Combination of dextran sulfate and recombinant trypsin on aggregation of Chinese hamster ovary cells

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Abstract In laboratory scale therapeutical protein production, cell clumps form typically in shake flasks, which hinders cell growth and decreases protein yield. To minimize clumps during the culture of Chinese hamster ovary cells, we employed the combination of two reagents, dextran sulfate (DS) and recombinant trypsin (r-trypsin). Our results showed that both DS and r-trypsin could diminish cell aggregation when adding them respectively, but clumps were still noticed obviously. In order to further mitigate cell agglomerate, a combination of 1.2 g/L DS and 8.0 mg/L r-trypsin was employed and no clumps were found under the bright field microscope. Strikingly, the highest viable cell density of combination group was increased from 5.12×10^6 to 7.13×10^6 cells/mL, while the integral of viable cells concentration was

raised from 35.13×10^6 to 60.87×10^6 cells-days/mL, and the culture period was prolonged by 4 days. In addition, the antibody integrity was maintained in the combination group compared with that of the control.

Keywords Aggregation · Chinese hamster ovary cells · Dextran sulfate · Fed-batch · Recombinant trypsin

Introduction

Chinese hamster ovary (CHO) cells are the main hosts employed for industrial recombinant protein production (Hacker et al. 2009). The performance of CHO cells in laboratory scale shake flask is an effective indicator of the behavior in bioreactors, which are the dominant container for the production of industrial proteins (Liu and Hong 2001). Production is tested in

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laboratory scale before large scale manufacture, during this process appropriate producer is selected, while the medium and culture process are optimized (Wurm 2004). However, in the process of laboratory scale protein production, many factors inhibit cell growth including aggregation. In shake flasks, CHO cells tend to gather into clumps when cultured in fed-batch mode. Besides, in perfusion cultivation, it also suffers cell aggregation (Liu and Goudar 2013).

Cell aggregation is a process of cell clumps forming during cell culture. It affects the accuracy of cell counting, and meanwhile hampers the efficiency of oxygen transport and nutrition diffusion, which usually leads to apoptosis and losses of productivity.

Physical approaches to solving the problem of cell aggregation have been proven to be less than satisfactory. For example, increasing shaking speed may be favorable to inhibition of cell aggregation, however, excessive speed may be accompanied with cell damage. Just like in bioreactor, increasing agitation rates mitigate cell aggregation, however, higher agitation rates fail to break up tightly bound aggregates and the concomitant higher shear rates bring about cell injury (Moreira et al. 1995).

Several commercially available anti-aggregation products are also claimed to alleviate aggregation such as anti-clumping agents supplied separately by Gibco and Lonza. Although they are advocated to decrease aggregation in CHO cell culture, the components are confidential and the concentration should be optimized by users individually.

A more rational solution is adding dispersing reagents such as suramin (Zanghi et al. 2000), heparin (Li et al. 2011), which are rich in sulfate group. They may alter the cell surface charge (Dee et al. 1997), leading to single cell suspension. Dextran sulfate, a kind of dispersing reagent with a high density of sulfate charge, has been exploited to prevent cell aggregation (Dee et al. 1997).

In addition, in the cell culture process trypsin is often used to detach cells, which can destroy the cell-to-cell adhesion molecules (Edwards et al. 1975; Steinberg et al. 1973), thereby enabling cells to stay in single-cell suspension state. Nowadays, there are still some commercial trypsins derived from animal pancreas, which may be toxic and cause infectious contamination to cultured cells (Golden et al. 2002). Recombinant trypsin expressed in *Escherichia coli* eliminates the risks mentioned above.

In this article, our results suggested that the addition of single reagent may not be proven effective enough to neutralize aggregation. When DS and r-trypsin were combined, minimized aggregation was obtained. The study offers an optimized and combined formula to avoid the formation of cell clumps in mammalian cell culture, and it is beneficial to cell culture and laboratory scale expression of recombinant proteins.

Materials and methods

Cell line, medium, and cell culture

CHO-SP is a cell line derived from CHO-K1(ATCC, Manassas, VA, USA) through adaption in serum contained medium and serum free medium repeatedly, which is preserved in our laboratory, and it is capable of switching between suspension in CHO-MB01 medium and adherence in 10 % fetal bovine serum contained medium. Cells were passaged every 4 days to a density of 5.0×10^5 cells/mL, and maintained in shake flasks at 37.0 °C and 150 rpm in a 5 % CO₂ humidified environment. To evaluate the effect of anti-aggregation regime on antibody productivity and antibody integrity, a cell line expressing a recombinant IgG1 antibody (CMAB-802) was employed, which was kindly provided by Shanghai Zhangjiang Biotechnology Co., Ltd (Shanghai, China).

