Cytotechnology **29:** 11–25, 1999.

Influence of osmolarity and pH increase to achieve a reduction of monoclonal antibodies aggregates in a production process

Rognoni Franco*, Gianesello Daniela, Maddalena Fabrizio, Giuntini Ilaria & Herbst Detlev
Research Department, Byk Gulden Italia S.p.A., Via Giotto 1, I-20032, Cormano (Mi), Italy

Received 15 September 1997; accepted 6 August 1998

Key words: aggregates, monoclonal antibodies production, osmolarity, pH

Abstract

Anti PSA monoclonal antibodies for diagnostic use were produced in an *in vitro* system. After purification using Protein G affinity chromatography a percentage of about 10% of antibody aggregates remained. The use of monoclonal antibodies containing aggregates as a capture antibody in a diagnostic kit reduces the performance of the test making it often unacceptable. The aggregates could be eliminated using gel filtration chromatography but, in that way, the final recovery of the whole production process was only about 50%. Aggregation is favoured when the working pH is near to the isoelectric point of the antibody. We varied the culture medium composition, modifying pH and osmolarity. We tested different values of pH and osmolarity: 7.1, 7.5, 8.0, 8.5 for pH, and 300, 340, 367, 395 mOsm/kg H_2O for osmolarity. By modification of the cell culture medium we obtained a significant decrease of monoclonal antibody aggregates in the production cycle. In this way we achieved higher recovery rate and could avoid gel filtration polishing step. The experiments were performed in two stages: first in culture flasks changing one parameter in each experiment, and then in spinner bottle using the best conditions obtained in the first stage. During scale up we used the modifications achieved from the experiment showed in this paper in our production by hollow fibre bioreactor with positive results.

Abbreviations: mAb, Monoclonal Antibody; IEF, Isoelectrofocussing; I.P., Isoelectric Point, PSA, Prostatic Specific Antigen; R.T., Retention Time

Introduction

For a protein to dissolve and remain in solution, its charged groups must interact with the molecules of the solvent. When such interactions are prevented from occurring, protein molecules interact with one another and form large aggregates which can also precipitate (Klein, 1982). Aggregation of antibodies in a solution can cause a loss of activity by steric interference of the antigen combining site and consequently a performance decrease of the immunological test. Aggregation of antibodies during a production cycle in an *in vitro* system can be a severe obstacle to achieve an enhanced product yield of bioactive molecules and a competitive and economical process.

We used a hollow fibre bioreactor system for growing hybridoma cells secreting monoclonal anti-PSA antibody for *in vitro* diagnostic use and we produced a large amount of antibodies during a bioreactor cycle. The bioreactor consists of hollow fibre membranes in an enclosed space, designed to hollow fluid flow through the lumen of the fibres. Cells are maintained outside the fibres in the extracapillary space. The semipermeable nature of the membrane allows nutrients and dissolved gasses to cross from the lumen of the fiber to the extracapillary space, and waste products and depleted medium from the extracapillary space to cross the membrane into the medium flow. Cells and secreted products with molecular weight higher than 10 Kd are retained in the extracapillary space. The mAbs were harvested in the harvest bottle to be later purified.

* Author for all correspondence.

Table 1. Results achieved in the first cycle. Osmolarity was increased up to 395 mOsm/kg H_2O from 300 mOsm/kg H_2O, which is the standard value detected in our cell culture medium, arriving to 395 mOsm/kg H_2O. Samples were analyzed for number of cells, viability, pH, glucose, lactate and ammonium. Determination of mAb purity and protein concentration were carried out after affinity chromatography. Growth and production rate were calculated and compared to identify general correlation of cells behaviour. The quantity and the purity of antibodies increased with higher osmolarity values

Flask 1 300 mOsm

		%	mg/ml	mg/ml	µg/ml	
Time	n° cells	Viability	Glucose	Lactate	NH_3	Det pH
0 h	1 x 10^7	90	3,59	0,15	13,2	7,1
24 h	1.3 x 10^7	100	3,47	0,29	21,49	7,2
48 h	5.7 x 10^7	96	2,7	0,94	30,5	7
72 h	1 x 10^8	94	2,38	1,58	30,1	6,75
96 h	1,5 x 10^8	82	1,66	2,01	32	6,45
µg after purification			1170	**purity**	86,90%	

Flask 2 340 mOsm

		%	mg/ml	mg/ml	µg/ml	
Time	n° cells	Viability	Glucose	Lactate	NH_3	Det pH
0 h	1 x 10^7	90	3,63	0,15	13,2	7,1
24 h	1.5 x 10^7	100	3,56	0,32	21,61	7,17
48 h	9 x 10^7	98	2,63	0,87	31,9	6,93
72 h	1.5 x 10^8	91	2,43	1,54	30,1	6,74
96 h	9,45 x 10^7	79	1,63	2	32,2	6,45
µg after purification			1290	**purity**	90,90%	
			mAb secretion		+ 10%	

Flask 3 367 mOsm

		%	mg/ml	mg/ml	µg/ml	
Time	n° cells	Viability	Glucose	Lactate	NH_3	Det pH
0 h	1 x 10^7	90	3,59	0,15	13,2	7,1
24 h	1.6 x 10^7	100	3,53	0,29	21,02	7,18
48 h	5.8 x 10^7	98	2,68	0,92	31,9	6,95
72 h	8.8 x 10^7	88	2,47	1,61	30,1	6,76
96 h	9.15 x 10^7	68	1,61	2,18	33,3	6,42
µg after purification			1410	**purity**	94,60%	
			mAb secretion		+ 20%	

Flask 4 395 mOsm

		%	mg/ml	mg/ml	µg/ml	
Time	n° cells	Viability	Glucose	Lactate	NH_3	Det pH
0 h	1 x 10^7	90	3,59	0,17	13,2	7,1
24 h	1.4 x 10^7	100	3,47	0,31	20,78	7,17
48 h	6.3 x 10^7	95	2,77	0,69	31,7	6,97
72 h	1 x 10^8	89	2,52	1,41	30,1	6,79
96 h	9.9 x 10^7	75	1,64	2,05	33,2	6,45
µg after purification			1470	**purity**	97,30%	
			mAb secretion		+25%	

.

After a continuous bioreactor cycle the antibodies were purified by affinity chromatography but, in all harvest batches, we found a percentage of about 10% or more antibody aggregates which caused a considerable decrease of purity. Eliminating the aggregates by gel filtration chromatography leads to a final yield of the process about 50%. (Guse et al., 1994).

The reduction of mAb's aggregates by size exclusion chromatography was very time expensive because the volume of the sample loaded onto the gel filtration

column should be only 5–10% of the total volume of the prepacked column. Usually at the end of a bioreactor cycle we achieved a quantity of mAb between 1 or 2 grams of purified mAb in a total volume of 500–1000 ml of cell culture supernatant. For the treatment of this volume with such a high concentration of mAb it is necessary to divide the sample for several chromatography steps increasing cost and time.

The aim of the present work is to prevent aggregates formation in an *in vitro* production process. The results of the experiments, as described below, allow the production of higher quantities of purified mAb, reducing also the costs of the production.

Before starting the project we investigated about the origin of the aggregates detected in the harvest batches of the production system, and we found that antibody aggregation started in the hollow fibre cartridge at pH 7.0. Increasing the pH, with a 500 mM bicarbonate buffer at values 7.5 and 8.0, only in the harvest bottle where the produced antibodies were collected, did not prevent aggregates formation. The increasing of pH in the harvest bottle was replicated more time, but we did not achieve positive results. For this way we decided to change the pH before harvesting the mAbs from the bioreactor.

