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Trehalose suppresses antibody aggregation during the culture of Chinese hamster ovary cells

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The aggregation of therapeutic antibodies during the manufacturing process is problematic because of the potential risks posed by the aggregates, such as an unexpected immune response. One of the hallmark effects of trehalose, a disaccharide consisting of two alpha-glucose units, is as a chemical chaperone with anti-aggregation activity. In this study, Chinese hamster ovary (CHO) cell line producing a diabody-type bispecific antibody were cultured in medium containing trehalose and the aggregation of the secreted proteins during the culture process was analyzed. An analysis of the various forms of the antibody (monomeric, dimeric, and large aggregates) showed that trehalose decreased the relative content of large aggregates by two thirds. The aggregation kinetics indicated that trehalose directly inhibited the polymerization and aggregation steps in a nucleation-dependent aggregation mechanism. Moreover, both specific and volumetric antibody production were increased in CHO cells cultured in trehalose-containing medium. Thus, the addition of trehalose to recombinant CHO cell cultures would offer a practical strategy for quality improvement in the production of therapeutic antibodies.

[**Key words:** Chinese hamster ovary cell; Cell culture; Antibody production; Antibody aggregation; Chemical chaperone; Trehalose; Bispecific diabody]

In the manufacture of therapeutic proteins, aggregation is a common problem. The aggregates take on diverse forms with respect to their size, reversibility, solubility, covalent/non-covalent interactions, and native/non-native conformations (1–3). These structural changes are important because they can cause a loss of potency of the intact proteins. Moreover, aggregation and misfolding can induce a new and cryptic epitope presentation, resulting in an undesirable immune response (4,5). Stable biological activities must be ensured in therapeutic proteins. Additionally, harmful immune responses following the administration of aggregated proteins must be avoided. These views highlight the need to control and prevent aggregation in the manufacturing process.

Chinese hamster ovary (CHO) cells are one of the most important industrial mammalian cell lines because of their use in GMP-certified recombinant protein production and in the development of industrial serum-free media as well as their reported production rates of more than 10 g recombinant antibody/L cultured cells (6–10). The aggregation of therapeutic proteins in cell culture has been reported in several studies (1–3). Aggregates are thought to form within the cell interior and following secretion of the recombinant protein into the medium. The over-accumulation of polypeptides in the endoplasmic reticulum leads to misfolding and aggregation, resulting in the induction of the unfolded protein response (UPR). Indeed, cell engineering with UPR-related genes is a useful strategy for preventing aggregation and enhancing the production of recombinant proteins (11–13). Secreted proteins can aggregate in response to physicochemical stresses, such as changes in the pH and osmolality of the medium or in the cultivation temperature and time. It is therefore important to control the culture conditions to suppress aggregation. However, culture conditions are typically optimized for cell growth and protein production rather than to suppress aggregation, which therefore remains problematic.

In contrast, the use of chemical chaperones is an attractive strategy to avoid aggregation. Osmolytes in the form of small organic additives, such as sugars, polyols, and amino acids, serve as chemical chaperones to stabilize proteins and inhibit aggregation (14–16). These properties suggest the use of these compounds to inhibit the formation of protein aggregates in culture media, as previously described in other studies (17,18). However, the mechanisms underlying aggregation and the ability of chemical chaperones to inhibit aggregate formation in cell culture are unclear. The osmolyte trehalose is widely distributed in many organisms,

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including bacteria, fungi, insects, plants, and invertebrates. A hallmark of this non-reducing sugar, in which two glucose units are joined by an α-1,1 linkage, is its chemical chaperone effects, as it protects biomolecules from dehydration, freezing, osmotic shock, oxidation, and heating (19–21). Trehalose is used as a protectant for several commercially available therapeutic antibodies and proteins, such as those marketed under the names Herceptin, Avastin, Lucentis, and Advate (22). Thus far, trehalose has been added only in the formulation process whereas its stabilization and anti-aggregation properties suggest its addition to the culture medium to protect secreted proteins against physicochemical stresses. In addition, trehalose should not be harmful to cell growth because, as noted above, it is a natural osmolyte with widespread occurrence.

