determined by SDS–PAGE according to the Laemmli method [9]. About 10 μg of antibody was reduced in boiling 1% DTT for 5 min and then loaded on 12% polyacrylamide gel. After electrophoretic separation proteins were visualized by staining the gel with Coomassie Brilliant Blue solution. Rainbow molecular mass markers (MW 14 300–200 000) (BioRad, Hercules, CA, USA) were used to estimate molecular mass.

3. Results and discussion

The architecture chosen for fabrication of a system that could both on-line monitor and harvest antibody from a fermenter had the following essential elements. First, membranes in the form of a hollow fiber bundle were used to interface the harvesting and analytical systems with the fermenter. The function of the membrane was to allow proteins to both enter and egress from the reactor while blocking the transfer of cells between the analytical/harvesting system and fermenter. Second, fermentation broth and permeate were continuously recirculated through the hollow fiber bundle in an effort to maintain concentration equilibrium of perfusible species on both sides of the membrane. The only species in which there was a net flux out of the fermenter was the monoclonal antibody being harvested. Third, a preparative affinity chromatography column directed to selective product adsorption was used for harvesting. The function of this column was to adsorb product selectively and allow other proteins to be returned to the membrane interface for reequilibration with the fermenter. Fourth, an analytical affinity column specific for IgG was used to quantitate mAb based on the fact that this approach provided the highest selectivity, fastest, and simplest analytical method. Fifth, the system design enabled product to be harvested either continuously or intermittently based on analytical data. And sixth, the same chromatography platform could be used for either analytical determinations or harvesting.

3.1. Interface

A cross-flow ultrafiltration type of interface, in the form of a hollow fiber bundle with a 500 kD exclusion limit, was chosen in these studies. This very high exclusion limit was chosen to facilitate efflux of antibodies from the fermenter. Broth was circulated through the lumen of fibers to continuously sweep membranes and in doing so minimized fouling. One possible drawback would be that breaches in the fiber membranes would allow contamination of the fermenter with foreign cells from the analytical/harvesting system. This was not found to be a problem during fermentation campaigns of a week in duration.

Protein flux across the membranes is a function of their pore size (or molecular mass cut-off), surface area and pressure drop. According to the manufacturer's specification, the hollow fiber bundle used in these studies had a molecular mass cut-off of 500 kD, a specific surface of 75 cm^2 and a volume of 23 ml. The ratio of the membrane surface area to the broth volume in this case was 0.326 m^2/l. Obviously increasing the ratio of the surface area to volume enhances the rate of protein clearance. In these studies, the system was operated with little or no pressure drop across the membranes. It will be shown below that this system was capable of clearing protein from a fermenter in 10 h. Based on the fact that (i) antibody production is spread out over a week, (ii) proteolysis is slow, and (iii) degradation by proteolytic enzymes is most likely to occur in a short time window at the end of fermentation, the decision was arbitrarily made that clearance of mAb under 12 h was acceptable. More rapid clearance would have required a more costly interface of a higher surface area to volume ratio.

3.2. Fermentation

A simple batch-mode of operation was chosen for the fermenter in this study. In this mode the supply of nutrients is limited, meaning that cell growth slows as nutrients are consumed and eventually stops as the medium is depleted. Cells die because of the constant consumption of nutrients and accumulation of waste products.

The fermenter and membrane interface were autoclaved after which sterile growth medium and cells

were introduced aseptically into the fermenter in a cell culture growth hood. Filters (0.2 μm) on both the CO_2/air inlet and outlet lines prevented contamination of the fermenter. Optimal cell growth conditions [3] were maintained by holding pH in the range of 6.9–7.5, dissolved CO_2 at 5–10%, and the temperature at 27°C. Although the rate of growth is higher at 37°C, maximum productivity was not the objective of these studies and it has been found that genetic stability is greater in some hybridoma cell lines at room temperature [10–12]. Sparging the culture with CO_2 provided buffer through dissolved HCO_3^-.

Fetal calf serum referred to commercially as 'Ultra-low IgG' was used in the growth medium instead of the normal fetal calf serum because it would provide minimal contamination (25 μg/ml of bovine IgG [13]) of the mAb with bovine IgG. The function of fetal calf serum is to provide essential nutrients for cell growth, i.e. hormones and growth factors not produced by hybridoma cells.

3.3. Chromatography

As noted above, Protein G affinity columns were chosen for both the analytical and preparative separations because of their extremely high selectivity. Protein G is widely used in the affinity purification of IgG where it has been reported to differentiate between classes of IgG from a variety of mammalian species [7]. A small 30×2.1 mm POROS G/M (20 μm particle) analytical column was used in these studies to maximize recovery and minimize analysis time. According to the manufacturer the column had a loading capacity of 2 mg at a linear velocity of 3600 cm/h. This was more than adequate for the 1–200 μg/ml concentration range encountered in the fermenter. Elution was achieved with a step gradient of 12 m*M* HCl. Fig. 3 shows that the analytical Protein G column resolved the mAb product into three components.

