Antibody Production in Plant Cell Cultures

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Abstract

Monoclonal antibodies (MoAbs) are a highly diversified class of proteins with major research and commercial applications such as diagnostics and therapeutics. Currently, the dominant method for producing MoAbs is through the hybridoma technique. However, this technique is slow, tedious, labor intensive, and expensive. The production of MoAbs in cultured transgenic plant cells can offer some advantages over that in the mammalian systems. The media to cultivate plant cells are well defined and inexpensive. Contamination by bacteria or fungi is easily monitored in plant tissue cultures. Furthermore, these contaminants are usually not potent pathogens to human beings.

In our interdisciplinary research efforts, heavy chain monoclonal antibody (HC MAb) was inserted into Ti plasmid vector and transferred into *A. tumefaciens* for the transformation in tobacco cells. It was found that 76% of the transformants produced HC MAb. The presence of HC MAb in the cell membrane fraction indicated that the signal peptide was functional and efficient. The change of the HC MAb concentration during a batch culture followed a similar trend as dry cell concentration, indicating that the production of HC MAb was growth related. The long-term repeated subcultures of 11 cell lines showed that there was no obvious trend of neither the decrease nor the increase of the productivity with the repeated subcultures.

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Introduction

Large-scale plant suspension cultures can be employed for the production of pharmaceuticals, flavors, food colors, etc. However, the slow growth rate and the low product yield of plant cells are the two major obstacles for their active commercial utilization. These limitations can be partially overcome by selecting fast growing and high yielding cell lines, by optimizing growth media, or by improving fermenter design.

Further improvements should be possible by employing recombinant DNA technology to either modify the metabolic pathways to overproduce a desired natural metabolite or to insert foreign genes to produce a protein product. The modification of metabolic pathways may be difficult due to their complex nature. On the other hand, current plant biotechnology has demonstrated the insertion of foreign genes into plant cells to produce foreign proteins such as chloramphenicol acetyltransferase,[9,12] neomycin phoshotransferase,[9] β-glucuronidase,[6,13] human serum albumin,[19,22] and monoclonal antibodies.[2,4,5,8,10,16,17,21]

The production of foreign proteins in cultured transgenic plant cells can offer some advantages over that in the mammalian systems. The media to cultivate plant cells are well defined and inexpensive. Contamination by bacteria or fungi, which does not contain potent pathogens to human beings, is easily monitored in plant tissue cultures. The protein products from recombinant plant cells may be more functional and potent as pharmaceuticals than those from microbial origin because a post translational modification is likely to occur in plant cells.

In this presentation, our efforts to produce monoclonal antibodies from genetically modified tobacco cells will be reviewed.

Monoclonal Antibodies

Monoclonal antibodies (MAbs) are a highly diversified class of proteins with major research and commercial applications. An increasing demand exists for MAbs in the areas of diagnostics and therapeutics. Currently, the

predominant method for producing MAbs in through the fusion of antibody-producing spleen cells and a myeloma cell line,[14] which is known as the hybridoma technique. However, this technique is slow, tedious, and labor intensive. Furthermore, the number of antibody producing hybrdoma lines per fusion is not large enough to provide for an adequate survey of the immunological repertoire. There are alsc significant limitations to producing an antibody with a precise amino acid sequence with this technique. It is also costly to cultivate large quantities of hybridoma cells because of their slow growth, low product yield, shear sensitivity, and expensive culture medium.

Recently, transgenic tobacco plants were used as hosts for the expression and production of completely assembled MAbs,[4,8,10] single-chain $F_V(scF_V)$ molecules,[5,17,21] or single heavy chain(V_H) domain.[2] The scF_v consists of the heavy and light chain variable domains of immunoglobulin and is known to bind to their cognate antigens with affinities similar to those of the parent antibody molecules.[17] The isolated V_H was also found to have good antigen-binding affinities.[2,23] Since tobacco cells grows very rapidly, it is an ideal plant system to express foreign genes for the production of valuable products.

However, most of these studies[2,4,10,17,21] used transgenic plants instead of suspension cultures. The latter will be a preferred method for the large scale production of MAbs. Even for those[5,8] that employed the suspension cultures, they did not report the antibody production level during batch cultures.

