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Rapid communication

A simple and reproducible approach to characterize protein stability using rheology

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ABSTRACT

As the utility of proteins in medical diagnosis and therapy becomes more fully realized, interest in characterizing proteins' stability continues to increase. This paper reports the merits of rheology as an approach to characterizing the stability of a model protein, immunoglobulin (IgG). A controlled-strain rotational rheometer equipped with parallel plates geometry was used to measure changes in the viscosity of an IgG buffer solution during a programmed temperature ramp. The results demonstrated that protein stability characterization is reproducible using the melting temperature (or unfolding temperature) of IgG ($T_m = 73.6 \pm 0.4\,^{\circ}C$). We have also identified an irreversible minor transition occurring at a temperature of 40–60 °C, which we believe to be the partial unfolding of some protein structures. Finally, we have determined that adding sugar, an established protein stabilizer, to the IgG buffer solution significantly increases the protein's melting temperature. Our results show the power and simplicity of rheology as analytical tool for characterizing protein stability through the measurement of viscosity changes during thermal denaturation.

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1. Introduction

Proteins are macromolecules composed of amino acids strung together in a definite sequence. They are generally folded into three-dimensional structures, which hydrogen bonds and other interactions hold stably, and which give proteins their functional ability. For example, immunoglobulins are proteins that have a molecular weight of ~150 kDa and a distinct Y-shaped, three-dimensional structure. The structure and functionality of immunoglobulin allow it to bind specifically to one or a few closely related antigens. This ability has led to increased therapeutic use of protein macromolecules. Also, because of their specific affinity for many ligands, proteins are used as probes for detecting molecules.

The structural stability of a protein formulation used diagnostically or therapeutically is critical: in such applications, molecules must be able to retain their folded structure. This has generated interest in characterizing the stability of protein macromolecules in solution, and understanding the effect of excipients in stabilizing their folded tertiary structure. The critical parameter of these explorations is the ability to measure quantitatively the stability of a protein in a simple, reproducible manner. Melting temperature (T_m) usually is used to quantify protein stability, and recent publications have outlined various techniques for accomplishing this, including: differential scanning calorimetry (DSC) (Baier, 2001; Badkar et al., 2006; Tang et al., 2007), Fourier transform infrared spectroscopy (FTIR) (Cauchy et al., 2002; Maury et al., 2005; Matheus et al., 2006a) and circular dichroism spectroscopy (Vermeer et al., 1998). However, many such approaches have their limitations in concentration regimes. For example, DSC typically needs 0.4–5 mg/ml of protein, while FTIR is reported to be unreliable when analyzing dilute solutions (Matheus et al., 2006a).

Rheology has been proved to be a powerful tool and been used widely in food and material science to detect viscoelastic changes in solutions containing macromolecules. One important parameter rheology provides is viscosity, which measures the resistance to flow. Flow viscosity generally changes progressively with temperature if there is no phase transition involved. However, when a transition such as protein unfolding occurs, viscosity usually changes abruptly mainly because of changes in the interactions between protein molecules. Even in extremely dilute solutions, this change can be more than one order of amplitude. The broadness of this viscosity change could offer an advantage in clearly monitoring the denaturation temperature of protein solutions.

In this paper, we employed a model protein – bovine serum immunoglobulin (IgG) – to explore the effectiveness of rheology in characterizing the stability of protein macromolecules. Also, we characterized the ability of trehalose to modulate the stability of IgG in solution. Our results establish rheology as a simple, reproducible tool to characterize protein stability, and a complement to other techniques.

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2. Materials and methods

We purchased bovine serum immunoglobulin (IgG) (product #: I9640, lot 016K7540, technical grade) and D(+)-trehalose dihydrate (product # 90210, CAS # 6138-23-4, lot 1250745, molecular weight: 378.33 g/ml) from Sigma–Aldrich, Inc. The IgG was supplied and used as a 10.4 mg/ml solution in a 0.01 M phosphate buffered saline (pH 7.2), containing 15 mM sodium azide as preservative. The purity measured by SDS electrophoresis was 93% (data from vender). Small (1 ml) samples of the solution were pipetted into plastic vials, and then stored in a −20 °C freezer. Nearly 2 h before rheology measurements were performed, the vials were removed and thawed at room temperature. To evaluate the effect of the addition of trehalose, the necessary amounts of trehalose were weighed and transferred into the sample vials, where they were allowed to dissolve completely for approximately 1 h before analysis.