Preparative separations were achieved on a 50× 4.6 mm POROS G/P (50 μm particle size) column with a 16 mg loading capacity. A short column with larger particles was used so that permeate from the hollow fiber bundle could be circulated through the column with a peristaltic pump. Nutrients not adsorbed to the affinity column were returned to the

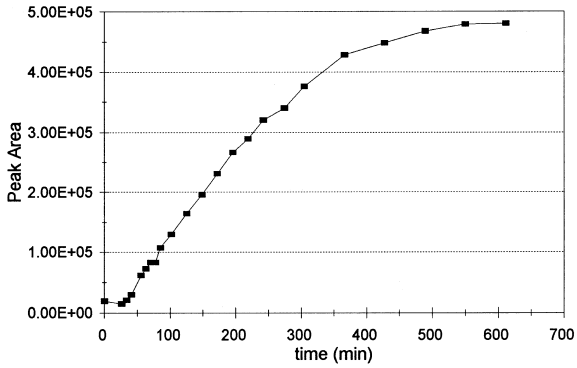

Fig. 3. Peak areas of anti-BSA plotted as a function of the sampling time in the simulation experiment.

membrane interface for reequilibration with the components in the fermenter. It is desirable that mAb adsorbed on the preparative column will be harvested at regular intervals for several reasons. One is that proteases circulating in the permeate will degrade the adsorbed antibody. Frequent harvesting minimizes exposure. A second is that under ideal conditions the fermenter could produce enough mAb to exceed the capacity of the preparative column. In this case, the fermenter was capable of producing 20 mg of antibody under ideal conditions.

The Protein G preparative column could also be used to remove bovine IgG from the growth medium. [Even the small quantities of residual IgG in 'ultra-low IgG' fetal calf serum can be removed.] Trapped bovine IgG, is purged from the preparative system before the onset of mAb production and product harvest. This precludes contamination of the mAb product with any bovine IgG.

3.4. Simulation studies

The procedures described below were designed to determine (i) how fast antibody from the fermenter equilibrated with the analytical/harvesting system, and (ii) the time required to clear antibody from the fermenter. Analytical monitoring was initiated immediately after the two peristaltic pumps began to deliver anti-BSA solution to the membrane interface and continued at 10 min intervals initially. As the system approached equilibrium, the sampling rate

was decreased to 30 min. Antibody quantitation on the analytical/harvesting side of the membrane was achieved by transferring an aliquot of permeate from the sampling valves to the analytical Protein G column, eluting the antibodies with acidic mobile phase, monitoring elution by absorbance at 225 nm, and integration of absorbance with the data analysis software of the system.

Clearance rate of IgG from the fermenter under ideal conditions was determined by loading the fermenter with 500 ml of buffered IgG (anti-bovine serum albumin) at a concentration of 100 μg/ml and determining how long it took to harvest the antibody. With the system shown in Fig. 1, IgG solution from the fermenter was circulated through the lumen of the hollow fiber cartridge and back to the fermenter at a flow-rate of 15 ml/min. Permeate from the hollow fiber cartridge was cycled through either the 250 μl sampling loop or the harvesting column at a flow-rate of 2 ml/min (see Fig. 2). Switching between sampling and harvesting was controlled by the two toggle valves. The preparative Protein G column and sampling loop were placed in parallel between these valves. When monitoring, the injection valve on the liquid chromatograph was in the loading position. By switching the injection valve on the instrument to the injection position, sample in the 250 μl sample loop could be pumped into the 200 μl sampling loop by the liquid chromatography pump. The reasons for transferring the sample to a second sampling loop were that (i) the toggle valves and unions in the analytical/harvesting system could not withstand the pressure required for injection into a high-performance liquid chromatography column, and (ii) the peristaltic pump could not force the sample through the high-pressure analytical column, due to its small particle size. Since the time required for sampling and analysis was less than 5 min, the measured mAb concentration still represented the concentration in the lumen of the hollow fiber interface.

Peak areas attributable to the antibody concentration in the interface, as a function of time after the simulation was initiated, are shown in Fig. 3. It may be concluded from the data that (i) it took approximately 40 min for anti-BSA to reach measurable levels in the lumen of the hollow fibers, and (ii) the system required roughly 10 h to reach equilibrium.

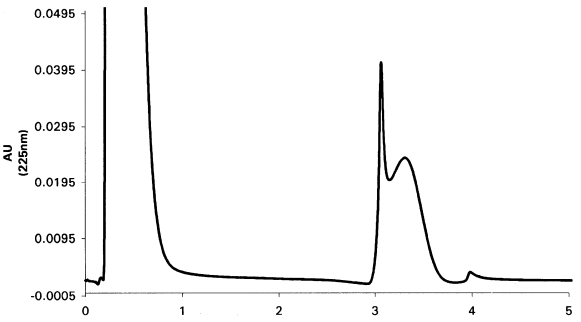

Fig. 4. Chromatogram from the sample at the beginning of fermentation.