The isoelectric point of the anti-PSA antibody is 6.1–6.5, so that an increase of pH up to a value of 7.5 or more should bring the net charge of the antibodies to further negative values and hence should raise the repulsive effect between the molecules. To promote this effect we increased also the osmolarity value, to achieve higher ionic strength in the cell culture medium (Scopes, 1997). Furthermore, the increase of osmolarity up to 300–400 mOsm/kg H_2O, by adding NaCl, raises the specific antibody secretion rate of hybridoma cells (Bibila et al., 1994).

In this work we present results demonstrating that a modification of the medium parameters, pH and osmolarity, can reduce the formation of antibody aggregates in a production system to a very low amount, greatly improving the economy of the process.

Materials and methods

Reagents

Cell culture medium (HB101) was provided by Irvine Scientific (Santa Ana, U.S.A.) , L-glutamine and glucose were bought from Sigma (St. Louis, U.S.A.). Sodium chloride was bought from Merck (Darmstadt, FR Germany).

Cell culture

Cells were a mouse hybridoma clone secreting anti-PSA monoclonal antibody of IgG2a subclass.

Cell culture work was divided in two stages.

Experiments of stage 1 were carried out in 175 cm^2 cell culture flasks containing 100 ml of culture medium. Cells were seeded at a concentration of 1×10^5 ml^{-1} in HB101, supplemented with 2 mM glutamine and 4 g/l glucose and were maintained in a 5% CO_2, 95% air atmosphere at 37 °C. After 72 h of culture 50 ml of complete culture medium were added to feed the cells.

Cell culture experiments of stage 2 were carried out in 1000 ml spinner bottle containing 500 ml of culture medium to scale up the results of stage 1. The cells were seeded at a concentration of 1×10^5 ml^{-1} in HB101 and maintained under continuous stirring (30 rpm) in a 5% CO_2, 95% air atmosphere at 37 °C. After 72 h of culture 250 ml of complete culture medium was added. At daily intervals, 5 ml aliquots of the suspension were harvested. Cells were counted and their viability was determined using trypan blue staining (Sigma). After centrifugation at $200 \times g$ for 10 min, the cell-free supernatant was used for subsequent determinations.

Sample analyses

For determination of glucose and ammonium concentration we used two enzymatic test kits (Sigma). The determination of lactic acid concentration was also made by an enzymatic kit (Boehringer Mannheim) using L-lactic acid as a standard. Osmolarity values were determined with an Osmometer by Fiske.

Purification of mAb

The sample was purified by affinity chromatography using a column prepacked with 10 ml of Gamma Bind Plus lined up in an FPLC system (Pharmacia). Binding conditions were 20 mM sodium phosphate, pH 7.4 with a flow rate of 3 ml/min for sample loading. To elute the bound antibody from the column a linear gradient was applied with a 100 mM Glycine buffer, pH 3.0 (0 to 100% in 5 min). The flow rate was 5 ml/min; monoclonal antibodies were eluted at pH 4.5 and were immediately neutralised with 1 M Tris HCl, pH 9.0. (Akerstorm et al., 1985; Guss et al., 1986).

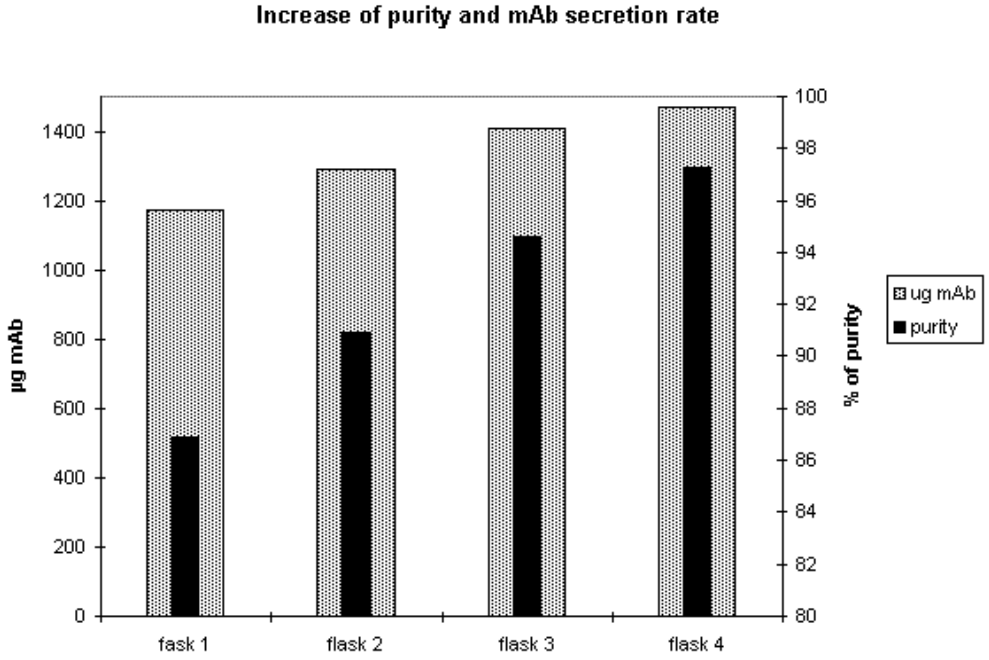

Figure 1. The increase of osmolarity with a NaCl solution (95.4 g/l) to values of 300, 340, 367 and 395 mOsm/kg H_2O, respectively (flask 1–4) caused an increase of purity at the end of purification procedure and an increase of mAb secretion rate.

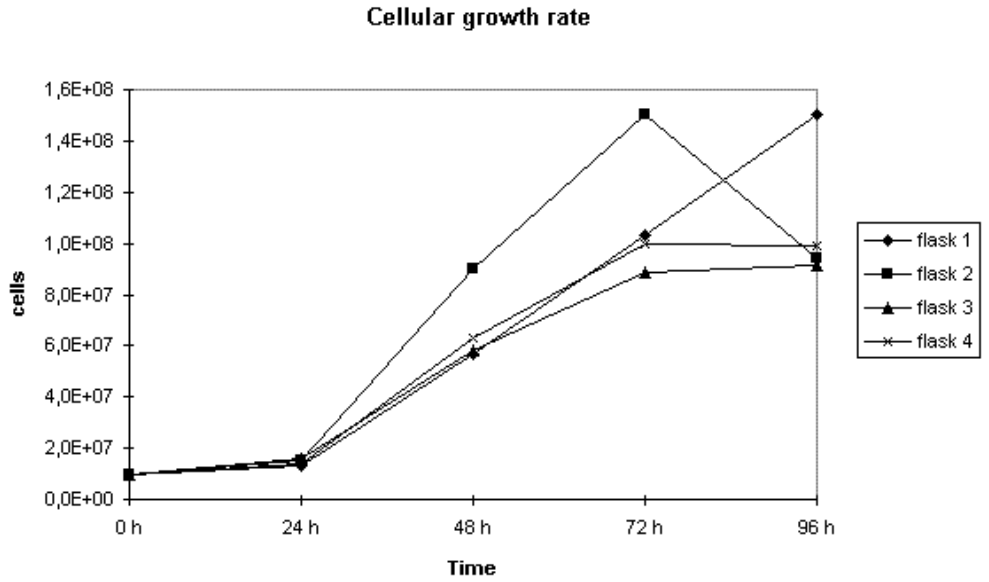

Figure 2. The increase of osmolarity at 340 mOsm/kg H_2O favours cellular growth more than standard condition (300 mOsm/kg H_2O). The increase of value higher than 340 mOsm/kg H_2O caused too much stress to the cell culture. Flask 1: 300 mOsm/kg H_2O; flask 2: 340 mOsm/kg H_2O; flask 3: 367 mOsm/kg H_2O; flask 4: 395 mOsm/kg H_2O.

