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NOTES

High-Resolution Particle Size Analysis in Biotechnology Process Control

John C. Thomas,*,†,‡ Anton P. J. Middelberg,§ Jean-Francois Hamel,∥ and Mark A. Snoswell⊥

Brookhaven Instruments Corporation, Holtsville, New York, Departments of Chemical Engineering and Biochemistry, University of Adelaide, Adelaide, SA, Australia, and Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts

Many industrially important proteins can now be expressed intracellularly as insoluble protein inclusion bodies. In production, large-scale centrifugation is commonly used to separate and recover the inclusion bodies. Recovery efficiency depends critically on the centrifuge feed rate, which must be optimized to minimize production costs. We have used a disc centrifuge photosedimentometer to make high-resolution measurements of the particle size distribution (PSD) of the supernatant during the production of porcine somatotropin (pST) inclusion bodies. These measurements readily monitor the breakthrough of inclusion bodies into the supernatant and allow the centrifugation operation to be optimized.

Introduction

Particle size is an important parameter in most industrial processes and is central to the optimization of centrifugal separation processes, which are used widely in biotechnological downstream operations. Centrifugation is often the most capital-intensive stage of production and this must be optimized to minimize the production cost. Many techniques are available for the measurement of particle size in the micrometer and submicrometer range (Dahneke, 1983; Barth, 1984; Provder, 1987). Sizing techniques return information that is "weighted" according to the physical principle used to make the measurement; i.e., different techniques measure different types of sizes. This makes it difficult to correlate "size" with the separation process. Sedimentation techniques return a Stokes' diameter, which is a measure of the rate at which particles settle through a viscous fluid and which is precisely the information required in order to optimize a centrifugal separation process.

The Brookhaven Instruments disc centrifuge photosedimentometer (BI-DCP) is a high-resolution particle sizing instrument based on centrifugal sedimentation. The instrument is typically able to measure submicrometer size distributions in less than 30 min and has found extensive application in the coatings industry (Thomas and Fairhurst, 1991; Thomas, 1991). In the following, the operation of the BI-DCP is outlined and its use in optimizing the recovery of protein inclusion bodies is described.

Experimental Procedures

Centrifugal Sedimentation. The BI-DCP determines particle size by monitoring the sedimentation of the particle in a viscous liquid under a centrifugal force in a spinning disc. The line-start method of operation, wherein all the particles are initially at the meniscus of the liquid and sediment radially toward the periphery of the disc, yields superior resolution to the homogeneous-start method, in which the starting condition is with the sample uniformly dispersed throughout the spin liquid (Coll and Searles, 1987). The BI-DCP uses the line-start technique. The time, t, for a spherical particle to travel from its initial position r_i (the meniscus) to a detector at r_d is given by Stokes' law:

$$t = \frac{18\eta \ln (r_d/r_i)}{\Delta\rho\omega^2 d^2} \quad (1)$$

Here η is the viscosity of the suspending liquid, $\Delta\rho$ is the density difference between the particle and suspending liquid, ω is the angular velocity of the spinning disc, and d is the particle diameter. For nonspherical particles, d is an equivalent Stokes' diameter, and since $t \propto 1/d^2$, excellent resolution is achieved as fractionation of the material occurs before it reaches the detector.

The detector system of the BI-DCP consists of a tungsten–halogen lamp and a PIN photodiode located behind narrow slits on opposite sides of the transparent spinning disc. As particles sediment and pass through the light beam, the intensity reaching the photodiode decreases due to scattering and absorption of the light. This extinction of the transmitted light is recorded by the data system as a function of time and is the basic signal measured. Thus the fundamental output of the BI-DCP is a curve of light extinction or turbidity versus time. The particle size distribution (PSD) is derived from these data by using Stokes' law above and by applying optical extinction corrections to convert turbidity to particle concentration. These corrections depend on the particle size, optical properties of the particle and suspending medium, and the spectral response of the instrument (Oppenheimer, 1983; Thomas, 1991; Tscharnuter et al., 1991).

† Brookhaven Instruments Corp.
‡ Current address: School of Applied Physics, University of South Australia, The Levels, SA 5095, Australia.
§ Department of Chemical Engineering, University of Adelaide.
∥ MIT.
⊥ Department of Biochemistry, University of Adelaide.