3.5. Monitoring and harvesting antibody during a fermentation campaign

Operational parameters during the fermentation campaign were the same as the simulation experiment except that the cell culture was cultivated in the fermentor. Fig. 4 shows the analytical chromatogram taken at the beginning of the campaign. The data analysis system was instructed to sum the peak area of the three antibody peaks based on the assumption that (i) the three peaks were variant forms of the same antibody, and (ii) they all had the same extinction coefficient. As shown in Fig. 5, continuous antibody production is clearly indicated. When antibody production reached a plateau at 60 h, harvesting was initiated. The analytical monitoring system was used to determine the peak areas as a measure of antibody concentration in the fermenter during harvesting. Analytical data in Fig. 6 show a

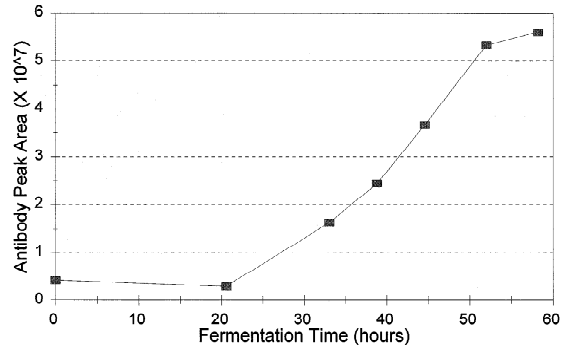

Fig. 5. Peak areas for IgG plotted as a function of fermentation time.

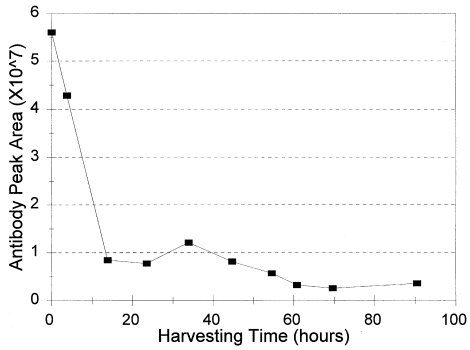

Fig. 6. Peak areas for IgG plotted as a function of time during harvesting.

reduction in antibody peak area in the fermenter of roughly 90% within the first 15 h of harvesting. Harvesting was terminated after 90 h, during which time 15 ml of antibody solution was collected. Concentration of the antibody solution was approximately 170 μg/ml as determined by the Warburg–Christian method described in Section 2. An SDS–PAGE analysis of the reduced antibody (Fig. 7) shows the heavy and light chains at 50 and 29 kD, respectively. No impurities could be seen.

Clearly this system efficiently harvests antibodies

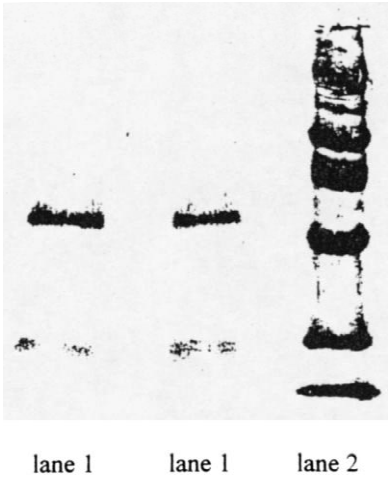

Fig. 7. SDS–PAGE of the harvested monoclonal antibody (lane 1) and molecular mass markers (lane 2). From bottom to top the molecular mass marker solution included lysozyme (14.3 kD), trypsin inhibitor (21.5 kD), carbonic anhydride (30.0 kD), ovalbumin (46.0 kD), bovine serum albumin (69.0 kD), phosphorylase b (97.4 kD) and myosin (200.0 kD). Lysozyme and trypsin inhibitor merged as a single band in the electrophoresis.

from the fermenter within approximately 20% of the time required for antibody production to plateau. Because the objective of this study was to test the harvesting rate of the system, harvesting was not initiated until antibody production had reached a maximum at 60 h. A much better approach for a production system would be to initiate harvesting shortly after fermentation begins. This would allow (i) the 150 h campaign time used in this study to be shortened, perhaps down to 60 h, and (ii) there would be less chance for antibody proteolysis as product accumulates in the fermenter.

It is interesting that antibody concentration appeared to increase slightly 30–40 h after harvesting was initiated (Fig. 6), i.e. 90–100 h after fermentation began. The reason for this phenomenon is unknown. It is unlikely that this represents a sudden burst in biosynthesis and excretion. The more probable explanation is that cell death during this time period caused antibody to be released into the medium [14].

4. Conclusions

It may be concluded that direct coupling of an integrated analytical monitoring and harvesting system to a fermenter has many advantages in the production of monoclonal antibodies relative to off-line systems. One is that the on-line system allows a relatively high degree of automation in both harvesting and monitoring. Another is that the potential for microbial contamination of the fermentor is diminished by eliminating the need for frequent sampling to carry out analytical determinations. Still another is that the product may be harvested while the cell culture is still producing, precluding the prospect of product proteolysis in the fermenter. Finally, it may be concluded that on-line systems are advantageous in terms of allowing automated, feedback controlled production of excreted proteins by fermentation technology.

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