In this study, we examined the effects of trehalose on the cultivation of recombinant CHO Top-H cell line (23,24) producing Ex3-scDb-Fc. This humanized immunoglobulin G (IgG)-like diabody-type bispecific antibody retargets lymphokine-activated killer T cells to attack cells expressing the epidermal growth factor receptor (25,26). Bispecific diabodies are promising candidates as next-generation therapeutic antibodies because of their dual functionality. By applying trehalose to the CHO cell culture process, we were able to suppress antibody aggregation and to gain insight into the possible mechanism.

MATERIALS AND METHODS

Cell culture, antibody production, and purification The CHO Top-H cell line producing Ex3-scDb-Fc was cultivated as a suspension culture in serum-free ExCD medium [mixture of ExCell 302 (SAFC Bioscience, St. Louis, MO, USA) and IS CHO-CD (Irvine Scientific, Santa Ana, CA, USA), supplemented with 1 μM methotrexate and 1 mM G418]. The cells were adapted to trehalose-containing ExCD medium in 500-mL polycarbonate Erlenmeyer flasks (Corning Inc., Corning, NY, USA) containing a working volume of 80 mL of the same medium. The flask was incubated at 37°C and 80 rpm in an orbital Climo-shaker ISF1-X (Kuhner, Basel, Switzerland). Trehalose was kindly supplied by Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan). The adapted cells were cultivated at a temperature of 37°C in a 1-L glass bioreactor (Biott, Tokyo, Japan) containing 750 mL of the above-described medium. The agitation speed was 70 rpm, the headspace of the vessel was aerated with air supplied at a flow rate of 100 mL/min, and the pH was maintained at 7.1. The dissolved oxygen (DO) concentration was measured by a DO sensor (InPro 6880, Mettler Toledo, Zurich, Switzerland) and was always kept above 40% of air saturation. Cell concentration was quantitatively determined using the Vi-cell XR cell viability analyzer (Beckman Coulter, Fullerton, CA, USA). Antibody concentrations in the cultivation medium were determined by a sandwich enzyme-linked immunosorbent assay (ELISA) or a chemiluminescent assay based on AlphaLISA technology (PerkinElmer, MA, USA). In the former, goat anti-human IgG-Fc and horseradish-peroxidase-conjugated goat anti-human IgG-Fc antibodies (Bethyl Laboratories, Montgomery, TX, USA) were used as the capture and detection antibodies, respectively. The absorbance change at 405 nm was measured with an Infinite M200 microplate reader (Tecan, Grödig, Austria). In the latter, the concentration of Ex3-scDb-Fc in the culture medium was determined using an EnSpire Alpha plate reader with the human IgG kit (PerkinElmer). Glucose and lactate concentrations were measured with the BioFlow BF-7 biosensor (Oji Scientific Instruments, Hyogo, Japan). The kinetic parameters [specific growth rate (μ), specific antibody production rate (ρ_{Ab}), specific glucose consumption rate (ρ_{Gluc}), specific lactate production rate (ρ_{Lac})] were calculated as previously described (27).

The Ex3-scDb-Fc antibody used in biophysical measurements was purified from the culture supernatant on a HiTrap protein A affinity chromatography with AKTA Prime Plus (GE Healthcare, Buckingham, UK). Eluted fractions, including the antibody, were dialyzed against equilibration buffer [25 mM Tris, 100 mM NaCl, 1 mM EDTA (pH 7.5)] and then analyzed by size-exclusion chromatography (SEC) using a Sephacryl S-300 column with AKTA Prime Plus system (GE Healthcare). The running buffer was the same as that in the dialysis step. The final purity was determined by SDS-PAGE. Purified Ex3-scDb-Fc concentrations were determined with the BCA protein assay kit (Thermo Scientific, Rockford, IL, USA).

Quantitative real-time PCR Total RNA was isolated from 5-day suspension cultures of CHO Top-H cells using the High Pure total RNA isolation kit (Roche, Basel, Switzerland). The cDNA was synthesized using the Prime Script II first-strand cDNA synthesis kit (Takara Bio, Otsu, Japan). Real-time PCR was performed using the Thunderbird SYBR qPCR mix (Toyobo Life Science, Osaka, Japan) and the Step One Plus real-time PCR system (Applied Biosystems, Foster City, CA, USA). The PCR primers were as follows: 5′-AGGAGTACAAGTGCAAGGTCTCCAC-3′ and 5′-ACCTGGTTCTTGGTCAGCTCATCC-3′ for Ex3-scDb-Fc; 5′-ACTCCTACGTGGGTGACGAG-3′ and 5′-AGGTGTGGTGCCAGATCTTC-3′ for CHO *ACTB* (β-actin). The efficiency of reverse transcription was verified by standardization with the housekeeping gene *ACTB* (β-actin), and mRNA levels were quantified from the Ct based on the standard curve method.