Our research group[16] studied the production of the heavy chain monoclonal antibody (HC MAb) during batch and semi-continuous suspension cultures of transgenic tobacco cells. We modified tobacco cells to express a murine gene coding for the HC MAb specific for *p*-azo-phenylarsonate (Ars). The transformed cells were cultivated as suspension cultures in shake flasks. The localization and secretion of heavy chain antibody were also studied by a cell fractionation technique. The reasons for the selection of the production of single HC MAb instead of fully assembled MAb or F_v fragment are: (1) the presence of the antigen binding affinity of the single HC MAb, (2) the ease of the genetic modification and expression, and (3) the applicability of the result for other more complex product including the fully assembled MAbs.

Plasmid Construction and Plant Transformation

Plasmid pHγ_1 360E containing a murine HC MAb cDNA specific for *p*-axophenylarsonate (Ars)[7,20] was inserted into plasmid pGA648 to form plasmid pGA1130. The HC MAb cDNA was subsequently introduced into a binary vector pGA748 to form plasmid pGA1132(Figure 1). In this construct, the heavy chain antibody cDNA was placed downstream of CaMV35S promoter and terminated by the T-DNA transcript 7 gene terminator. The plasmid pGA1132 was transferred by the freeze thaw method[1] into *A. tumefaciens*,[11] and co-cultivated for 3 days with *Nicotiana tabacum* (NT-1) suspension cells.[1]

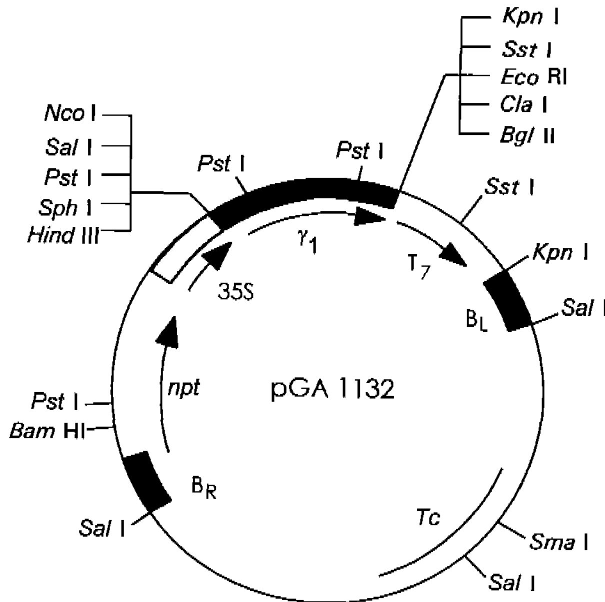

Fig. 1. Construction of plasmid pGA1132 carrying the 93G7 HC MAb cDNA (1.75 kb, noted as γ 1), which is a binary vector carrying a kanamycin resistant gene(npt) and a tetracycline resistant gene (Tc). The HC MAb cDNA (γ 1) was placed under the control of CaMV 35S promoter and T7 terminator. BL and BR are the left and the right border of a T-DNA.

The genetically modified cells were plated onto MS agar medium containing 50 μg/mL kanamycin for the selection of transformants. Suspension cultures were developed by subculturing the transformed callus in a liquid medium and cultivated in 250-mL Erlenmeyer flasks (working volume: 60 mL) at 29℃ on an orbital shaker (150 rpm). The suspension cell lines were sub-cultured weekly with the 5% inoculum of seven-day old cells.

Batch Suspension Cultures in Shaker Flasks

Eighty-six independently transformed calli were selected and maintained on agar medium. Seventy six per cent of the cell lines were positive in ELISA with the production level up to 15 μg per of HC MAb g of dry cells, of which eleven cell lines expressed more than 8 μg per g of dry cells. Seven transformed calli with different levels of HC MAb production were selected and developed into suspension cultures. Six cell lines produced higher than 1.2 μg of HC MAb per g of dry cell whereas one cell line showed no detectable level of the HC MAb production.

