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CHEMISTRY

Determining the Second Virial Coefficient (B_{22}) by Self-Interaction Chromatography (SIC)

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The purpose of this experiment was to validate and utilize a novel method of determining the second virial coefficient, B_{22}, to measure the effect of various solution additives and precipitants on protein interaction. The B_{22} value is a parameter that measures the total intermolecular attraction of proteins in a given solution. Based on the value of this dilute solution property and the concept of the crystallization slot, researchers can determine whether a protein will crystallize, remain stable, or precipitate in a given solution. The B_{22} value can revolutionize two area of research—protein crystallography and protein stabilization for pharmaceutical formulations—by eliminating the guess work and current shotgun approach employed in these processes and by allowing for a quantitative approach to be used. The B_{22} value can rapidly increase the rate in which protein structures and drugs targets are identified, saving millions of dollars annually. Despite the advantages, the B_{22} value has never been utilized because determining the value is tedious and time consuming.

In this project, self-interaction chromatography (SIC) is used to obtain the B_{22} value. In SIC, a small diameter column is packed with beads to which the experimental protein is bound. The solution being tested flows through the column, and a sample of protein is injected onto the column. The retention time of the injected protein sample is directly related to the B_{22} value. The results of this experiment indicate that SIC can be used to quickly determine B_{22} value, and can aid in elucidating the effect of various additives on protein self-association.

INTRODUCTION

Most structural biologists, biochemists, and pharmaceutical companies currently attempt to develop drugs through a process called structure-based drug design. Structure based drug design takes on a systematic approach to rationally designing drugs. The basis of this process is that protein structure can be determined using diffraction pattern data collected from shooting an x-ray beam at a crystal. The angles and intensity of the diffracted x-rays are then used to determine the exact position in an x,y,z coordinate system of all the atoms within the molecule. Determining the structure is crucial because it provides key information about a protein's function and how it may possibly interact on the molecular level with other substances (McPherson, 2004). This knowledge is invaluable because it enables a rational and direct approach to developing drugs.

The bottleneck of structure based drug design is the crystallization step. The crystallization of a protein is the most difficult step in the process due to the many factors that influence crystallization. Currently, crystallization conditions for a particular protein are found by screening a wide range of conditions then fine screening hits to optimize crystal results. Even with the aid of robotics this process can take much time and possible crystallization conditions can go undetected (Henry, et al., 2003).

The second virial coefficient value, B_{22} , is a property of dilute solutions such as boiling point elevation, freezing point depression, and other colligative properties. The B_{22}, in particular, measures the total protein-protein interactions in a given solution. The B_{22} places a numerical value on the extent of intermolecular attractions of a protein in a solution (Tessier, et al., 2002). The second virial coefficient can be used to improve two major areas of research: x-ray crystallography and pharmaceutical protein stabilization.

The second virial coefficient may provide an easier way to

find crystallization conditions and eliminate the bottleneck in the structure based drug design process. All crystallization solutions that have been studied have been found to have B_{22} values within a narrow range, between –1 *10^-04 and –8*10^-04 mol-mL/g^2. This narrow range of slightly negative B_{22} values is known as the "crystallization slot" (George & Wilson, 1994). The concept of the crystallization slot allows for predictions to be made on whether a protein will crystallize in a certain solution condition.

Many pharmaceutical companies spend 1 to 2 years at a cost of approximately 100 to 200 million dollars to find a solution in which a protein is stable. As of now most companies have no way of efficiently determining if or how long a protein will stay in a given solution. The second virial coefficient can also be used to determine protein stability; increasingly positive B_{22} values are directly correlated to increased stability of a protein in solution. The B_{22} allows for a quantitative approach and enables pharmaceutical companies to choose solutions that will maximize the shelf life of formulations.

Despite the apparent advantages to using B_{22} values to determine crystallization and protein stabilization conditions, B_{22} values have not yet been utilized because of the tedious and time-consuming methods from which B_{22} values are traditionally determined. One such method, static light scattering, although accurate requires a large protein sample size and a lot of time (Valente, 2005).

A relatively a new type of affinity chromatography, self-interaction chromatography (SIC) has been proposed as an excellent candidate to rapidly determine B_{22} values (Tessier et al., 2002). In SIC, a column is packed with beads to which the experimental protein is bound. The buffer solution being tested flows through the column and a sample of protein is injected onto the column. This method directly measures protein-protein interaction between the sample and the immobilized protein. The retention time of the protein sample injected onto the column is directly related to the B_{22}.