CHO-MB01 is a proprietary serum free medium for cell maintenance and subculture, containing 3.5 g/L glucose, and additional 4 mmol/L glutamine is supplemented before use (all purchased from the State Key Laboratory of Medicine and Target Therapy (Shanghai, China)). CHO-MS01 is another proprietary serum free medium for supplementation in fed-batch culture.

Effect on cell aggregation

Dextran sulfate (DS, 5,000 Da, Wako Pure Chemical Industries, Ltd, Osaka, Japan) and recombinant trypsin (r-trypsin) were employed to examine the potential function of anti-aggregation. Recombinant trypsin (Human recombinant trypsin 2, Shanghai Yaxin Biotechnology Co., Ltd, Shanghai, China) is a 24 kDa protein expressed in recombinant *E. coli*. Both DS and r-trypsin were dissolved in phosphate buffered saline (PBS) and filtered through a 0.22 µm filter before use.

CHO-SP suspension cells were cultured in 100 mL shake flasks with a working volume of 30 mL.

Fed-batch experiments

For experiments, exponentially growing cells were inoculated at a concentration of 5.0×10^5 cells/mL in CHO-MB01 containing various concentrations of DS and r-trypsin, ranging from 0 to 1.4 g/L and 0 to 16.0 mg/L, respectively. After cultured for 72 h, 3 % of CHO-MS01 was supplemented. Meanwhile, glucose concentration in the culture broth was assayed and replenished to 2.5 g/L daily by adding a 20 % concentrated glucose solution from day 3 onwards. In addition, 1 mmol/L glutamine was supplemented every other day from day 3 onwards. Approximately 0.5 mL of culture suspension was sampled daily from the flask for cell counting, microscopy, photography and other analyses. The culture process was terminated when cell viability declined below 70 %.

Antibody quantification and SDS-PAGE

The culture supernatant containing the recombinant antibody was collected by centrifuging the whole cell culture at 1,000 rpm for 5 min, and the yield supernatant was then centrifuged at 1,000 rpm for 10 min before it was used for further antibody productivity quantification and SDS-PAGE analysis. The culture supernatant was purified on a HiTrap Protein A HP column (GE Healthcare, Pittsburgh, PA, USA) following the manufacturer's instructions. Briefly, 1 mL of supernatant was diluted with 3 volumes of 0.02 mol/L sodium phosphate (pH 7.0) loading buffer and was loaded on pre-equilibrated column, and the column was washed with loading buffer and eluted with 0.1 mol/L citric acid (pH 3.0). The eluate was neutralized with 1.0 mol/L Tris-HCl (pH 9.0). Then, analysis of antibody expression was performed according to the absorbance at 280 nm.

Non-reducing SDS-PAGE was performed. Proteins were mixed with $2 \times$ non-reducing loading buffer, and heated at 100 °C for 10 min. A volume of 20 μ L was loaded and the proteins were separated on 8 % polyacrylamide gel before staining by coomassie brilliant blue G-250.

Analytical and calculation methods

Cell density was determined using a hemacytometer, and viable cells were distinguished from dead cells

Fig. 1 The profiles of the cell growth, cell viability and IVCC under conditions of various concentration of DS. In the fed-batch, DS was added at the time of inoculation, i.e. day 0. **a** Viable cell density, **b** viability. Open square control, filled inverted triangle 1.0 g/L DS, open circle 1.2 g/L DS, filled triangle 1.4 g/L DS. Triplicate experiments were performed. Error bars represent standard deviation

using the trypan blue exclusion method. Glucose concentration was assayed with glucose assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), following the manufacturer's instructions.

Integral of viable cells concentration (IVCC, 10^6 cells-days/mL) was calculated using the following adapted formula (Dorai et al. 2009):

$$\text{IVCC} (d_{n+1}) = (\text{VCD} (d_n) + \text{VCD} (d_{n+1})) / 2 + \text{VCD} (d_n)$$

Fig. 2 The morphology of CHO cells treated with different reagents. Photographs were taken on the day 7 of every fed-batch and magnified by 10×10 under bright field microscope.

a Control, **b** 1.2 g/L DS, **c** 8.0 mg/L r-trypsin, **d** combination of 8.0 mg/L r-trypsin and 1.2 g/L DS

IVCC (d_{n+1}) means the IVCC on day $n + 1$; VCD (d_{n+1}) is the viable cell density on day $n + 1$; VCD (d_n) indicates the viable cell density on day n .

