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Effect of Shear on Human Insulin in Zinc Suspension

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Abstract

Human insulin in zinc suspension was used as a model protein to test the effect of shear on the settling rate of proteins, a possible inference for protein denaturation. The rate of settling was determined directly in a spectrophotometer. Shear effects are important in retaining the activity of proteins and are present in bubble, foam, and droplet protein fractionation processes. A simple test, such as that conducted here, may even be useful for monitoring changes in protein structure caused by commercial shipping of the protein. The settling rate for insulin was continuously monitored in the original bottle by spectrophotometric absorbance changes as a function of time. A settling curve was determined following each shear experiment, which included shaking the "worked" insulin solution in a vortex mixer for different lengths of time. It was determined, when comparing long shaking times with short ones, that the initial settling rate was less for the long-term shaking of the insulin samples and greater for the short-term shaking. The secondary effects of light and heat, along with shaking, apparently did not produce differences from shaking alone.

Index Entries: Insulin; human insulin; settling rate; shear; shear stress; shear stress on proteins.

Introduction

The role of hydrodynamic shear on the activity and structure of proteins is a topic of much interest because proteins are important products used in industry *(1,2)*. Recently, Elias and Joshi *(3)* summarized some of these applications and why shear is important:

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During production and recovery, proteins are subjected to fluid forces which arise due to operations such as stirring, pumping and centrifugation. The resulting hydrodynamic shear forces may cause damage to the large molecular weight proteins, resulting in denaturation and inactivation of the protein. This is a major concern as it affects the overall efficiency of protein recovery and final yield of the product. A considerable amount of research has been devoted to studying the effects of hydrodynamic effects of proteins, especially to the enzymes.

The effect of shear on proteins is also an important part of the separation of proteins through foam fractionation or droplet fractionation *(4,5)*.

Our experiment was performed by shaking a 10-mL commercial bottle of human insulin in a vortex mixer and measuring the absorbance vs time with a spectrophotometer whose well was modified to hold the bottle, as if it were a test tube. Human insulin used was a genetically engineered form of Humulin L© of rDNA origin in zinc suspension, produced by Eli Lilly, Indianapolis, IN. Genetically engineered insulin has a shelf life of >7 mo (according to the stated expiration date), but comes with specific instructions to determine whether it is good for human injection even before the expiration date. The check step for acceptable product is to observe whether there is precipitate at the bottom of the bottle that does not go into suspension with the solution after gentle shaking or rotating. It is this check step that inspired the development of the settling rate of insulin as a means for determining possible denaturation. The settling rate was presumed to be a simple measure to characterize, at least one aspect, of changes in the three-dimensional (3D) structure of insulin. The question of what role the settling rate plays with the product's medical effectiveness is an open question. Insulin is a globular protein with discrete secondary structures and forms self-aggregates, such as dimers, tetramers, and hexamers *(6–8)*. Denaturation of insulin does not necessarily break a sulfur bond, but it may alter its 3D structure, and thus its globular and self-aggregating properties, resulting in an altered settling mechanism. It is also unknown how this settling rate correlates with other measures of the protein's 3D structure, as determined by circular dichroism. Because an abnormal settling rate may indicate less effective human insulin, a valid test of the shear effect on human insulin may possibly turn out to be a predetermined change in the settling curve after the product is shaken for different time intervals. Further studies are necessary to quantify the extent of protein structural change and its resulting correlation with the measured settling rate of that protein. Here, the preliminary question to be answered is qualitative: Can the extent of shear alter the settling rate of the insulin protein?

Measuring the same sample for several different shaking times requires prolonged exposure to both heat and light from the spectrophotometer. Also, using different bottles presents significant problems when comparing results. Therefore, one sample (Sample A) was used for all the settling/denaturation measurements, and another (Sample B) was used to measure the effect of light and heat on the human insulin, to mini-

mize these experimental errors. Particle size determinations were also performed on the samples to determine whether the effect of shear on the insulin aggregate size itself could be observed. In this article, we report on the shear effect on human insulin with the emphasis on one variable: the time of shaking.