Certain chemical compounds or small molecules are known to have dramatic effects on the success with which individual proteins crystallize. Determining the second viral coefficient through self-interaction chromatography enables the effects of commonly used additives to be analyzed (McPherson and Cudney, 2006). In this study, SIC was used to measure the B_{22} values of nicotinamide mononucleotide adenylyl transferase (NMNAT) as a function of three excipients: arginine, glutamic acid, and trehalose. Additionally, B_{22} values that were measured as the concentration of the crystallization precipitants, ammonium sulfate and polyethylene glycol 400, varied.

METHODS

Coupling chemistry

40 mg of AF-Tresyl-650M chromatography beads were washed with 50mM Tris, pH 7.0, 100mM NaCl, 10% glycerol three times. 40 microliters of the protein, NMNAT, at a concentration of 4.7 mg/mL was added to the chromatography particles, and allowed to rotate overnight at 22 degrees. 1mM DTT (1,4-Dithio-DL-threitol) was added to the buffer and the beads were washed again 3 times the following day. 5 microliters of chromatography beads were assayed using Pierce BCA Protein Assay to determine the concentration of protein that bound to the chromatography particles.

Column Construction

The chromatography beads were loaded into 22 cm of 1/16 x 0.03 in. tubing, packed and then cut to an 18 cm length. Frits and ferrules were connected to both ends of the tubing to keep the chromatography beads in the tube. One column was loaded with the chromatography beads that had the protein bound. The second, dead column, was loaded with chromatography beads without the protein bound.

Chromatography procedure and data

A Shimadzu micro-scale HPLC system was used to conduct chromatography experiments and analyze the data. All experiments were set to run at a flow rate of 0.06 mL/min, 1.0 µl injection of filtered protein onto the column, and experiments were done in triplicate. Data was collected at a wavelength of 280 nm. 3% acetone was also injected onto both live and dead columns as a marker. Arginine, glutamic acid, and trehalose were tested individually at the concentrations of 50mM and 100mM in the running buffer (50mM Tris, pH 7.0, 100mM NaCl, 10% glycerol, 1mM DTT). The effects of arginine and glutamic acid, arginine and trehalose, in addition to glutamic acid and trehalose were tested at concentrations of 50mM in the buffer. Finally, the effects of all three excipients (arginine, glutamic acid and trehalose) were tested at a concentration of 50mM in the running buffer. The vapor diffusion reservoir that crystallized the protein, 2.0M Ammonium Sulfate and 2% PEG 400, was diluted with the Tris buffer and ran at 75, 50, 25, and 12.5 % dilutions

Determination of Second Virial Coefficient Values

B_{22} values were calculated using the average retention time of each set of experiments and the following equation:

$$B = (B_{HS} - \frac{K'}{\phi_1 * \varphi}) * (NA / MW^2)$$

RESULTS

When arginine, glutamic acid and trehalose were added to the protein buffer, the protein crystallized in vapor diffusion mixed 1 to 1 and equilibrated against a reservoir of 2M Ammonium Sulfate and 2% PEG 400 (Figure 1).

In the first part of the experiment, self-interaction chromatography was used to measure the effect of excipients on the protein Nicotinamide Mononucleotide Adenylyl Transferase (NMNAT). The first chromatogram displays the retention times for the experiments ran at 50 mM concentrations of arginine, glutamic acid, and trehalose (Figure 2). The second chromatogram displays the retention times for the 100 mM excipient concentrations (Figure 3). The retention times for the 100mM concentrations were shorter than the 50mM.

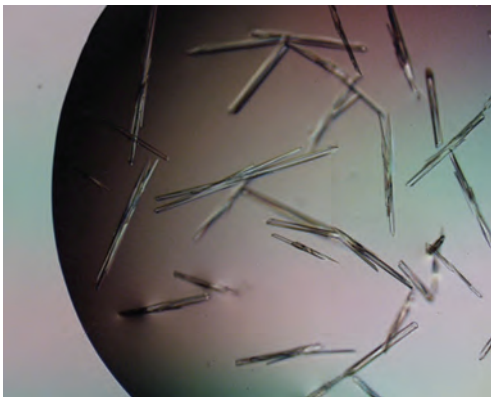

Figure 1. Crystals of NMNAT, a nuclear enzyme essential for NAD synthesis.

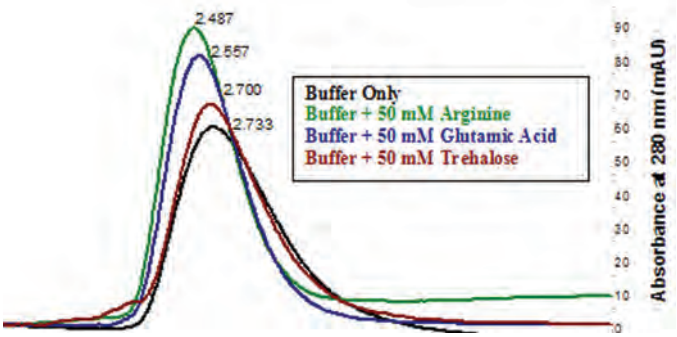

Figure 2. Comparison of the Effect of 50mM Arginine, Trehalose, and Glutamic Acid on the Retention Time (in minutes) of NMNAT.