Protein concentration

The protein concentration was determined after affinity chromatography using spectrophotometric method (Harlow and Lane, 1988).

Aggregates determination

The amount of aggregation was determined using an analytical gel filtration column (HR Superdex 200 from Pharmacia) and a 150 mM sodium chloride,

Table 2. Results achieved in the second cycle increasing the pH from 7.1 to 8.5. Daily determinations were the same as in the first cycle. The final purity of antibodies raised from 90 to 96% when the pH was changed from 7.1 to 7.5. At pH values higer than 7.5 secretion of mAb is reduced

Flask 1 ph7,1

		%	mg/ml	mg/ml	µg/ml	
Time	n° cells	Viability	Glucose	Lactate	NH_3	Det Osm
0 h	1 x 10^7	90	3,86	0,33	13	303
24 h	3.5 x 10^7	89	3,63	0,42	25,3	306
48 h	7.9 x 10^7	88	2,5	1,5	30,7	310
72 h	not det	not det	not det	not det	not det	not det
96 h	5.7 x 10^7	42	1,6	2,4	32,2	310
µg after purification			1122	**purity**	90%	

Flask 2 ph 7,5

		%	mg/ml	mg/ml	µg/ml	
Time	n° cells	Viability	Glucose	Lactate	NH_3	Det Osm
0 h	1 x 10^7	90	3,88	0,32	13	317
24 h	2.9 x 10^7	90	3,49	0,47	25,5	318
48 h	7.3 x 10^7	87	2,6	1,5	30,8	330
72 h	not det	not det	not det	not det	not det	not det
96 h	7.2 x 10^7	55	0,36	3,9	32,2	330
µg after purification			1068	**purity**	96%	

Flask 3 ph 8,0

		%	mg/ml	mg/ml	µg/ml	
Time	n° cells	Viability	Glucose	Lactate	NH_3	Det Osm
0 h	1 x 10^7	90	3,66	0,32	13	340
24 h	1.8 x 10^7	78	3,26	0,38	26,3	339
48 h	5 x 10^7	87	2,3	1,38	30,7	348
72 h	not det	not det	not det	not det	not det	not det
96 h	6.3 x 10^7	42	0,59	3,4	32,1	348
µg after purification			660	**purity**	96%	

Flask 4 ph 8,5

		%	mg/ml	mg/ml	µg/ml	
Time	n° cells	Viability	Glucose	Lactate	NH_3	Det Osm
0 h	1 x 10^7	90	3,58	0,33	13	352
24 h	6.4 x 10^6	60	3,32	0,39	24,1	385
48 h	8 x 10^6	42	3	0,6	30,8	400
72 h	not det	not det	not det	not det	not det	not det
96 h	0	0	1,95	0,32	32	400
µg after purification			not det	**purity**	not det	

.

50 mM sodium phosphate buffer at pH 7.2. The absorbance was read at 280 nm.

The molecular weight of the aggregates was determined using a gel filtration calibration kit from Pharmacia (Aldolase MW 158 000, Ferritine MW 440 000 and Thyroglobulin MW 669 000) (Cooper et al., 1985; Hangel, 1993).

Results

Stage 1

The first stage is divided in three cycles: in the first cycle we tested the influence of increased osmolarity in the cell culture medium using four flasks, each containing 100 ml of HB101 pH 7.1, with an osmolarity of 300, 340, 367, 395 mOsm/kg H_2O, respectively.

Table 3. Results achieved in first cycle with the increase of osmolarity and pH to confirm previous results. Also in this case the increasing of osmolarity caused the increase of mAbs secretion. Furthermore, the increase of osmolarity around 350 mOsm and pH at 7.5 limited aggregation of antibodies in the production cycle and did not cause too much stress to the cells

Flask 1 315 mOsm ph 7,5

		%	mg/ml	mg/ml	µg/ml	
Time	n° cells	Viability	Glucose	Lactate	NH_3	Det pH
0 h	1 x 10^7	90	3,09	0,27	13	7,5
24 h	2.1 x 10^7	75	3,36	0,46	24,3	7,37
48 h	4.5 x 10^7	90	2,6	1,56	31,3	7,19
72 h	8.25 x 10^7	84	1,98	2,37	31,5	6,9
96 h	1.12 x 10^8	74	1,12	3,07	33	7,5
µg after purification			1134	**purity**	92,00	

Flask 2 340 mOm ph 7,5

		%	mg/ml	mg/ml	µg/ml	
Time	n° cells	Viability	Glucose	Lactate	NH_3	Det pH
0 h	1 x 10^7	90	3,09	0,27	13	7,5
24 h	1.7 x 10^7	80	3,43	0,55	23,8	7,37
48 h	5.8 x 10^7	85	2,58	1,37	31,5	7,19
72 h	1.6 x 10^8	82	1,94	2,79	32	6,9
96 h	9.45 x 10^7	61	1,18	3,25	33,5	7,5
µg after purification			1248	**purity**	96,00	
			mAb secretion		+10%	

Flask 3 368 mOsm ph7,5

		%	mg/ml	mg/ml	µg/ml	
Time	n° cells	Viability	Glucose	Lactate	NH_3	Det pH
0 h	1 x 10^7	90	3,09	0,27	13	7,5
24 h	1.6 x 10^7	69	3,37	0,47	24,5	7,35
48 h	4.8 x 10^7	94	2,67	1,32	32	7,2
72 h	1.11 x 10^8	85	2,14	2,8	32,5	6,9
96 h	7.35 x 10^7	64	1,33	3,45	33,5	7,5
µg after purification			1362	**purity**	96,00	
			mAb secretion		+20%	

Flask 4 400 mOsm ph 7,5

	n°/ml	%	mg/ml	mg/ml	µg/ml	
Time	n° cells	Viability	Glucose	Lactate	NH_3	Det pH
0 h	1 x 10^7	90	3,09	0,27	13	7,5
24 h	2 x 10^7	71	3,34	0,54	24	7,35
48 h	4 x 10^7	88	2,82	1,52	31,2	7,24
72 h	7.5 x 10^7	83	2,3	2,11	31,5	7,01
96 h	6.45 x 10^7	61	1,42	2,64	32	7,5
µg after purification			1482	**purity**	96,00	
			mAb secretion		+30%	

.

The increase of osmolarity was performed by adding a solution of 95.4 g/l sodium chloride (3281 mOsm/kg H_2O) to the culture medium. At daily intervals number of cells, viability, pH, glucose, lactate and ammonium concentration were determined. After 5 days of the culture, 120 ml of cell culture supernatant from each flask experiment were loaded onto the Gamma Bind Plus column. After the affinity chromatography the mAb concentration was determined by spectrophotometric method to detect variations of mAb production during the experiments. The percentage of mAb's aggregates was determined by gel filtration chromatography using a purified sample.