To investigate whether the antibody production correlates with the expression of mRNA level, total RNA was isolated from 4-day-old suspension cultures and mRNA level of the HC MAb gene was measured by the Northern blot analysis. Equal amounts of total RNA was loaded in each lane of the gel as determined by ethidium bromide staining of the 18S and 28S ribosomal RNAs in each sample (data not shown). All transformed cell lines expressed the antibody mRNA except the clone No. 27, for which no HC MAb was detected by ELISA. Most of the antibody yields determined by ELISA assay were proportional to mRNA expression levels. The major hybridizing bands were approximately 2.2-kb which is an expected transcript size, starting at the 35S promoter and ending at the T7 terminator sequence. Figure 2a shows the change of cell and HC MAb concentrations during a batch suspension culture of the cell line No. 70 in 250-mL Erlenmeyer flask. The cell concentration changes (solid line) showed that there was about 1 day of lag phase which was followed by and exponential growth and stationary phases. The HC MAb concentration (μg/L medium, broken line) followed a similar trend as dry cell concentration and reached a maximum value at the same time. This indicated that the production of HC MAb was growth related. The concentration of HC MAb was decreased during the stationary phase indicating the degradation of the produced HC MAb. Another potential reason for the decline is the insolubilization of produced HC MAb due to the hydrophobic nature of the variable region of the molecule.[18] The amount of HC MAb in the medium ranged from 4 to 10% of the total HC MAb as shown in Figure 2a.

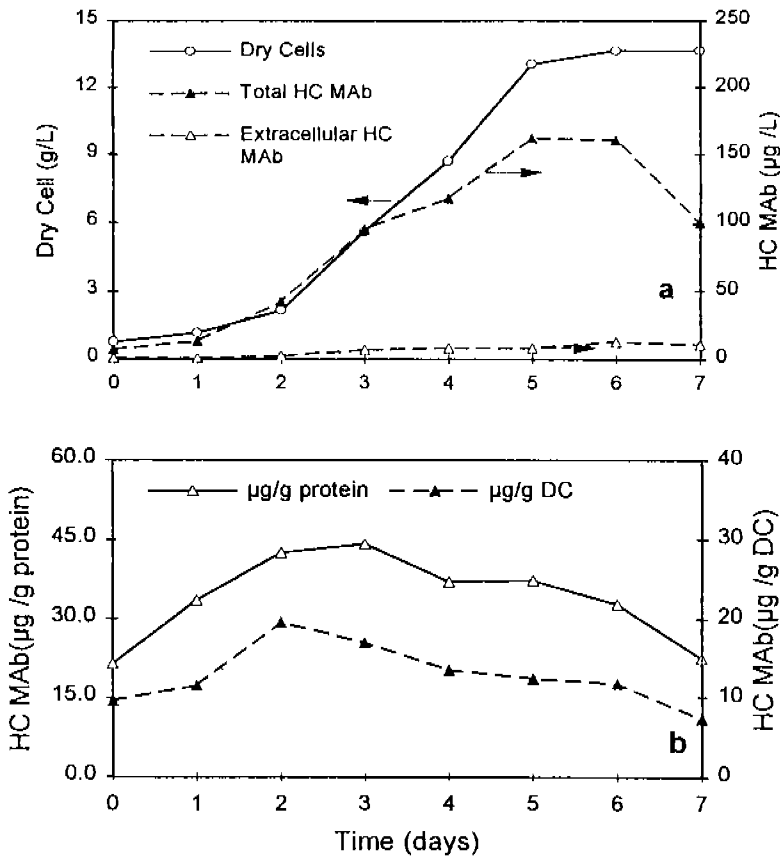

Fig. 2. Batch culture of transformed tobacco suspension cells (No. 70) in a 250-mL Erlenmeyer flask: (a) The change of dry cell and HC MAb concentrations (total and extracellular) based on unit medium volume, (b) The HC MAb productivity per gram of total soluble protein and of dry cells.

Figure 2b shows the change of the HC MAb productivity with time for the same batch. The productivities based on unit amount of protein and on that of dry cell weight showed the same trend giving their maximum values after 2 or 3 days of culture. This is the time when the lag phase ended and the exponential growth period started. It has been known that cells produce various enzymes to metabolize available nutrients after inoculation.[15] Therefore, it is postulated that the HC MAb gene is actively expressed during this lag period along with other proteins necessary for the cell growth and multiplication.