The concentration of the trehalose (c, mmol/ml) in protein solution was calculated by dividing the weight of trehalose ($w_{\text{Trehalose}}$, mg) added in solution with the molecular weight of trehalose (M_{w}, 378.33 g/ml) and the total volume (ml) of the final solution

$$\text{Trehalose concentration } c\,(\text{mmol/ml}) = \frac{w_{\text{Trehalose}}(\text{mg})/M_{\text{w}}}{\text{Total volume (ml)}}$$

where the total volume of the solution after trehalose addition was determined by dividing the total weight of the solution with the protein solution density, which was measured by the following method.

First, the solution was thawed at room temperature for over 2 h. Then 250 μl of solution was measured using a calibrated pipette, and dispensed into a pre-dried and weighed vial. The combined weight of the solution and vial then was measured with an analytical balance (Mettler Toledo XP205, precision: ±0.01 mg). Triplicate measurements were performed for each sample. The densities (d) of the samples at room temperature were calculated by dividing their weight with their volume (d = weight (g)/volume (ml)). The room temperature during testing was recorded with a calibrated thermometer.

The measured density and trehalose concentration are summarized in Table 1. All the measured densities are repeatable with relative standard deviations (%RSD) of less than 0.5%.

Viscosity was measured using a controlled-strain rotational rheometer (ARES, TA Instruments, Inc.) equipped with parallel plates fixtures and a Peltier temperature controller. About 0.5 ml of protein solution was sandwiched between two parallel plates. To prevent water evaporation at elevated temperatures, a layer of low viscosity silicone oil (η = 50 cP, Brookfield Engineering Laboratory, Inc.) was applied around the solution to seal it. Since this silicone oil is not reactive under experimental conditions, its viscosity would not change transitionally with temperature during measurement. So while the addition of a sealant might introduce error to observed viscosity values (by increasing them), it would not affect the accuracy of transition temperature measurements. The sealed fixture then was shielded with a plastic cover to mitigate solvent evaporation, and to ensure uniform temperature distribution within the heating cell.

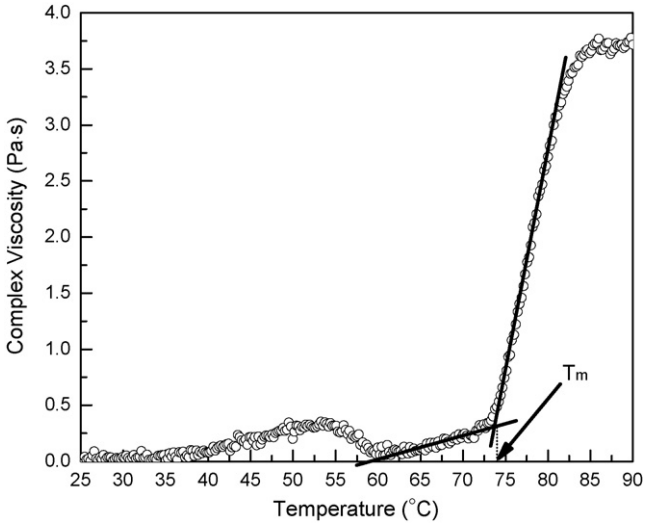

Fig. 1. Viscosity of 1 wt% immunoglobulin solution in phosphate buffer as a function of temperature when heated from 25 °C to 90 °C at 1 °C/min. Linear regression was used before and after the major thermal transition to calculate the melting temperature.

A dynamic temperature sweep test was used to measure the complex viscosity at 10% strain and 1 rad/s oscillation frequency, which was within the viscoelastic region of the solution as proved by dynamic strain sweep tests. Protein solution samples were heated from 25 °C to 90 °C at a rate of 1 °C/min and then cooled to 25 °C at same rate to determine if the transitions observed were reversible. The temperature was calibrated and no thermal lag was observed in the measuring range.

3. Results and discussion

Fig. 1 shows a typical viscosity profile when a 1 wt% IgG protein solution is heated from room temperature to 90 °C at 1 °C/min. Up to a temperature of approximately 40 °C, the solution is water-like, with a viscosity below 0.1 Pa s. During this phase of heating, the properties of the solution do not appear to change; it is believed that the protein retains its native, folded structure. However, when the temperature of the solution reaches about 40–60 °C, a small bump appears in the figure, indicating a definite change in the protein structure. Heating the solution above 70 °C leads to a second thermal transition, one that increases viscosity by more than an order of magnitude. This region is identified as the irreversible denaturation of the IgG in solution.