We decided to detect the mAb concentration at the end of the culture, after affinity chromatography, in order to have enough antibody to make a complete characterization (determination of aggregates).The performance of the culture was followed by

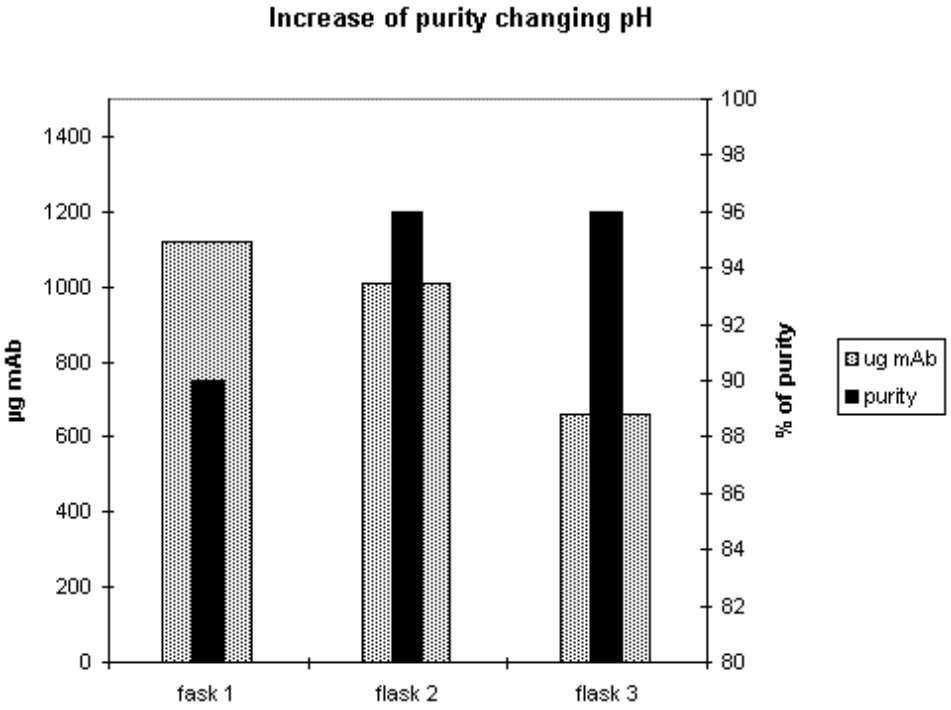

Figure 3. The increase of pH from 7.1 (flask 1) to 7.5 (flask 2) improves the mAb purity at 96%. Subsequent increase of pH to 8.0 (flask 3) did not cause an extension of mAb purity up than 96%. Furthermore in the experiment performed at pH 8.5 the cells died and no mAb production was observed.

daily determinations of metabolic parameters (number of cells, viability, glucose, lactate and ammonium); in this way we could analyze the effect of pH and osmolarity modifications on the cells.

Table 1 shows the results obtained in a typical experiment with increased osmolarity values in the medium. The amount of secreted antibodies was increased in the supernatant samples with higher osmolarity values (Ozturk and Palsson, 1991). Furthermore, samples with increased osmolarity showed a reduction of aggregates in the monoclonal antibody solution. The increase of osmolarity at 340 mOsm/kg H_2O caused an increase of the mAbs purity to 90%; furthermore an increase of about 10% on the mAbs secretion was observed. Increase in the osmolarity to 367 and 395 mOsm/kg H_2O gave rise to further improvement of mAb secretion, respectively, of 20 and 25% and an increase of mAb purity to 94 and 97%. Figure 1 shows an increase of purity and mAb secretion achieved in a first experimental cycle in culture flasks with different osmolarity values. As illustrated in Figure 2 the increase of osmolarity to 340 mOsm/kg H_2O improves the cellular growth rate, but after 72 h the number of viable cells begin to decrease. In comparison at 300 mOsm/kg H_2O (standard conditions) the cellular growth rate was rather constant until 96 h. The increase of osmolarity to values higher than 340 mOsm/kg H_2O, on the contrary, showed a lower cellular growth rate than the conditions in flask 1 and 2. Probably such high osmolarity values may cause stress to the cells in culture. (Sridhar et al., 1992).

During the experiment we tested also the parameters of glucose, lactate and ammonium, but the results described in Tables 1–3 never become critical conditions for a good cellular growth, because the results achieved with a modified condition were very similar to the results achieved in normal condition. In the second experimental cycle we tested the influence of pH increase on aggregates formation. Also this cycle was performed in four culture flasks containing 100 ml of HB101 300 mOsm/kg H_2O each with a pH of 7.1 (normal condition), 7.5, 8.0, and 8.5, respectively. The daily determinations were the same as in the first cycle. Table 2 shows the results from this experiment. An increase of antibody purity from 90 to 96%, after affinity chromatography, was observed when the pH was raised from 7.1 to 7.5. That means the percent-

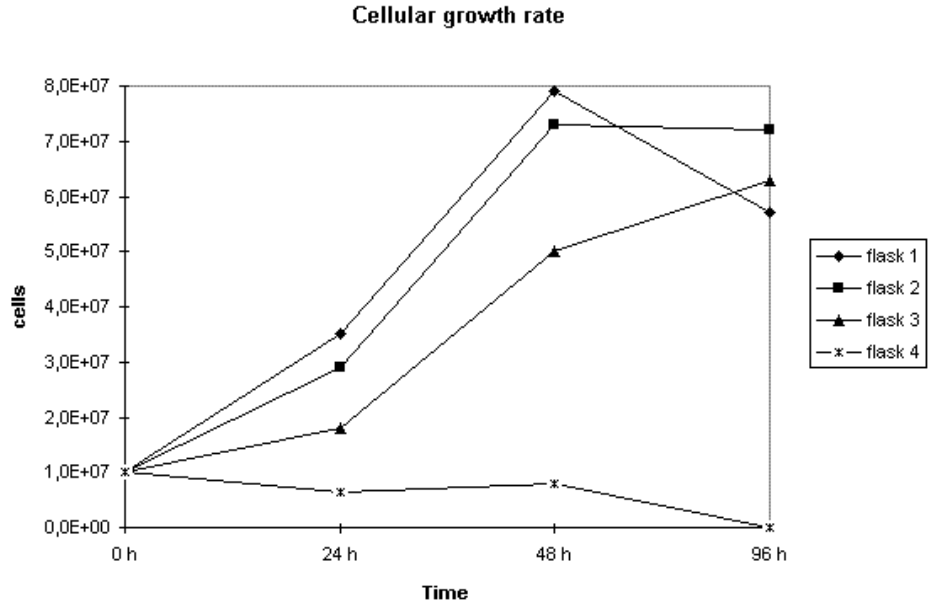

Figure 4. In the experiment of cycle 2 the increase of pH to a value of 7.5 (flask 2) does not limit the cellular growth. pH values of 8.0 and 8.5 (flask 3 and 4), on the contrary, cause a reduction of cell growth.