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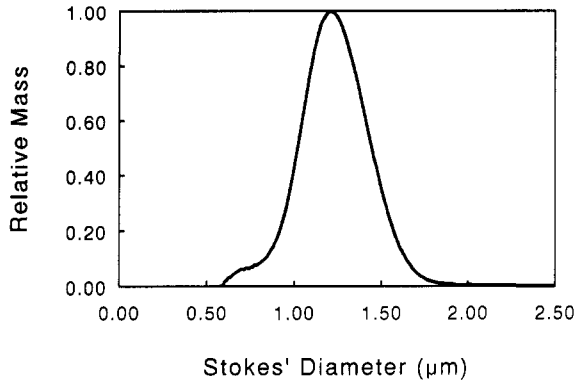

Figure 1. Particle size distribution by mass of intact *E. coli* cells determined by centrifugal sedimentation with the BI-DCP.

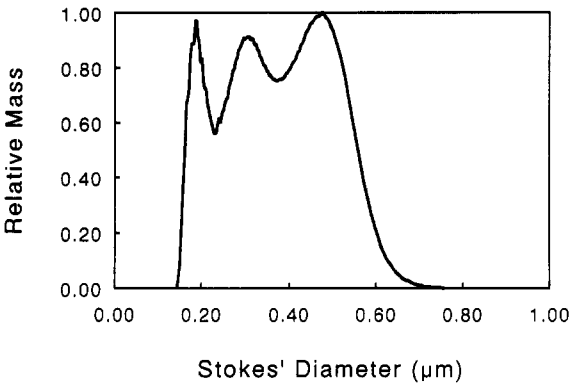

Figure 2. Particle size distribution by mass of the homogenate determined by centrifugal sedimentation with the BI-DCP.

Monitoring Inclusion Body Centrifugation. Proteins are typically much too small and their density is too low to take advantage of the array of techniques available for characterizing and separating colloidal particles. However, many proteins can now be expressed intracellularly as insoluble protein inclusion bodies (Shoner et al., 1985; Hoare and Dunnill, 1986a,b; Marston, 1986; Kane and Hartley, 1988). An advantage of expressing them in this form is that, with typical sizes from a micrometer or so down to a few hundred nanometers and densities in the region 1200–1400 kg m^{-3}, inclusion bodies have dimensions similar to those of colloids (Schoner et al., 1985; Taylor et al., 1986) and one can apply a number of popular colloidal techniques to study them. The electrical sensing zone technique and centrifugal sedimentation, which are routinely used in the characterization of colloids, have both been used to measure the PSD of purified inclusion bodies (Taylor et al., 1986; Middelberg et al., 1990). Note, however, that the electrical sensing zone technique is really only applicable to purified inclusion bodies since any cellular debris present will foul the orifice. Thus this technique is not suitable for particle size monitoring during intermediate production steps. Centrifugal sedimentation does not suffer adversely from the presence of cellular debris and it is ideally suited for monitoring the inclusion body recovery process. Recombinant porcine somatotropin (pST) has been expressed in *Escherichia coli* as an inclusion body (Vize and Wells, 1987). This protein, when administered to pigs in active form, is able to increase their growth rate by up to 25% with a concomitant increase in animal feed efficiency. In addition, the carcass fat of the pig is greatly reduced (Hohmann, 1986). There is currently a great deal of interest in producing pST in commercial quantities.

E. coli cells containing pST inclusion bodies were fermented at the MIT bioprocess separations laboratory. The inclusion bodies were released by homogenization at 1 kbar with an APV-Gaulin high-pressure homogenizer. Fractionation of the inclusion bodies and the cellular debris was achieved by centrifugation with an Alfa-Laval BTPX-205 continuous discharge disc stack centrifuge. A range of feed rates was tested, and the supernatant from each feed rate was analyzed with the BI-DCP to determine the effect of feed rate on the efficiency of the separation process.

The supernatant contains a mixture of cellular debris and pST inclusion bodies. The cellular debris is heterogeneous in size, density, and optical properties (refractive index). The inclusion bodies have a better defined size and optical properties, but these are quite different from those of the cellular debris. In this situation the questions of which density and optical corrections should be used to extract the PSD from the raw BI-DCP data are not easily answered. For convenience we have used the density of the inclusion bodies, ρ = 1260 kg m^{-3}, and the optical extinction corrections for polystyrene latex spheres. The justification for this is that this has been done previously (Taylor et al., 1986; Middelberg et al., 1990), although it seems improbable that the brownish-gray inclusion bodies would have similar optical properties to white polystyrene latex spheres. For measurements on whole cells we used a density of 1050 kg m^{-3} and the same optical corrections. Note that even though the PSDs obtained are unlikely to be the actual mass distributions, we are primarily interested in comparative results, the conclusions of which are unaffected by the choice of extinction correction, density, etc.

