P-062 **Cell immobilization for anti-TNFα antibodies production**

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INTRODUCTION AND OBJECTIVES

Today cell immobilization in microcapsules increasingly attracts attention of the researches working in the field of biotechnology. One of the most known applications of this method is a cultivation of genetically modified cells to obtain recombinant products, namely peptides, enzymes, proteins as well as monoclonal antibodies (MAbs) (Seifert 1999). The microencapsulated cells were shown to maintain their viability for a long time (20 days or more), providing high production of biologically active compounds (Orive 2003), e.g. MAbs against tumor necrosis factor.

Tumor necrosis factor-alpha (TNFα) is a cytokine implicated in the pathogenesis of a variety of human diseases including septic shock, cachexia, graft-versus-host disease and several autoimmune diseases. Anti-TNFα MAbs provide an attractive mode of therapeutic intervention in these diseases (Knight 1993).

Anti-TNFα MAbs production could be performed in different ways, while cell microencapsulation seems to be one of the most promising techniques. The main advantage of microencapsulation is a production of a high quantity of genetically modified cells within microcapsule volume resulting in high yield of MAbs.

The objective of this research was to form stable alginate-oligochitosan microcapsules and to show a capability of immobilized genetically modified CHO cells to produce anti-TNFα chimeric antibodies.

MATERIALS AND METHODS

Chemicals: Sodium alginate (medium viscosity), EDTA and $CaCl_2$ were from Sigma. Chitosan degraded sample (MM 3900 Da) obtained by radical degradation with hydrogen peroxide was kindly provided by prof. A. Bartkowiak (Poland). All solutions for cell immobilization were prepared with 0.9% NaCl.

Fractionation of chitosan degraded sample: To remove all low molecular mass impurities, five methods of fractionation of degraded chitosan were tested:
A) Precipitation in NaCl (15 mg of the sample was solubilized in 5 ml of NaCl saturated solution for 30 min at 5°C and was re-precipitated in acetone (10 ml));
B) Precipitation by increasing pH value (15 mg of the sample was solubilized in 5 ml of distilled water, titrated with NaOH in the cold (pH 6.64) and placed at 5°C for 1h).
C) Precipitation in diethyl ester : 2 ml of the sample solution (37.5 or 75 mg/ml) was solubilized in 4 ml of cold diethyl ester and placed at 5 °C for 1h).
D) Precipitation in acetone : 2 ml of the sample solution (37.5 or 75 mg/ml) was solubilized in 4 ml of cold acetone and placed at 5°C for 1h. After precipitation all the oligochitosan samples were freeze-dried.

Cells and cell cultivation media: A stable genetically modified Chinese hamster ovary cell line (CHO) producing chimeric antibodies against TNFα were chosen for this study. Cells were obtained by transfection of cell line CHO/DG44 (OptiCHO antibody express kit, Invitrogen) using two plasmids (pcDNA3.3-HC and OptiCHO-LC). Cells were cultured in CD OptiCHO medium with 500 γ/ml G418, 8 mM L-glutamin without thymidine and hypoxanthine. The cells were cultivated in suspension at 130 rpm at 37°C in a humidified 5 % CO_2 / 95 % air atmosphere.

Cells microencapsulation: Cell precipitate (6 x 10^6 cells) was mixed with 2 ml of a sterilized sodium alginate solution, and the mixture was extruded using an electrostatic bead generator into 0.5 % (w/v) $CaCl_2$ by peristaltic pump. The obtained hydrogel microbeads were incubated with 0.2 % (w/v) oligochitosan solution for 7 min, in order to form an alginate-oligochitosan membrane on microbead surface. Then the microbeads were washed 3 times with physiological saline. In order to get hollow microcapsules, the microbeads were incubated in a 50 mM EDTA solution for 10 min and then again washed and transferred into the cultivation medium.

Cultivation of microencapsulated cells: The cells entrapped in microbeads and microcapsules were cultivated in 50-ml flasks on a roller set (60 rpm). To measure cell concentration in the microcapsules, the aliquot (0.1 ml slurry) was mechanically destructed to release cells, and the cells were calculated by Trypan blue assay.

Determination of antibodies production: MAbs quantification in cultivation medium of encapsulated cells was measured using ELISA, as described earlier with minor modifications (Catty 1989). Briefly, 96-well immunoplates were coated with 100 µl of TNFα (1 µg per well) and then incubated overnight at 4°C. Then samples diluted in PBS were added (1 µl per well), and detection was carried out with TMB One (Immunotech, Russia) and horseradish peroxidise conjugated with mouse MAbs

(2A11) against constant domain of the human IgG heavy chain (Immunologiya, Russia). Conjugate was diluted 1:25000.