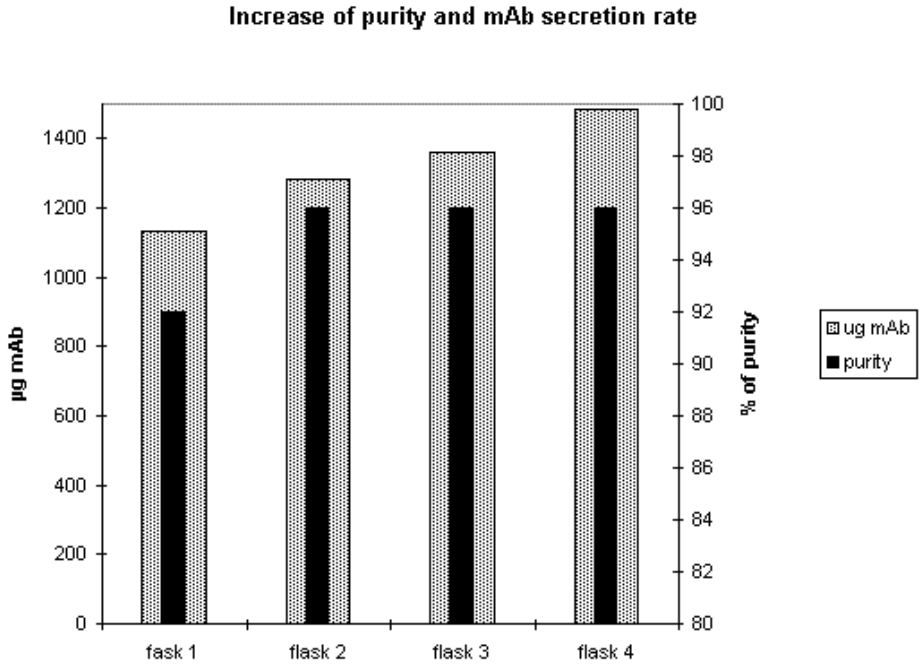

Figure 5. Changing osmolarity and pH at the same time led to a reduction of mAb aggregates. The pH was adjusted to 7.5 and the osmolarity was increased from 315 to 340, 368 and 400 mOsm/kg H_2O (respectively flask 1, 2, 3 and 4). Increasing the osmolarity to values higher than 340 mOsm/kg H_2O does not improve the mAb purity higher than 96%. Osmolarity values from 368 to 400 mOsm/kg H_2O were not favourable for cellular growth.

age of aggregates in the flask was reduced from 10 to 4%. Increasing the pH to values more than 7.5, however, does not raise the purity higher than 96% and the secretion of mAb is even reduced. These results are shown in Figure 3.

As illustrated in Figure 4 also the cellular growth

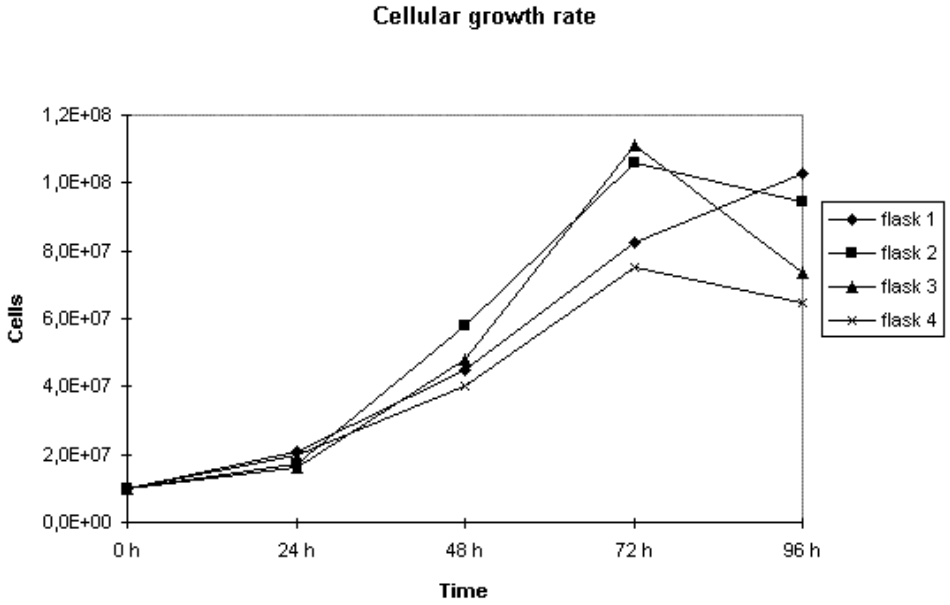

Figure 6. The increase of osmolarity to 350 mOsm/kg H_2O does not cause a beforehand death of the cells. Furthermore the osmolarity at 350 mOsm/kg H_2O favours cellular growth more than standard condition. All these data confirm the results achieved in the first two cycles. Flask 1 = 315 mOsm/kg H_2O and pH 7.5; flask 2 = 340 mOsm/kg H_2O and pH 7.5; flask 3 = 368 mOsm/kg H_2O and pH 7.5; flask 4 = 400 mOsm/kg H_2O and pH 7.5.

Table 4. Results achieved in the batch production cycle at standard condition. This cycle was carried out in order to compare the results achieved in this production with another batch under modified conditions (see Table 5). At daily intervals number of cells, viability, pH, glucose, lactate and ammonium concentration were determined as in Table 1. Also protein concentration and mAb purity were determined as in Table 1

Condition of cell culture medium

Osmolarity	300 mOSm	pH	7,2

Daily monitoring of the cycle

Time	n° cells	% viability	mg/ml Glucose	mg/ml Lactate	µg/ml NH3	mOsm/KgH2O Det Osm	Det pH
0 h	$5x10^7$	88	4,1	0,01	8,11	300	7,2
24 h	$6x10^7$	80	3,45	0,5	18,5	300	7,2
48 h	$1.5x10^8$	84	2,69	0,6	33,45	300	7,2
72 h	$4.2x10^8$	72	2,25	0,99	31,2	300	7,2
96 h	$5.3x10^8$	70	2,03	1,23	34	300	7,2

mg mAb after purification:	15.75 mg
Determination of purity	88%

rate was unfavourable under conditions of pH higher than 7.5. The increase of pH to a value of 7.5 did not limit the cellular growth and the mAb secretion was very similar to the results achieved in the normal condition. Consequently the increase of pH at values higher than 7.5 reduced to a half the mAb secretion. In the experiment carried out at pH 8.5 the cells were dead after 72 h and no mAb production could be observed.

During the experiments a constant pH value was maintained by addition of 500 mM bicarbonate buffer, when required after daily determination. During the

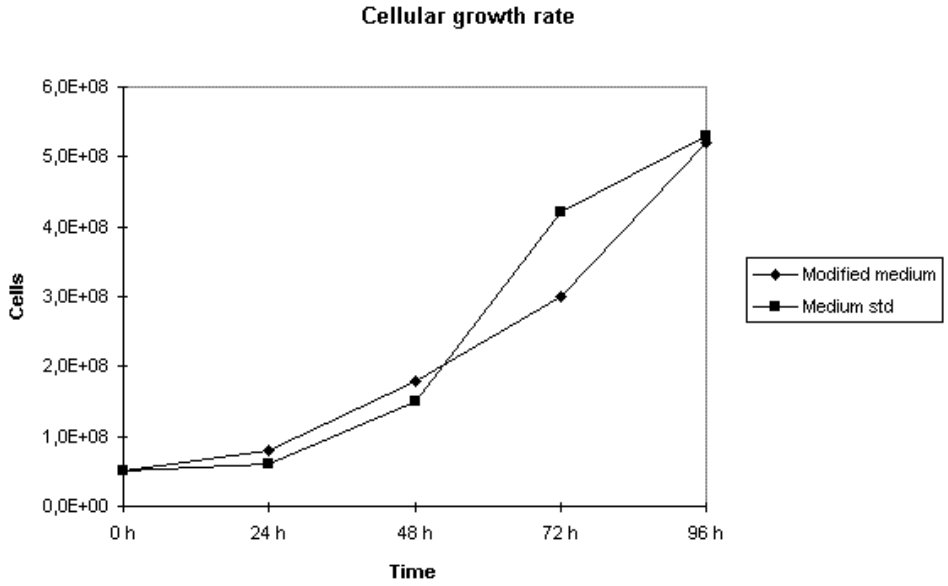

Figure 7. From the comparison between cellular growth rates in two batches, one with modified medium and the other under standard conditions, does not emerge such a great difference. On the other hand the cellular growth at modified condition was lightly higher. That means the increase of osmolarity and pH does not cause stress to the cells neither in a production batch nor in culture flask.

experiments it was necessary to add bicarbonate buffer for flasks 1, 2 and 3 but the increase of osmolarity was ineffectual for the performance of the experiments. On the contrary for the flask 4, it was necessary to intervene every day to maintain the pH value which caused an increase of osmolarity to 400 mOsm/kg H_2O.