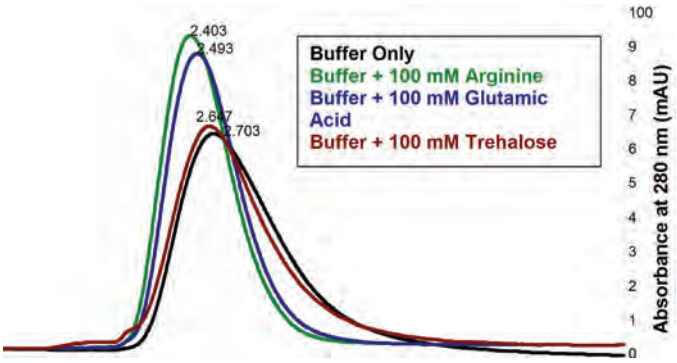

Figure 3. Comparison of the Effect of 100mM Arginine, Trehalose, and Glutamic Acid on the Retention Time (in minutes) of NMNAT.

In the second part of the experiment, the effects of different combinations of arginine, trehalose, and glutamic acid on the retention time of NMNAT were investigated. The retention times of all combinations were shorter than any of the excipients alone at a 50mM concentration (Figure 4).

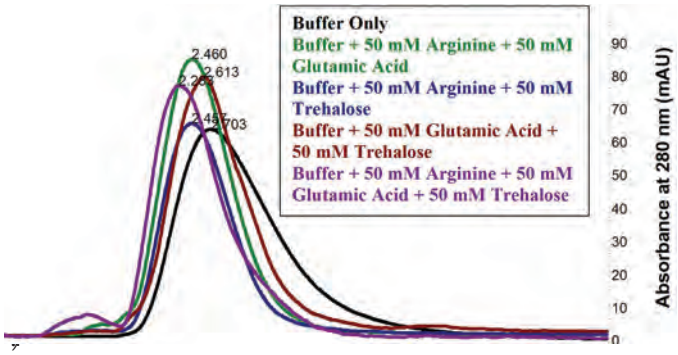

Figure 4. Effect of Combinations of Arginine, Trehalose, and Glutamic Acid on the Retention Time (in minutes) of NMNAT.

The second virial coefficient (B_{22}) values were calculated for the self-interaction chromatography experiments done with the stabilizing additives. For experiments 2 through 7, the solutions with the higher excipient concentrations have larger and more positive B_{22} values. The B_{22} values for experiments 8 through 11 are more positive than any of the individual experiments done with one excipient at a 50mM concentration (Table 1).

Table 1. Calculated Second Virial Coefficient (B_{22}) Values

	Solution	B_{22} * 10-4	Std Dev* 10-4
1	Buffer Only (50mM Tris, pH 7, 100mM NaCl, 10% glycerol, 1mM DTT)	0.2411	0.5373
2	Buffer + 50mM Arg	6.987	1.188
3	Buffer + 100mM Arg	11.118	0.2350
4	Buffer + 50mM Glu	9.700	0.6654
5	Buffer + 100mM Glu	6.135	1.948
6	Buffer + 50 mM Trehalose	0.8279	0.4617
7	Buffer + 100mM Trehalose	2.614	0.3532
8	Buffer + 50mM Arg+ 50 mM Glu	9.320	0.9273
9	Buffer + 50mM Arg + 50 mM Tre	9.173	0.3176
10	Buffer + 50mM Glu + 50 mM Tre	3.765	0.2350
11	Buffer + 50mM Arg + 50 mM Glu +50 mM Tre	14.386	0.527

NMNAT crystallized in a vapor diffusion experiment when mixed 1 to 1 in a reservoir containing of 2M ammonium sulfate and 2% PEG 400. Self-interaction chromatography experiments were run at 75, 50, 25, and 12.5% dilutions of the crystallization condition with the buffer (Figure 5). As the

concentration of ammonium sulfate and PEG 400 increases, the calculated second virial coefficient (B_{22}) values become more negative and enter the crystallization slot; however, once the concentration reaches 1M Ammonium Sulfate and 1% PEG 400 the B_{22} becomes largely negative and is outside the crystallization slot (Table 2).