RESULTS AND DISCUSSION

For our work, oligochitosans were obtained by chitosan radical degradation in hydrogen peroxide and five fractionation techniques were proposed to remove toxic low-molecular oligochitosan fraction. The comparison of the purified degraded samples leads to a general conclusion that precipitation in saturated NaCl and by increasing pH value is highly ineffective, since one can observe even 85 % weight lost. In the case of the samples precipitated in acetone and diethyl ester, the observed yield was over 55 and 80 % weight, respectively.

Additionally, the effectiveness of both precipitation methods was verified by their ability to form mechanically stable microcapsules. Microcapsules based on all the fractionated samples were prepared, and it was shown that all the microcapsules were mechanically stable.

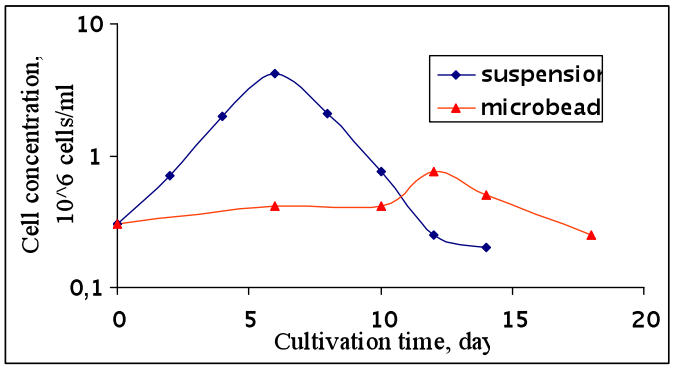

Figure 1 : CHO cell growth curve in suspension and alginate microbeads

In order to study genetically modified CHO cell growth inside the proposed alginate-chitosan microcapsules and in alginate microbeads, the cells were immobilized and cultivated for 20 days. As can be seen from Figure 1, cell concentration in the suspension culture increased for the first 6 days, but then the cell death was observed, while the concentration of cells entrapped in microbeads remained permanent during the whole cultivation time and did not exceed 0.8 x 10^6 cells/ml.

With the aim to examine anti-TNFα MAbs production by CHO cells, the samples of culture medium were collected every 2 days, and MAb concentration was determined by ELISA (figure 2). The cells cultivated in the suspension were used as a control. CHO cells entrapped in microbeads presented a high-level antibody production compared to the suspension culture.

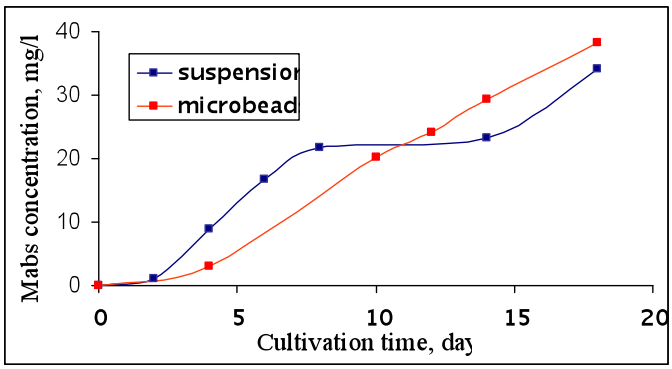

Figure 2 : accumulation of anti-TNFα MAbs produced by CHO cells in the cultivation medium

MAbs at concentration 38.2 mg/L were tested in the cultivation medium after 18 days of cell cultivation in alginate microbeads (figure 2). These results confirmed that immobilization of transfected cells is an effective strategy for anti-TNFα antibodies production.

CONCLUSIONS

Mechanically stable microcapsules based on alginate and purified oligochitosans obtained by various fractionation techniques of chemically destructed chitosan sample, were formed. The alginate-oligochitosan microcapsules and the alginate microbeads were demonstrated to provide the growth and proliferation of genetically modified CHO cells. The long-term cultivation allowed to provide anti-TNFα MAbs production for more than 20 days. Thus, anti-TNFα MAbs obtained by cultivation of immobilized genetically modified cells could be considered as a promising approach, whereas a variety of biocompatible polymers could expand the potential of the immobilization technique.

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