To confirm the results achieved in cycles 1 and 2, in a third test we used a pH of 7.5, and an osmolarity value of 315 mOsm/kg H_2O in the first flask and 340, 368, 400 mOsm/kg H_2O in the other three flasks, respectively. We avoided a pH higher than 7.5 because the cellular growth rate was very low in such conditions, as shown before. Also in this case an increase of osmolarity leads to a reduction of aggregates increasing the final purity of the antibodies to 96% (Table 3). The mAb secretion was higher in flasks where culture medium with increased osmolarity was used (see Figure 5). Furthermore, in the third experimental cycle, the cellular growth rate was better at 340 mOsm/kg H_2O than at 315 mOsm/kg H_2O. However, increasing the osmolarity of the medium to 368 mOsm/kg H_2O and 400 mOsm/kg H_2O reduces the cell growth after 72 h, as shown in Figure 6.

Although the increase of osmolarity to 368 and 400 mOsm/kg H_2O improved the mAb secretion of 20 and 30%, respectively, the cellular growth showed a decrease after 72 h. This result was not advantageous to perform a long time production.

The increase of osmolarity to values higher than 340 mOsm/kg H_2O does not improve the mAb purity and causes stress to the cells. For this reason it was suitable to use culture medium with an osmolarity value at 340 mOsm/kg H_2O.

Also in this experiment the comparison of the results obtained from the daily determination of glucose, lactate and ammonium in the flask carried out in normal condition was very similar.

At the end of this experiment we found that an increase of osmolarity to 350 mOsm/kg H_2O and a pH of 7.5 limited the aggregation of anti-PSA antibodies in culture flask to an amount of 4% and did not cause too much stress to the cells. The achievement of this situation, as described before, is particularly important for maintaining the cell culture in a production process (hollow fibre bioreactor) for a long time. It is very important to bring only the essential changes in the medium without using the extreme conditions because, in this way, the cell proliferation is not compromised from the stress situation.

Stage 2

In the second stage we verified the results obtained in culture flask (stage 1) making a scale up in a batch production cycle using a 1 l spinner bottle. The first experiment in spinner bottle (cycle 1) was carried out

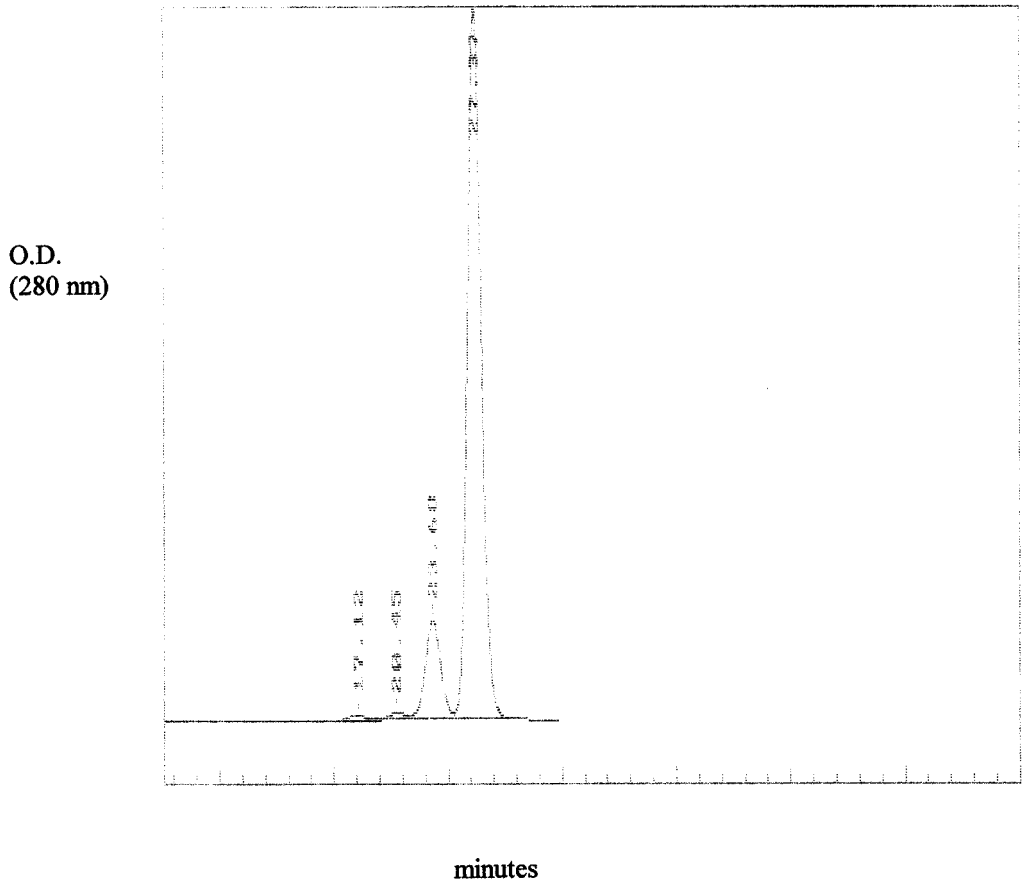

Figure 8. Gel filtration chromatography with Superdex 200 HR 10/30 (Pharmacia) of anti-PSA purified by affinity chromatography from Spinner Bottle cycle using a culture medium in standard condition (300 mOsm/kg H_2O and pH 7.2). RT 23.68 min: mAb aggregates (about MW 350 kD) determined with high molecular weight gel filtration standard (Pharmacia). RT 27.37 min: Monoclonal Antibody anti-PSA Eluent: 50 mM Phosphate, 150 mM NaCl ph 7.4; Flow 0.5 ml/min At the end of purification procedure we achieved a percentage of 13% mAb aggregates. The aggregate's molecular weight was determined using high molecular weight gel filtration standard kit (Pharmacia). (Aldolase MW 158 kD, RT 27.5 min; Ferritine MW 440 kD, RT 23.60 min and Tyroglobulin MW 669 kD, RT 20.68 min.)

under standard conditions (300 mOsm/kg H_2O and pH 7.2) in order to compare the results with the second cycle in spinner under modified conditions of 350 mOsm/kg H_2O and pH 7.5, following the results developed in stage 1.

We decided to use an osmolarity value at 350 mOsm/kg H_2O and not 340 in order to simplify the manufacture procedure during the preparation of the media.

In the experiments of stage 2 the cell number, viability, pH, glucose, lactate and ammonium concentration were determined daily. The pH value was maintained at 7.5 with little additions of 500 mM bicarbonate buffer when required. After the additions of bicarbonate buffer the osmolarity value was checked. At the end of batch production 600 ml of cell culture supernatant were loaded onto the Gamma Bind Plus column for each cycle and, after the affinity chromatography, the mAb concentration was determined using the same method described in the stage 1.

Table 4 shows the results achieved in the spinner bottle experiment using standard conditions. As in stage 1 (first cycle, flask 1) the cellular growth (Figure 7). rate does not show a decrease until 96 h The other values monitored in spinner bottle, like glucose, lactate, ammonium, etc, were never critical.