Materials and Methods

At all times the insulin solution was kept in the same airtight, impermeable, sanitary 10-mL glass bottle that it was packaged in from Eli Lilly. The tests were performed during May and June of 1998 in bottles with control no. 1MR35M and expiration dates of February 1, 1999. When not being subjected to shear or photometric monitoring of the sedimentation rate, the samples were stored at their recommended storage temperature of 5°C. All shaking was done in an S/P Vortex Mixer (cat. no. S8223-1; American Scientific Products, McGaw Park, IL) set at a power of eight. All absorbance readings were performed in a Bausch & Lomb Spectronic 20 spectrophotometer (Bausch & Lomb, Rochester, NY), whose well was specially fitted for the diameter and depth of the bottle. The particle size was measured in a Coulter Particle Analyzer (Delsa 440SX Zeta potential sizer analyzer; Coulter, Miami, FL).

Before any measurements or shaking was performed on the human insulin sample in the bottle, it was removed from the refrigerator and allowed to warm to room temperature (about 22°C) for a minimum of 30 min. The bottle was initially prepared by removing the label so that the bottle could transmit light in the spectrophotometer, much like a cuvette. The outside of the bottle was cleaned with alcohol and methanol to remove any contaminants present, especially the residue from the label. Shaking was done in the vortex mixer for intervals of 5 min each, while holding only the top of the bottle. The vortex mixer was designed for test tubes and vials and had 10 speed settings. Another sample was shaken by a no. 2866 Tyler portable sieve shaker (W.S. Tyler, Cleveland, OH) designed to produce wider, less vigorous shaking on the sample. This shaker held the sample within polyurethane mounted on top of a sieve at the bottom of a pendulum and was swung back and forth manually.

To ensure that all samples were evenly mixed before measurements were taken, each sample lay untouched for 5 min and was then gently inverted four times immediately before being placed in the spectrophotometer light pathway. The reference curve for the bottle in the spectrophotometer was calibrated at 610 nm using a bottle filled with deionized water. All bottles were wrapped with masking tape along the top to a diameter equal to that of the spectrophotometer well diameter (25 mm). Once placed in the spectrophotometer, the bottle was not moved for 50 min during which absorbance measurements were read at time intervals ranging from 30 s to 10 min. For each time interval being measured, four absorbance vs time readings were performed. These readings were averaged together to

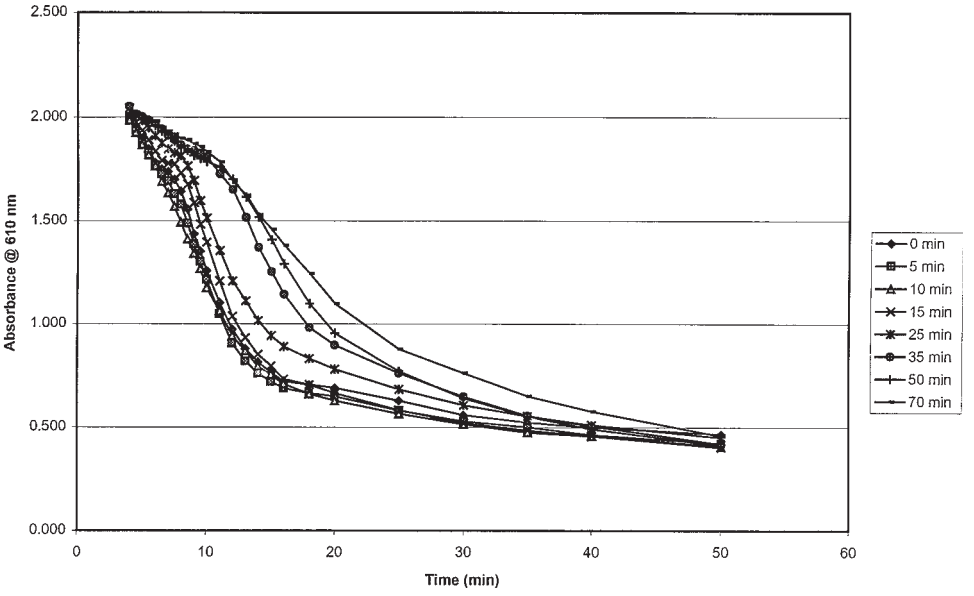

Fig. 1. Settling curve averages for different shaking times in the same bottle shaken by a vortex mixer.

reduce the magnitude of the random deviations that occurred from the true absorbance vs time curve.