Paired samples Student's t test was performed to evaluate the significance of difference between two groups. A p value <0.05 was considered statistically significant.

Results and discussion

The anti-aggregation effect of DS

To investigate the effect of DS on cell aggregation, we tested the various DS concentrations from 0 (control) to 1.5 g/L. Preliminary study indicated that 1.0–1.5 g/L DS obviously inhibited cell aggregation in shake flasks

(data not shown). Then, we further compared the effect of DS concentration of 1.0, 1.2 and 1.4 g/L, and a blank control was added. The maximum viable cell density reached up to 5.88×10^6 , 6.42×10^6 and 5.76×10^6 cells/mL when 1.0, 1.2 and 1.4 g/L DS were supplemented, respectively, while the control group reached only 5.10×10^6 cells/mL (Fig. 1a). The maximal viable cell density with 1.2 g/L DS was significantly higher than that with 1.0 g/L DS ($p < 0.05$), while the difference was not significant compared with that supplemented with 1.4 g/L DS. In addition, cells treated with various concentrations of DS (1.0, 1.2 and 1.4 g/L) showed significantly higher viability than the cells of the control group on days 7 and 9 (Fig. 1b). On day 11, the viability in the group supplemented with 1.2 g/L DS was significantly higher than that with 1.0 g/L DS ($p < 0.05$), however,

the difference was not significant in comparison to that with 1.4 g/L DS. The cultures supplemented with 1.2 g/L DS displayed the maximum cell density and highest viability throughout the process. Besides, in the group of 1.2 g/L DS, processing of the culture was prolonged from 9 to 11 days. With 1.2 g/L DS treated, cell aggregation attenuated, however, evident clumps were still observed (Fig. 2a, b).

Dextran sulfate, a highly sulfated polyanion, has been successfully applied to mitigate the CHO aggregation and maintain stable single cell suspension of BTI-TN5B1-4 cells (Dee et al. 1997). It was reported that DS treatment decreased the expression of cadherin-11 gene in the cDNA microarray analysis, which indicates that DS decreases gene expression of such cell–matrix adhesion factors and prevents cell adhesion (Takagi et al. 2005). Our results showed that DS was able to attenuate aggregation, increase viable cell density and cell viability. However, cell aggregation could not be dissociated completely when DS was administrated alone.

The anti-aggregation effect of r-trypsin

Trypsin, as a protease, is known to degrade membrane glycoproteins. Trypsin has been widely used in the cultivation of mammalian cells, for instance, dissociating primary cells to obtain single cells from tissues and organs (Shibeshi et al. 2008), yet no reports indicate its application in addressing aggregation issues in suspension CHO cells.

Tentative studies indicated that the effect of anti-aggregation was less than satisfactory when the concentration of r-trypsin was below 2.0 mg/L, and obvious cell injury was observed when the concentration was above 16.0 mg/L (data not shown). In the present study, we compared the effects of 4.0, 8.0 and 16.0 mg/L r-trypsin on aggregation of CHO cells in shake flasks. We found that r-trypsin facilitated the increase of viable cell density. On day 5, the viable cell density reached a maximum value of 6.39×10^6 cells/mL with 8.0 mg/L r-trypsin, and increased by 18.77 % compared with 5.38×10^6 cells/mL of the control. Whereas cell density of the groups with 4.0 and 16.0 mg/L r-trypsin were 6.03×10^6 and 6.08×10^6 cells/mL, respectively, slightly lower than that with 8.0 mg/L r-trypsin, however the differences were not significant (Fig. 3a). On day 10, the viable cell density with 8.0 mg/L r-trypsin was significantly higher than

Fig. 3 The profiles of the cell growth, cell viability and IVCC under conditions of various concentration of r-trypsin. In the fed-batch, r-trypsin was added at the time of inoculation, i.e. day 0. **a** Viable cell density, **b** viability. Open square control, filled inverted triangle 4.0 mg/L r-trypsin, open circle 8.0 mg/L r-trypsin, filled triangle 16.0 mg/L r-trypsin. Triplicate experiments were performed. Error bars represent standard deviation

that with 4.0 mg/L r-trypsin ($p < 0.05$), however, the viable cell density was not significantly improved compared with that of 16.0 mg/L r-trypsin. In addition, a relatively higher viability was observed in the group supplemented with 8.0 mg/L r-trypsin at late phase, and the highest viability was 87.01 % on day 7, while that of the control group was 74.41 % (Fig. 3b): the difference was significant ($p < 0.05$). Given the above, 8.0 mg/L r-trypsin was selected for further research. However, some cell clusters still formed in