However, the percentage of antibody aggregates in the spinner bottle cycle with 300 mOsm/kg H_2O culture medium and pH 7.2 was 13% after purification as determined by gel filtration. Figure 8 shows a chromatogram of a gel filtration chromatography of the antibody produced in spinner batch. To con-

Table 5. Results achieved in a production batch under modified conditions (350 mOsm/kg H_2O and pH 7.5). At the end of purification procedure by affinity chromatography the purity of mAb was 98%: furthermore there was an increase of about 20% in mAbs secretion. Data obtained confirmed the results of the first step

Condition of cell culture medium

Osmolarity	350 mOSm	pH	7,5

Daily monitoring of the cycle

		%	mg/ml	mg/ml	µg/ml	mOsm/KgH$_2$0	
Time	n° cells	viability	Glucose	Lactate	NH3	Det Osm	Det pH
0 h	$5x10^7$	70	4,1	0,03	9,01	350	7,5
24 h	$8x10^7$	74	4,03	0,49	22,2	350	7,5
48 h	$1.8x10^8$	62	3,26	0,8	30,45	350	7,5
72 h	$3x10^8$	74	3,15	1,22	31,4	360	7,49
96 h	$5.2x10^8$	72	2,1	1,77	34,2	360	7,1

mg mAb after purification:	19,56 mg	**mAb secretion:**	+20%

Determination of purity	98%

firm that the shown peak with a retention time of 23.68 min was aggregated antibody we performed an SDS electrophoresis gel analysis under reducing and non reducing conditions (results not shown). Under non-reducing conditions there was found a band with a molecular weight of 158 Kd which corresponds to the monoclonal antibody and a band of MW much higher than the 212 Kd standard. This high molecular weight band represents aggregated mAbs. Under reducing conditions only bands of light and heavy chains with a molecular weight of 25 and 50 Kd were visible on the gel. We present the results achieved with gel filtration chromatography because in this way it was possible to detect the right percentage of mAb's aggregate. The peaks obtained from gel filtration were fractionated separately and the IgG subclass of the samples was determined. The two peaks with RT of 23.68 min and 27.37 min showed the same result of IgG 2_a subclass.

As described in Table 5, in spinner bottle cycle 2 we used the best conditions developed in stage one to verify that an increase of osmolarity to 350 mOsm/kg H_2O and increase of pH to 7.5 reduced the amount of mAb aggregates. To avoid the decrease of the pH value during the culture, after 72 h were added 1.2 ml of 500 mM bicarbonate buffer, and the osmolarity was checked again. The addition of bicarbonate buffer caused an increase of osmolarity to 360 mOsm/kg H_2O. After purification of the supernatant we found a mAb purity of 98% and an aggregate percentage of only 2% (see Figure 9). The antibody secretion rate was enhanced by 20% (Øyaas et al., 1989). These results were confirmed in other two spinner bottle cycles under the same conditions (results not shown). Table 5 summarises the results of the experiments carried out in a batch production cycle using modified conditions in spinner flask. It was confirmed that an increase of the osmolarity to 350 mOsm/kg H_2O and a pH of 7.5 did not cause any reduction of the cellular growth.

Discussion

Anti-PSA monoclonal antibodies were produced in hollow fibre bioreactor in our laboratory. To obtain antibodies of very high purity ($\geq$95%) for diagnostic use we employed affinity chromatography (Gamma Bind Plus Sepharose FF, Pharmacia), but during the characterisation of the purified product we found a percentage of 10–15% aggregated mAbs. Initially we used a gel filtration column packed with Superdex 200 Prep Grade (Pharmacia) to eliminate these aggregates, but in such way the final yield of the purification procedure was very low. In the elution profile of the preparative gel filtration purification step the peaks relative to aggregates (MW about 350 kD) and not aggregated mAbs were partially overlapping: therefore a complete separation of the fractions containing the mAb's aggregate from the fractions containing mAb would led to a loss of antibody product. Clearly this effect was proportional to the aggregates concentration.

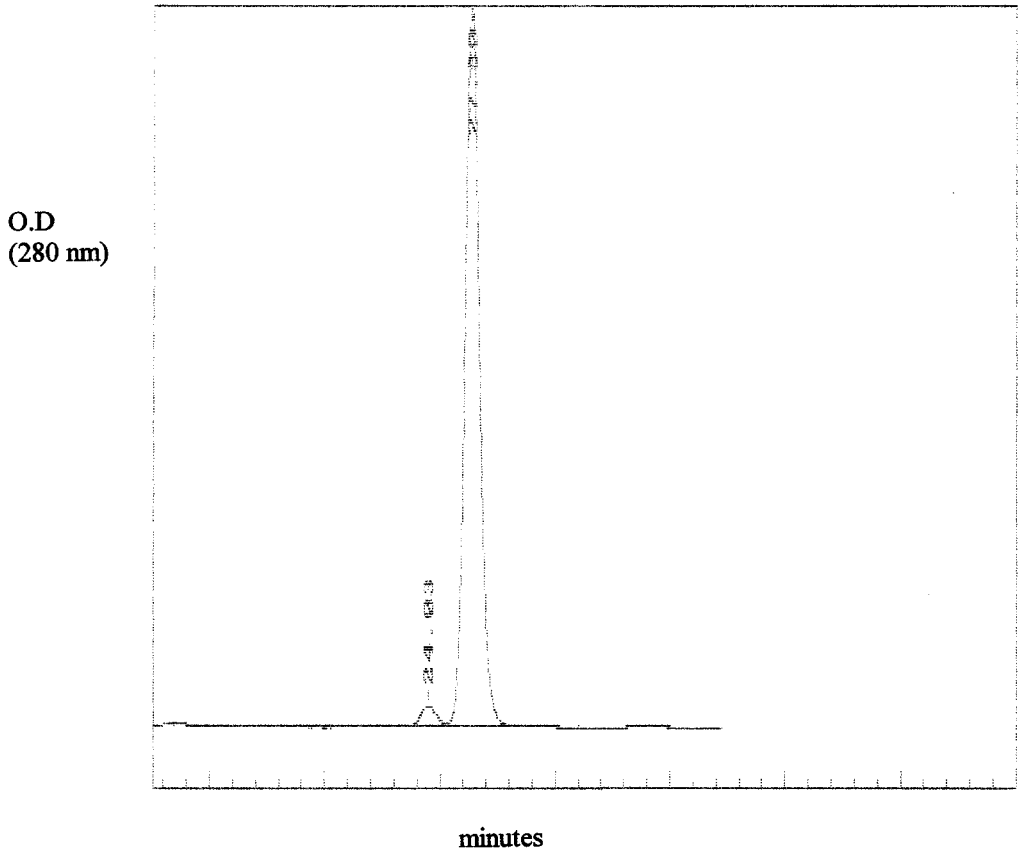

Figure 9. Gel filtration chromatography with Superdex 200 HR 10/30 (Pharmacia) of anti-PSA purified by affinity chromatography from Spinner Bottle cycle using medium culture under modified conditions (350 mOsm/kg H_2O and pH 7.5). RT 24.03 min: mAb aggregates (MW 350 kD) determined with high molecular weight gel filtration standards (Pharmacia). RT 27.80 min: Monoclonal Antibody anti-PSA Eluent: 50 mM Phosphate, 150 mM NaCl pH 7.4; Flow 0.5 ml/min At the end of purification procedure we achieved a percentage of 2% mAb aggregates.

Furthermore the gel filtration polishing step was used with good results in a small scale production but not to process the mAb obtained from the bioreactor cycle. To process the mAb solution eluted from Gamma Bind column with a gel filtration chromatography it was absolutely necessary to make more chromatographies using a column dimension too high and reducing the final recovery of the mAb.