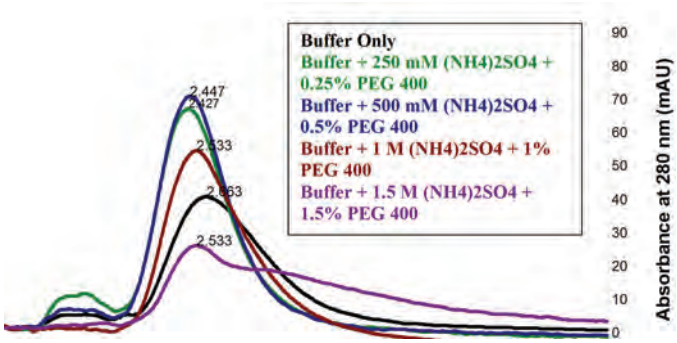

Figure 5. Chromatogram (in minutes) of Ammonium Sulfate and PEG 400 Gradient.

Table 2. Calculated Second Virial Coefficient (B_{22}) Values.

	Solution	B_{22}	Std Dev
1	Buffer Only (50mM Tris, pH 7, 100mM Nacl, 10% glycerol, 1mM DTT)	-5.936E-04	2.781E-05
2	Buffer + 250mM (NH4)2SO4 + 0.25% PEG 400	3.771E-04	6.113E-05
3	Buffer + 500mM (NH4)2SO4 + .5% PEG 400	2.7110E-04	7.484E-05
4	Buffer + 1M (NH4)2SO4 + 1 % PEG 400	-4.628E-05	4.847E-05
5	Buffer + 1.5M (NH4)2SO4 + 1.5 % PEG 400	-9.517E-05	7.451E-05

DISCUSSION

NMNAT did not crystallize unless the excipients arginine and glutamic acid were added to the protein buffer. NMNAT was also very unstable; however, when the excipients were added to the protein buffer, the protein crystallized in vapor diffusion mixed 1 to 1 and equilibrated against a reservoir of 2M Ammonium Sulfate and 2% PEG 400 (Figure 1). The results from the crystallization screens and vapor diffusion indicate that the excipients have a stabilizing effect when added to NMNAT that is critical to crystal formation. Self-interaction chromatography and the second virial coefficient allow for the stabilizing effect of the excipients to be assessed quantitatively.

The retention times for the 100 mM concentrations of arginine, glutamic acid, and trehalose were all shorter than the retention times of the 50mM concentrations. The shorter retention time indicates that increased excipient concentration decreases the protein self-interaction between the NMNAT bound to the stationary phase and the NMNAT in the mobile phase. The stabilizing effect of the higher excipient concentrations is also reflected in the calculated B_{22} values. The second virial coefficient values for the 100 mM concentrations of arginine, glutamic acid, and trehalose were larger and more positive than the 50 mM concentrations.

The results of the experiment testing the effect of multiple combinations of excipients indicate that arginine has the largest effect on protein intermolecular attraction. The B_{22} for the arginine-trehalose combination and the arginine-glutamic acid combination at is 9; however, the glutamic acid-trehalose combination only has a B_{22} value of 3.

The self-interaction chromatography data reinforces the dynamic light scattering data: without the addition of trehalose, arginine, and glutamic acid, NMNAT is insoluble and incapable of being purified nor crystallized. The small molecules must establish stabilizing, intermolecular, non-covalent crosslinks in protein crystals and thereby promote lattice formation.

The second virial coefficient (B_{22}) values for the ammonium sulfate and PEG 400 gradient indicates that a concentration between 500mM (NH4)2SO4 + 0.5% PEG 400 and 1M (NH4)2SO4 + 1% PEG 400 is the crystallization condition. The ammonium sulfate and PEG 400 have a combined effect of increasing protein self attraction and therefore producing a negative B_{22} value.

CONCLUSIONS

SIC appears to be a powerful method for determining the second virial coefficient of NMNAT as well as other proteins. The B_{22} values measured using self-interaction chromatography agrees with the data obtained from dynamic light scattering and crystallization screens, thereby strengthening the validity of this approach. Self-interaction chromatography can be used to determine B_{22} values quickly compared to traditional methods such as static light scattering. In addition, SIC works with variety of solution conditions which are difficult or impossible to measure by using static light scattering.

Continuing studies include cross-checking B_{22} values with results from static light scattering experiments to further validate the method and utilizing SIC to determine crystallization and stabilization conditions of membrane proteins. Also SIC can be used to screen and detect crystallization conditions for proteins that are difficult and have not been crystallized yet using the current shotgun approach. Furthermore, the results of SIC experiments are being integrated with a neural net predictive technology, and the engineering of a device that will miniaturize SIC and enable high throughput studies is underway.

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