The samples were run in a spin fluid of 20 mL of water with a buffer fluid of 1.5 mL of 20% (v/v) ethanol in water by the external gradient method (Holsworth et al., 1987). The samples were suspended in 0.5 mL of 20% ethanol and injected onto the meniscus. The measurements were made at 6000 rpm and ambient temperature (23 °C), and run times were typically 20–25 min.

Results

Figure 1 shows the PSD obtained for the intact *E. coli* cells from the fermenter prior to being fed to the homogenizer. The PSD is unimodal, extends from ~0.6 to ~1.8 μm, and is centered around 1.2 μm. There is no material in the size range below ~0.6 μm.

Figure 2 shows the PSD obtained after one pass through the homogenizer. The whole PSD is shifted to smaller sizes and three peaks are apparent. The two peaks at smaller sizes are attributed to cellular debris and the one at 0.4–0.5 μm is due to the inclusion bodies. The cellular debris probably extends down to very small sizes and does not cut off sharply at ~150 nm. The cutoff is due to termination of the measurement and positioning of the baseline. After one pass through the homogenizer, the homogenate was fed to the disc stack centrifuge.

In Figure 3 we show the PSD of the supernatant from the disc stack centrifuge obtained for various feed rates. Curve (a) is for a feed rate of 100 kg h^{-1}. There is only a single peak at around 0.16 μm and no significant amount of material in the 0.4–0.5-μm range, where the inclusion bodies are expected to appear. Thus the supernatant contains substantially cellular debris and no inclusion bodies are present. Curve (b) is the PSD of the supernatant obtained for a feed rate of 180 kg h^{-1}. There are two distinct components present. The major peak occurs at precisely the same size as the peak in curve (a) and corresponds to the cellular debris. The minor peak occurs near 0.4–0.5

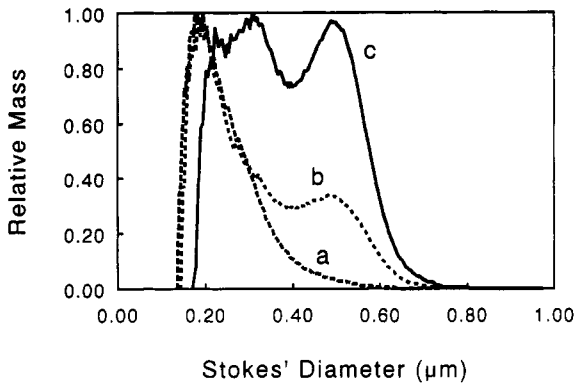

Figure 3. Effect of stack centrifuge feed rate on the particle size distribution of the supernatant. (a) Feed rate 100 kg h^{-1}; (b) feed rate 180 kg h^{-1}; (c) feed rate 240 kg h^{-1}.

μm and is due to the presence of pST inclusion bodies in the supernatant.

Finally, curve (c) shows the PSD that results with a feed rate of 240 kg h^{-1}. The large peak near 0.5 μm is evidence of a large breakthrough of inclusion bodies into the supernatant. Furthermore, this PSD is similar to that of the unfractionated homogenate shown in Figure 2. There are peaks at ~0.2, 0.31, and 0.5 μm, just as in Figure 3; i.e., the same three components are present, albeit in slightly different proportions. Thus, at a feed rate of 240 kg h^{-1}, the inclusion bodies and the cellular debris are not being separated substantially.

So, at a feed rate of 100 kg h^{-1}, no inclusion bodies break through into the supernatant. At 180 kg h^{-1}, inclusion bodies are evident in the supernatant, and at 240 kg h^{-1}, no significant recovery of the inclusion bodies occurs. In order to obtain a reasonable fractionation, some loss of inclusion bodies to the supernatant is unavoidable (Middelberg et al., 1989). From the data presented here, it is apparent that the optimum feed rate is in the vicinity of 180 kg h^{-1} for the present process.

Discussion

By examining a practical bioseparation process, the isolation of pST inclusion bodies, we have shown the importance and utility of having available particle sizing data of the material both before and after the centrifugal separation process. To optimize the recovery process, particle sizing information is paramount. Furthermore, the technique used to measure the particle size must be based on the same physical principle as the separation process, i.e., in this case sedimentation.

In the case of protein inclusion bodies, which are relatively large and dense, a large range of physical techniques may be applied to their characterization. However, disc centrifuge instruments, such as the BI-DCP, are ideally suited to this application since they provide high-resolution PSD information on a time scale of half an hour and they are based on sedimentation.

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