By fitting a linear background to the slope of the curve before and after this thermal transition, we obtained a numerical measure of the melting temperature (T_{m}) characteristic of the denaturation of the IgG protein (Fig. 1). Using eight different samples, we observed the melting temperature for IgG in solution (10 mg/ml) to be 73.6 ± 0.4 °C. This agrees with the T_{m} values for IgG that have been reported in literature (Vermeer et al., 1998; Matheus et al., 2006a). Viscosity continues to increase beyond 74 °C, saturating around 85 °C before diminishing slightly at greater temperatures.

Table 1
Summary of the density, trehalose concentration, and unfolding temperatures of the protein solutions.

Sample ID	Trehalose added (mg) (based on 1 ml protein solution)	Density at 21 °C (g/ml)	Trehalose concentration (mmol/ml)	Unfolding temperature (°C)
IgG-0	0	1.00	0	73.6 ± 0.4 (SD)
IgG-50	50	1.01	0.13	76.0 ± 0.4
IgG-200	200	1.06	0.47	78.7 ± 0.3
IgG-500	500	1.12	0.99	82.9 ± 0.2

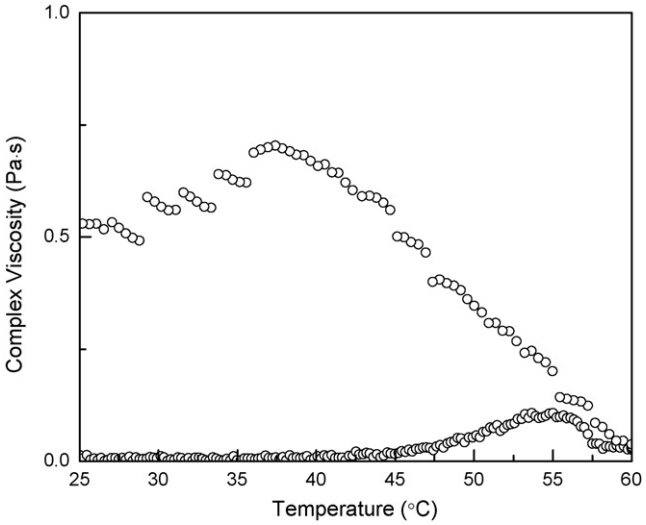

Fig. 2. Illustration of the irreversibility of the minor thermal transition around 40–60 °C by heating to 60 °C and then cooling back to room temperature. The top curve shows that viscosity continues to increase throughout cooling, and indicates that the transition at 40–60 °C is irreversible.

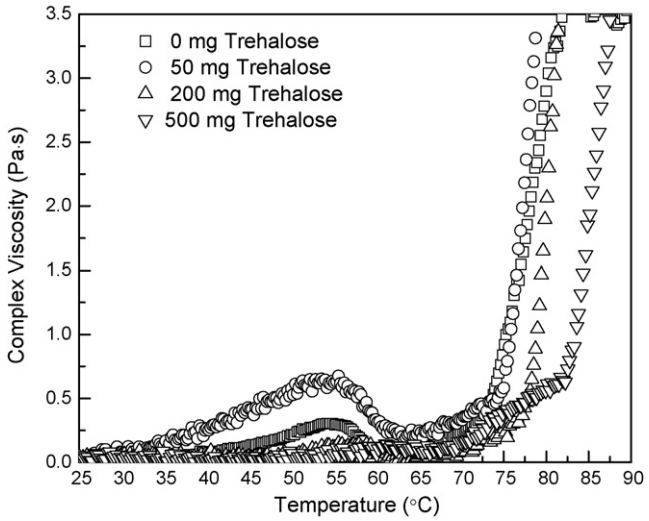

Fig. 3. Effect of trehalose on the stability of the IgG solution. The protein solution shows increased stability as evidenced by the significant increase in unfolding temperatures.

Upon cooling, the viscosity of the solution increases and never recovers (data not shown), confirming the protein's irreversible denaturation. After unloading the up fixture, a milky colored suspension can be observed on the plate, further demonstrating that irreversible protein denaturation occurred during the heating.