Localization of HC MAb

In order to find the location of the produced HC MAb in cells, the proteins in the cells were extracted in two steps. First, to extract proteins from cytoplasmic fraction, the cells were sonicated in a low salt extraction buffer and centrifuged at 15,000 g. Then, the resulting pellet containing the cell protoplasmic membrane, cell wall and organelle fractions was washed in the PEB buffer and was sonicated in high salt buffer to obtain the HC MAb concentration in the membrane fraction. If the HC MAb is secreted, it has to be found not only in the extracellular fraction, but also in the membrane fraction because signal peptide-mediated translocation across the endoplasmic reticulum membrane is the first step in the transport route common to vacuolar, ER, or Golgi resident proteins and those that are secreted.[3]

Table 1. Location of HC MAb and soluble proteins in the cells and medium after 4 days of suspension culture (cell line No. 70). The intracellular fraction was divided by two general regions: cytoplasmic and membrane fraction.

Fraction	HC MAb μg/g DC	Protein mg/g DC	HC MAb μg/g protein
Cytoplasm	7.5	270	28
Membrane	3.8	116	33
Medium	0.50	3.3	152

Table 1 shows the amount of HC MAb and total soluble proteins in each fraction from a 4 day old suspension culture (cell line No. 70). Though only 4% (0.50 μg/g dry cell) of the produced HC MAb was found in the medium, about 30% (3.8 μg/g dry cell) was in the membrane fraction and the rest 66% (7.5 μg/g dry cell) in the cytoplasm. The HC MAb was targeted by the leader sequence to cell endomembrane after its synthesis. The abundant detection of the antibody heavy chain in the cell membrane fraction indicated that the signal peptide was functional and efficient.

Table 1 also shows that only 0.8% of protein was found in the medium whereas about 4% of HC MAb was located extracellularly. Therefore, the amount of HC MAb per g of protein in the medium was very high (152 μg /g), which was 5 times higher than those in intracellular fractions. If HC MAb leaked out instead of secretion, other proteins should also be found in

the culture media. Therefore, this is another indication that cells are selectively secreting out HC MAb.

Genetic Stability of Transformed Cell Lines

Eleven transformed cell lines with different levels of HC MAb production were selected to study the effect of long-term repeated subcultures of the HC MAb production. Though the HC MAb production fluctuated (data not shown), there were no obvious trend of either the decrease or the increase of the productivity with the repeated subcultures for all 11 cell lines, which indicated the genetic stability of the cell lines. This stability is observed because foreign genes integrate into nuclear chromosome in plant cells instead of self replicating plasmids as seen in the case of microbial systems as shown in the previous section.[6] The fluctuation of the HC MAb concentration may be due to the variability of culture conditions, sampling times, and cell metabolisms.

Semi-continuous Fermentation

From the batch cultures of the HC MAb producing tobacco cells,we found that HC MAb was produced during the exponential growth phase and it was degraded during the stationary phase (Figure 2). The maximum productivity per unit amount of dry cell was reached during the early exponential phase. In this type of growth related production, the continuous mode of culture may be advantageous to the batch mode by prolonging the exponential growth phase. Furthermore, a continuous process reduces the cost of plant cell cultivation by eliminating the lag phase and the down time of the reactor between batches. However, the operation of a small-scale continuous plant cell cultivation is impractical because the continuous pumping of a small amount of cell suspension out from a fermenter is difficult due to the formation of cell aggregation and foaming.

Therefore, we tested a semi-continuous mode of operation by removing a

portion of culture broth periodically and replacing it with fresh media after 4 days of batch culture in 250 mL shaker flasks. Figure 3 shows the results of two runs: one-third of culture broth replaced with fresh medium every 12 hour and one-half replaced every 24 hours. The latter case showed the maintenance of the same level of HC MAb concentrations at the replacement time, though the rate of production dropped gradually. On the other hand, when one third was replaced every 12 hour, the HC MAb concetration was accumulated in increasing measure and reached a maximum. However, for both cases, the productivities dropped after about 4 days of semi-continuous runs possibly due to the accumulation of old cells. The replacement of one-half of medium broth might be too drastic for cells to avoid the shock of the fresh medium. The sudden change of cell medium environment is known to be the major reason for the presence of lag phase in batch cultivation.