Results and Discussion

As Fig. 1 shows, the effects of shaking on the settling rate are easily observable. All curves represent the same sample shaken for different lengths of time. At first, as the amount of shear energy applied to the system was increased, the settling curve appeared to fall slightly, and then as the amount of energy increased it rose. The original reference curve (the unshaken or no shear case) has two distinct regions. First, all of the insulin particles in the solution were falling and the change in absorbance over time was relatively constant. Then, after about 12–15 min, there was a sharp break in the curve as it flattened out. After this break, the curve seemed to slowly decay asymptotically to its lowest settling value. One day after being shaken for 30 min, the insulin samples showed the same settling curves as 30 min after shaking, suggesting that these shifts in the settling curve were permanent.

The settling curves for these samples, which were shaken for just a short time (5–10 min), are very close to the curve of the unshaken/no shear case. Although these moderately shaken samples appeared to be slightly lower than the no shear case, the difference appears to be insignificant and suggests no real change in the settling curve after light to moderate shaking. For the insulin samples shaken for 15 min, there is a noticeable shift outward in the settling curve that suggests some sort of critical shaking time. Once this shake time was extended to 25 min, then 35 min, and then

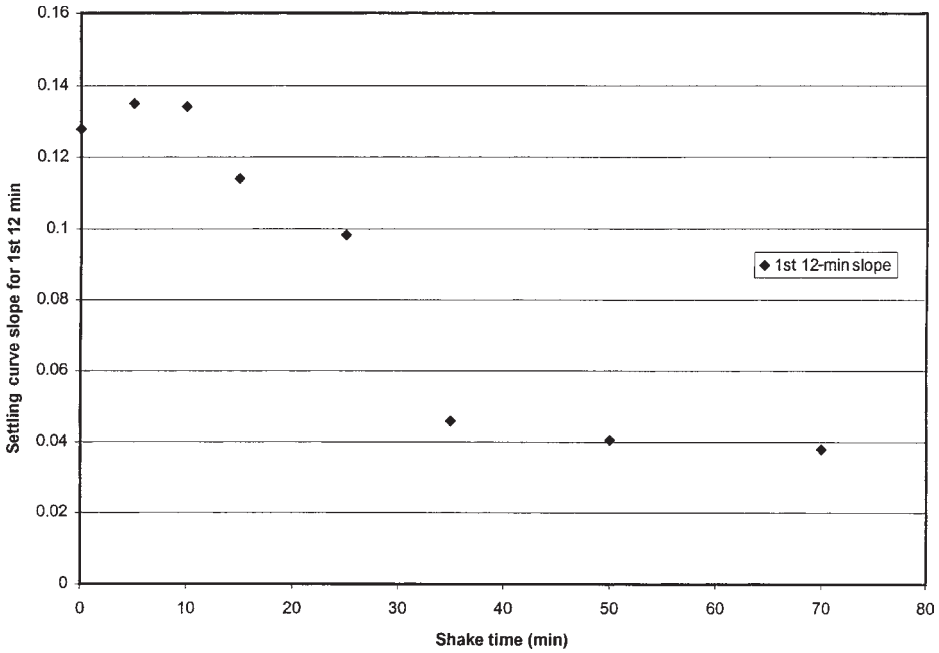

Fig. 2. Change in slope over the first 12 min of the settling curve over the range of different shaking times.

incrementally up to 70 min, the settling curve was observed to shift outward even farther as the shear rate increased.

Another interesting factor to note in how the settling curve shifts is how the two distinct regions originally observed in the unshaken settling curve shift. For samples shaken for 70 min, the characteristic settling curve does not appear to have these two distinct regions. If one looks at all of the curves, though, a pattern in the shifting of the two distinct regions readily becomes apparent. It is as if the breaking point from the original curve is shifting up and outward with the two end points (at time = 0 for the first line and time = ∞ for the second line) of the lines being the same. This results in what appears to be a curve representing samples shaken for a long time much closer to a straight line than the unshaken curve. It is observed, therefore, that these distinct patterns in the settling curve may characterize the amount of work expended on the insulin solution. If it is assumed that the change in protein settling time correlates with the amount of denaturation, these patterns may then determine (at least qualitatively) the extent of denaturation of the insulin. As the shake time increases, the curve shifts from its original steep then flat pattern to a steadier decline in the settling curve. Figure 2 shows how the settling rate over the first 12 min shifts as the time the insulin sample has been shaken increases. The settling rate appears to asymptotically approach a final value of about 0.03.