Fig. 4 The profiles of the cell growth, cell viability, IVCC and proteins in the culture supernatant treated with different reagents. In the fed-batch, both DS and r-trypsin were added at the time of inoculation, i.e. day 0. The experiments in **a–c** were performed in CHO-SP, while the experiment in figure **d** was conducted in CHO cells expressing an IgG1 antibody in fed-batch culture. **a** Viable cell density, **b** viability, **c** IVCC,

d non-reducing SDS-PAGE of the supernatant proteins on day 7, DS + r-trypsin indicates 1.2 g/L DS and 8.0 mg/L r-trypsin. The arrow indicates the position of intact IgG1 antibody. *Open square* control, *filled inverted triangle* 8.0 mg/L r-trypsin, *open circle* combination of 8.0 mg/L r-trypsin and 1.2 g/L DS, *filled triangle* 1.2 g/L DS. Triplicate experiments were performed. Error bars represent standard deviation

shake flask under this condition from day 7 onwards (Fig. 2c).

Trypsin destroys the cell-to-cell adhesion molecules such as adhesion receptor, and enables cells a single-cell suspension state which makes it an effective reagent to detach adherent cells (Edwards et al. 1975; Steinberg et al. 1973). Recombinant trypsin is more suitable for the production of pharmaceutical proteins because it is non-toxic and free of virus contamination. Here, we proved that r-trypsin could also be used to decrease aggregation in suspension cell culture. Our results indicated that when 8.0 mg/L r-trypsin was added, the viable cell density, viability and IVCC were greater than those with 16.0 mg/L r-trypsin, while obvious aggregation was still observed.

The anti-aggregation effect of combination of DS and r-trypsin

The given results indicated that both DS and r-trypsin showed the property of anti-aggregation, which increased the viable cell density and prolonged life span. Despite these advances, apparent clumps still exist (Fig. 2b, c). To completely solve aggregation problems, we investigated the effect of a combination of 1.2 g/L DS and 8.0 mg/L r-trypsin, which was optimized in the above mentioned studies. Besides, the other three conditions were employed including the blank control and separate administration of 1.2 g/L DS and 8.0 mg/L r-trypsin.

No apparent cell clumps were observed under microscope throughout the cell culture process in the

culture broth containing a combination of 1.2 g/L DS and 8.0 mg/L r-trypsin. Maximum discrepancy of aggregation appeared on day 7 among the four different conditions (Fig. 2). Moreover, the combination group achieved the highest viable cell density, and the peak value reached up to 7.02×10^6 cells/mL on day 6, while the cell density of the three other groups was $<6.00 \times 10^6$ cells/mL (Fig. 4a), and the difference in the viable cell density was significant ($p < 0.05$). On day 10, the viable cell density was significantly higher in the combination group than those in the other groups ($p < 0.01$). Cell viability appeared to decline gradually after day 7. On day 10, the cell viability of the combination group was significantly higher ($p < 0.05$) than that of the other two groups (Fig. 4b).

More strikingly, the maximum IVCC of the combination group could reach 60.87×10^6 cells-days/mL, 31.83 % ($p < 0.05$) and 50.76 % ($p < 0.05$) higher than that of groups supplemented with DS and r-trypsin, respectively, and the culture time was prolonged to 13 days (Fig. 4c).

Antibody productivity was measured in CHO cells expressing CMAB-802 antibody in the condition of 1.2 g/L DS and 8.0 mg/L r-trypsin treatment as well as the control. The antibody concentration was 497 ± 15 mg/L on day 7 in the DS and r-trypsin treated group, and the difference in antibody productivity was not statistically significant ($p > 0.05$) compared with that of the control, which was 473 ± 12 mg/L. Meanwhile, the supernatant proteins were analyzed by non-reducing SDS-PAGE (Fig. 4d). The antibody of interest are localized above the band of 170 kDa protein, and distinct bands of other proteins are localized below the band of the antibody. The difference in profile of proteins on the non-reducing SDS page gel was not significant between the combination group and the control.

Conclusion

Dextran sulfate and r-trypsin were used to antagonize aggregation of CHO cells in shake flasks. Both dextran sulfate and r-trypsin could minimize cell aggregation when they were employed separately, however evident clumps were still observed. A regimen combining 1.2 g/L DS and 8.0 mg/L r-trypsin completely solved the problem of cell aggregation in laboratory scale culture of Chinese hamster ovary cells in shake flasks. In addition, the antibody integrity was maintained in

the combination group compared with that of the control.

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