Circular dichroism and fluorescence spectrum measurements Far-UV circular dichroism (CD) spectra were measured using a Jasco J-820 spectropolarimeter (Jasco, Tokyo, Japan) with a quartz cell of 1-mm path length. Antibody concentrations were prepared at 0.4 mg/mL in 25 mM Tris, 100 mM NaCl, and 1 mM EDTA (pH 7.5). The temperature of the samples was kept at 20°C by a Peltier temperature controller (PYC-347WI; Jasco). Fluorescence spectra were measured at an excitation wavelength of 445 nm using a Hitachi F-7000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). Each sample contained 0.1 mg antibody/mL and 10 μM thioflavin-T [(ThT) Wako Pure Chemicals (Osaka, Japan)] in the above-described buffer. In aggregation kinetics experiments, concentrations of monomeric Ex3-scDb-Fc were prepared at 0.1 mg/mL in 25 mM sodium citrate, 100 mM NaCl, 1 mM EDTA and 10 μM ThT (pH 4.1) with and without 200 mM trehalose.

RESULTS

Adaptation of recombinant CHO cells to trehalose-containing medium and bioreactor cultivation The anti-aggregation effects of 200 mM trehalose during the culture process were examined in CHO Top-H cells cultured in serum-free medium. However, these cells, which were directly inoculated cells into the medium, were apparently subjected to abrupt hyperosmotic stress and cell growth was thereby prevented. We therefore attempted to adapt the cell line to trehalose-containing medium by increasing the concentration of trehalose stepwise in 50-mM increments. At each concentration, the time at which the specific growth rate and the passage interval became constant was defined as adaptation. Accordingly, for proliferating cell cultures we chose a final trehalose concentration of 150 mM, corresponding to a culture medium osmolality of 480 mOsm/kg compared with 319 mOsm/kg in the medium without trehalose. The adaptation to 150 mM trehalose required about 40 days; this concentration was the maximum concentration that resulted in adaptation.

The time courses of viable cell density and antibody productivity in flask cultures are shown in Fig. 1A and B, respectively. The estimated kinetic parameters of the recombinant CHO cell culture, the specific growth rate (μ; 1/h), and the specific production rate (ρ; pg/cell/day) are listed in Table 1. The properties of cells cultured in 150 mM trehalose included: (i) a decrease in both the specific growth rate and the maximum cell density, (ii) a prolonged life span, and (iii) increased specific and volumetric antibody productivity. These are common characteristics of mammalian cells cultured in a hyperosmotic medium (18,28–30). We then cultured the same cells in a bioreactor and monitored the effects of trehalose on culture performance under controlled conditions. The viable cell density, Ex3-scDb-Fc concentration, and glucose and L-lactate concentrations are shown in Fig. 1C–E, respectively. The estimated kinetic parameters of the culture are summarized in Table 1. Bioreactor operation using a medium containing 150 mM trehalose qualitatively reproduced the properties of the flask cultures; that is, a decrease in the maximum cell density and enhanced antibody production. However, there were quantitative differences in the performance of the two culture systems. Thus, while in the bioreactor, the specific cell growth rate in the presence of 150 mM trehalose was almost identical to that without trehalose (Table 1); the rate of specific antibody production was enhanced to a lesser extent than in the flasks (Table 1). The reasons for the attenuated effects of trehalose on culture performance are unclear but may involve the pH and DO concentration of the controlled bioreactor conditions. Metabolic parameters were also changed by trehalose addition (Fig. 1E and Table 1). Specifically, the rates of glucose consumption and L-lactate production were enhanced, suggesting that CHO Top-H cells require a high energy supply under hyperosmotic conditions and that osmotic stress shifts cell metabolism to the increased production of L-lactate. In both the flask and the reactor cultivations, the presence of 150 mM trehalose greatly