To avoid such a loss of antibody during a purification procedure using a gel filtration step we decided to act on the cell culture medium to reduce the formation of aggregates during the production cycle. The formation of antibody aggregates occurred directly in the hollow fibre cartridge at pH values about 7.0. We decided to modify the culture medium by changing the pH into the harvest bottle. Changing the parameters of the culture medium was the only way to act before the mAbs secretion.

Ionic strength and pH are two of the main parameters which can be manipulated to alter the solubility of a protein in an aqueous solution. The I.P. of our anti-PSA mAb is 6.1–6.5. In standard production conditions we work at a pH 7.1–7.2. At this pH the antibody carries a negative net charge, but there are still some positive charges on the molecule. The electrostatic forces between positive and negative charges on other mAb molecules favour the formation of aggregates in solution. To limit this phenomenon we increased the pH to such a degree that the net charge of the antibody should be sufficient to effect a repulsion between the molecules in solution. Furthermore, to enhance the solubility, we increased the ionic strenght of the medium by adding a solution of sodium chloride (98 g/l) to achieve an osmolarity value of 350 mOsm/kg H_2O.

The principle aim of this work was to develop a

method which can be used for the bioreactor production avoiding the size exclusion chromatography step during the purification procedure.

The modification of pH and osmolarity values could be a stress condition for the cell culture. The daily monitoring of the metabolic parameters was important to verify that the medium modifications did not cause a negative effect on the cells.

The experiments were performed in two stages: in the first phase of stage 1, cycles 1 and 2, we examined the increase of pH and osmolarity separately. In a second phase, cycle 3, we tested the effect of increasing osmolarity and pH simultaneously. We found that an increase of the pH to 7.5 and the osmolarity to 350 mOsm/kg H_2O limited the formation of mAb aggregates to a value of 4% as shown by HPLC analysis at the end of the purification procedure. Purified monoclonal antibodies with a purity of 96% can be used in a diagnostic test kit without additional purification step.

In this experiment we showed that the increase of pH and osmolarity, to values of 7.5 and 350 mOsm/kg, respectively, does not cause stress for the cellular growth and the secretion of mAbs was stimulated. The increase of mAb secretion was quantified about 10%. Afterwards, in stage 2, we tested the conditions developed in stage 1, using a batch production system. The results from the batch cycle carried out in standard and modified conditions reconfirmed that the increase of osmolarity and pH, as described before, allows to reduce the mAb's aggregate formation. In this way it is possible to obtain the purified mAb in only one purification step and to reduce the costs of the process.

Increasing pH and osmolarity to values higher than 7.5 and 350 mOsm/kg H_2O stimulated the mAbs secretion of the cells more than 10%, but limited the cellular growth rate after 72–96 h. Since the aim of this work was the development of a method to transfer in the long time production cycle performed in hollow fibre bioreactor, we decided to bring only the essential changes to avoid stress condition for the cell proliferation.

During the experiment of the stages 1 and 2 we tested also the concentration of glucose, lactate and ammonium to control the influence of modified osmolarity and pH on the cellular growth.

In all the experiments the metabolic parameters were never critical: we can declare it because the metabolic values achieved from the tests, described before, were compared with the values obtained from the culture of PSA cells grown in normal condition as shown in the tables dedicated to the daily monitoring.

In all experiments the glucose concentration was never critical for the cell culture, because we started with 4.0 mg/ml, the same concentration used in the bioreactor. The ammonium concentration was never higher than 34.2 μg/ml; the toxic concentration for the cells was 76 μg/ml (4.5 mM). (Reuveny et al., 1986).

A value higher than 3.0 mg/ml of lactate concentration could cause a reduction of cell growth but not a decrease of antibody production (Kromenaker and Srienc, 1994). The lactate concentration was higher than 3.0 mg/ml only during the flask experiment in the cycle with pH modification and in the cycle with osmolarity and pH modification.

An increase of concentration over the limit was observed only after 96 h: for this reason we have not considered critical the lactate concentration achieved. Furthermore during the scale up in spinner bottle the lactate concentration was never over 1.77 mg/ml.

After the trial stages we used the modified medium in large scale production cycles carried out with hollow fiber bioreactor systems. The results found at the end of purification procedure confirmed that the aggregation of mAbs can be greatly reduced changing pH and osmolarity of the medium as described before.

This approach allowed to increase the quality as well as the final yield of anti-PSA mAbs produced in our laboratory.

At the end of the bioreactor cycle we reduced the volume of the harvest using an ultrafiltration membrane with a cut off of 30 Kd to 250 ml. After the concentration step the supernatant was loaded onto a column packed with Gamma Bind Plus Sepharose FF (Pharmacia). The purity of mAb obtained during the characterization was 97%. The mAb was then tested as a capture antibody in a diagnostic kit and the performance of the test (sensitivity and CV%) was very satisfactory.

Acknowledgements

The authors like to thank Mrs Ornella Barbieri for performing the determinations of glucose, lactate and ammonium.

References

Akerstrom B, Brodin T, Reis K and Bjorck L (1985) Protein G: a powerful tool for binding and detection of monoclonal and policlonal antibodies. J Immunol 135: 2589.

Bibila TA, Ranucci CS, Glazomitsk, K, Buckland BC and Aunins JG (1994) Monoclonal Antibody Process Development using Medium Concentrates. Biotechnol Prog 10: 87.

Cooper EH, Turner R, Johns EA and Crockson RA (1985) Identification and measurement of paraprotein polymers by high performance gel filtration chromatography. Biomed Pharmacother 39: 78.

Guse AH et al. (1994) Purification and characterization of an anti-CD4 monoclonal antibody for human therapy. J Chrom 661: 12.

Guss B, Eliasson M, Olsson A, Uhlen M, Frei AK, Jornvallm, Flock JI and Lindberg M (1986) Structure of the IgG – binding regions of streptococcal protein G. EMBO J 5: 1567.

Hangel L (1993) Size-exclusion chromatography in an analytical perspective. J Chromatogr 648: 19.

Harlow E and Lane D (1988) Antibodies – A Laboratory Manual, Cold Spring Harbour Laboratory, p. 673.

Klein J (1982) Immunology – The Science of Self-Nonself Discrimination, John Wiley & Sons Inc., p. 245.

Kromenaker SJ and Srienc F. (1994) Effect of lactic acid on the kinetics of growth and antibody production in a murine hybridoma: secretion patterns during the cell cycle. J Biotechnol 34(1): 13.

Øyaas K, Berg TM, Bakke O and Levine DW (1989) Hybridoma growth and antibody production under conditions of hyperosmotic stress, In: Spier, RE, Griffiths, JB, Stephenne, J and Crooy PJ (eds) Advances in Animal Cell Biology and Technology for Bioprocesses, pp. 272–278, Butterworths, Sevenoaks, U.K.

Ozturk SS and Palsson BO (1991) Effect of medium osmolarity on hybridoma growth, metabolism and antibody production. Biotechnol Bioeng 37, 989.

Reuveny S, Velez D, Macmillan JD and Miller L (1986) Factors affecting cell growth and monoclonal antibody production in stirred reactors. J Immunol Methods 86: 53.

Scopes KR (1987) Protein Purification – Principles and Practice, p. 42–44.

Sridhar R, Bauer Kenneth D and Miller WM (1992) Determination of antibody content in live versus dead hybridoma cells: Analysis of antibody production in osmotically stressed culture. Biotechnol and Bioen 40: 947.

Address for correspondence: Rognoni Franco, Research Department, Byk Gulden Italia S.p.A., Via Giotto 1, I-20032, Cormano (Mi), Italy