When the protein macromolecules in solution are heated, the weak bonds between amino acids holding the tertiary folded structure are the first to break. As this structure deforms, the internal functional groups of the macromolecule become exposed to the buffer solution and begin to interact with water or ions in it, forming new hydrogen bonds in the peptide backbone of the protein. Also as a result of heating, individual proteins might interact with one another to form aggregated microstructures. This unfolding and the subsequent protein–protein interactions affect the solution's viscosity and viscoelasticity, thereby changing dramatically the protein's rheological properties around melting temperature. For this IgG solution, viscosity increased more than one order of magnitude after structure unfolding, making the measurement sensitivity much better than that of any other method reported in literature.

Fig. 2 shows the result of exploring the irreversibility of the minor peak in viscosity around 40–60 °C. The IgG solution was heated to 60 °C at a rate of 1 °C/min., and then allowed to cool at 2 °C/min. The data reflect clearly that the solution could not return to its original viscosity, indicating that this minor transition indeed is irreversible. While we attribute this change to a partial unfolding of the IgG structure, it remains unknown to us whether it results in any loss of the protein's functionality. Future experiments might aim to ascertain the nature of the tertiary structural changes brought about by this transformation.

To explore the stability of IgG proteins, we characterized the effect of trehalose addition, which is known to stabilize proteins (Liao et al., 2002; Maury et al., 2005; Matheus et al., 2006b). Fig. 3 shows the viscosity of the IgG solution as a function of temperature. With the addition of 50 mg of trehalose to a 1 ml IgG solution (0.13 mmol/g) comes a measurable increase in the T_m value of the solution to 76 ± 0.4 °C. Adding more sugar to the IgG buffer solution raises T_m further, until at 500 mg/ml trehalose (0.99 mmol/ml), a significant 9 °C increase could be achieved.

Fig. 4 plots the measured T_m of the IgG solution as a function of the trehalose addition's concentration, demonstrating the sugar's

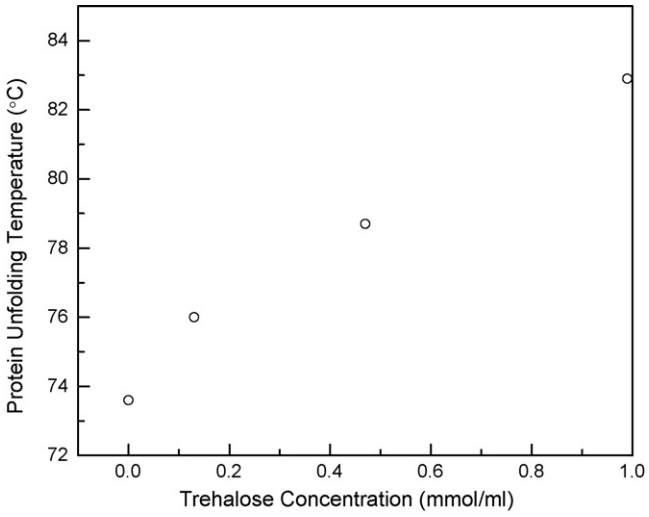

Fig. 4. Correlation of the protein unfolding temperatures to the trehalose concentration.

dramatic influence on protein structure stabilization as measured by the increase in T_m. With the exception of the first data point, which represents no trehalose addition, the figure illustrates good linearity.

It is notable that although the presented setup already is much more sensitive than other analytical methods documented in literature, the parallel plates geometry described herein was not optimized for measuring such low viscosity materials. Concentric cylinders geometry (also known as Couette viscometry) would increase sensitivity significantly, thereby improving the gathering of quantitative information, especially that related to the protein's minor transition at 40–60 °C.

4. Conclusion

Thermal stability of an immunoglobulin buffer solution has been investigated by a method using controlled-strain rotational rheometry. We found rheology to be a simple and reproducible method to characterize the stability of proteins by monitoring viscosity

changes as a function of increases in temperature. We further observed that trehalose, when added to stabilize IgG in solution, did influence protein stability significantly. The addition of about 500 mg/ml (0.99 mmol/ml) of trehalose increased the melting temperature of IgG by 9 °C. Another important finding of this rheological method was the detection of a minor, irreversible transition (protein unfolding) at around 40–60 °C.

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