The structure of the insulin is such that it is tough to break the bonds, but the shear stress may nevertheless denature the molecule. Particle size

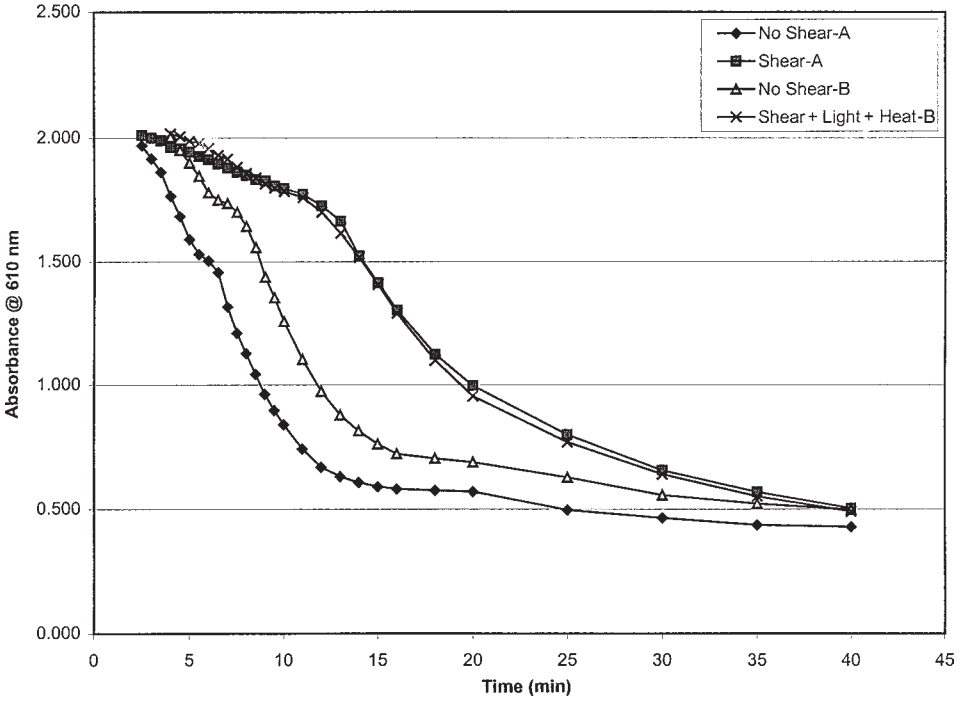

Fig. 3. Comparison of the curve shift of only shear stress applied to the system vs shear stress plus the added effects of heat and light. A represents sample bottle A and B represents sample bottle B, both of which remained unopened.

analysis tests were performed prior to settling as a baseline to determine how much insulin clumps/particle sizes were affected by the applied particle size instrument. Since the particle size analyzer could measure only particles of diameter >50 nm and <300 nm, it appears that the clumped insulin molecule particle size is in a range >300 nm owing to the largeness of the insulin molecule. The altered settling rate suggests an altered 3D structure. The particles in the bottle, when observed by the naked eye, were seen to aggregate to sizes large enough for the naked eye to see before settling in the bottle. An altered 3D structure would prevent the molecule from forming self-aggregates in the same manner as before the shear stress was applied.

Over the course of the experiment, the insulin bottle was subjected to exposure to light and heat for significant periods of time from the spectrophotometer. To obtain all the desired measurements, the sample was exposed to about 4 h of light and heat, reaching temperatures of about 35°C. To rule out the settling curve's shifts being the result of overexposure to heat and light, a second "fresh" sample (Sample B) was used to compare the 70-min shake averages (directly obtained rather than cumulatively as in the previous incremental experiments) and observe any noticeable shift in the curve other than the shifts observed from the original sample. As seen in Fig. 3, the shifts in the sheared sample not exposed to heat and light