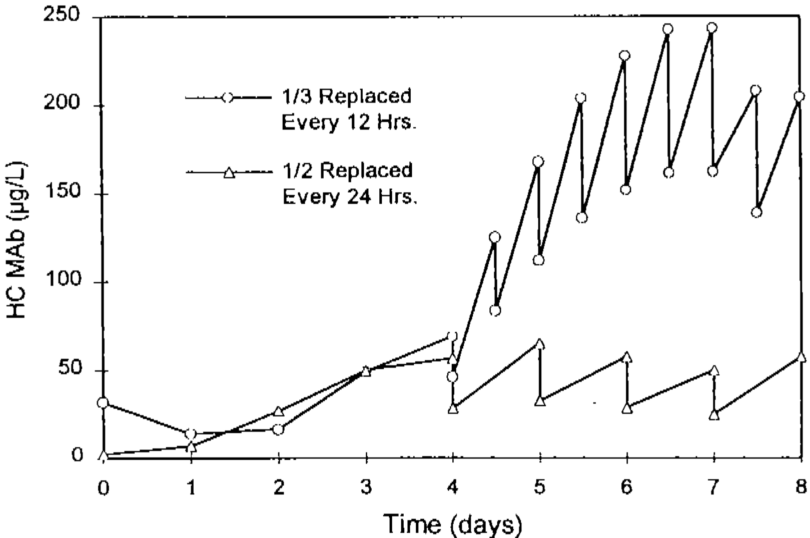

Fig. 3. Semi-continuous cultures of transformed tobacco suspension cells(No. 70) in a 250-mL Erlenmeyer flasks (working volume: 60 mL). After 4 days of batch cultures, one third of culture broth replaced with fresh medium every 12 hours or one half replaced every 24 hours.

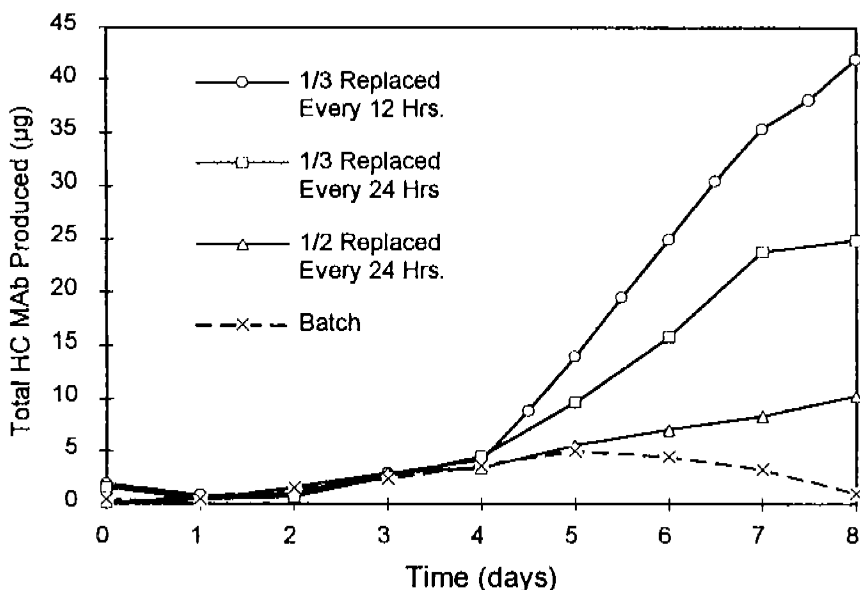

Fig. 4. Comparison of the total amount of HC MAb produced from three different semi-continuous runs and a batch run (working volume: 60 mL).

Figure 4 shows the comparison of the total amount of HC MAb produced from three different semi-continuous runs and a batch run (working volume: 60 mL). As expected, the total amount of HC MAb produced in one run was increased by almost 10 folds by operating the culture semicontinuously. The optimum condition was found to be one third of culture broth replaced every 12 hours. These results show the possibility of the dramatic improvement of the production rate by optimizing the mode of fermenter operation when this type of process is commercialized.

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