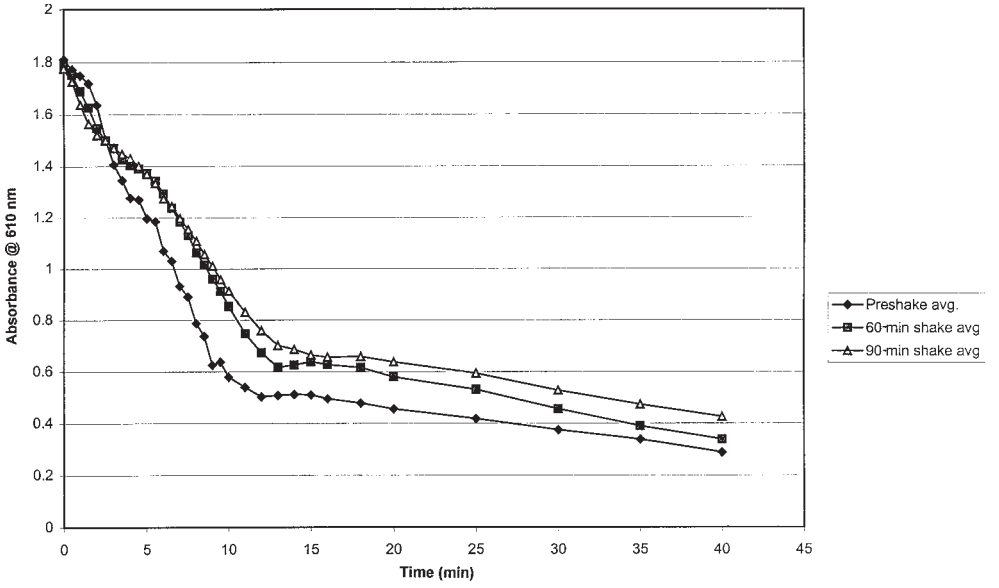

Fig. 4. Comparison of shake averages for the sieve shaker showing a shift in the settling curve.

(Sample A) were very similar to the shifts from the sheared sample (Sample B) cumulatively exposed to heat and light. This suggests that the shifts in the settling curve are primarily related to the shear stress from the vortex mixer and not to the exposure to heat and light encountered in these experiments.

A third sample was measured in a test tube and shaken gently. The shaking involved wider, slower motions in the portable sieve shaker and was done for 60 min and then an additional 30 min, for a total of 90 min. The results of these experiments (*see* Fig. 4) showed shifts in the curve similar to those observed for the vortex mixer in Fig. 3, but not nearly as pronounced. The shear rate relative to that of the vortex mixer was not determined but, based on the less intensive mixing, was presumed to be lower than the vortex mixer's shear rate. The gentler shaking did not "denature" the insulin to the extent of the vortex mixer, suggesting that the strong agitation of the vortex mixer was also a strong contributing factor to the presumed denaturation.

Although it is not clear whether the insulin's covalent bonds were broken during shear, it is clear that the shear stress of a vortex mixer has a significant effect on the insulin sample's settling rate, suggesting at least an alteration of its structure and/or agglomeration of insulin molecules caused by shear stress. The applied energy was similar to what a bottle of insulin might undergo when being shipped via truck or train and shows that excessive shaking causes the insulin to settle differently. It is not known how the medical effectiveness of the molecules is affected, but the settling rate is greatly affected, suggesting that measures may need to be taken to keep the molecules from grouping together before settling in the solution.

The analysis presented here could be used as the basis for a simple quality control measure for the "activity" of insulin both at the manufacturing plant and by the druggist or consumer prior to usage. Corroborated with another method, such as circular dichroism, the settling results could perhaps provide a low-cost method of determining structural changes in the insulin owing to the effect of shear.

Conclusion

The settling time profiles for the late shaking times indicated that changes either in the insulin molecule or in its coagulation are affected by the shear. It remains to be determined what the relationship is (if any) between shear and the extent of the denaturation of the insulin samples. The globular and self-aggregation properties were apparently altered as a result of the shear stress placed on the system, as observed by differences in the settling rate. On the other hand, use of the profiles describing the early time trajectories (little work had been expended on the insulin solution), in conjunction with circular dichroism, light scattering, or other physical measurements, may provide a simple and inexpensive means to validate complete or near complete retention of the insulin activity.

Acknowledgment

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