Selecting Temperature for Protein Crystallization Screens Using the Temperature Dependence of the Second Virial Coefficient

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Abstract

Protein crystals usually grow at a preferable temperature which is however not known for a new protein. This paper reports a new approach for determination of favorable crystallization temperature, which can be adopted to facilitate the crystallization screening process. By taking advantage of the correlation between the temperature dependence of the second virial coefficient ($B_{22}$) and the solubility of protein, we measured the temperature dependence of $B_{22}$ to predict the temperature dependence of the solubility. Using information about solubility versus temperature, a preferred crystallization temperature can be proposed. If $B_{22}$ is a positive function of the temperature, a lower crystallization temperature is recommended; if $B_{22}$ shows opposite behavior with respect to the temperature, a higher crystallization temperature is preferred. Otherwise, any temperature in the tested range can be used.

Introduction

After the successful accomplishments of the Human Genome Project (HGP), more and more scientists have concentrated considerable interest on solving molecular-based diseases, which can be treated by structure-based rational drug design. Obtaining the 3-dimensional structure of the target biomacromolecules, which are often proteins, is the key to success in achieving this goal. Due to the potentially important applications of the structural information in human health, researchers have been making broad efforts to investigate the structures and functions of many proteins. It is well known that X-ray crystallography is the most widely used method to determine the 3-dimensional structure of proteins. More than 85% of the structures in the PDB (www.pdb.org) were determined by this method, which requires high quality protein crystals as diffraction targets. However, due to the complexity in crystal nucleation and growth, obtaining satisfactory protein crystals is often the rate-limiting step for structure determination. For example, over 60% of the targets for most commercial therapeutic drugs are membrane proteins [1,2], which are usually hard to crystallize. Therefore, growing high quality protein crystals is an important task for structural biologists [3,4].

Generally, it is accepted that if we could understand more clearly the behaviors of crystal nucleation and growth under various conditions, we would better know how to proceed with crystallization. To determine the structure of a protein using X-ray crystallography, the first step (after purification of the protein) is to find suitable conditions for crystallization (crystallization screening). Then, based on the screening results, the goal is to optimize the crystal quality for the purposes of high resolution diffraction. Since there are still no general guidelines for growing high quality protein crystals, these steps are often based on trial and error, which consumes time, money and manpower. To expedite the process and reduce the cost, rational protein crystallization has been proposed, and many efforts have been made [5–13]. Among these efforts, DLS (Dynamic Light Scattering) and SLS (Static Light Scattering) are often used. A famous coefficient of a protein solution, the second virial coefficient ($B_{22}$), is familiar to protein crystal growers [14–16] and is derived from the SLS method. $B_{22}$ is a static parameter that is related to the molecular weight of the protein [17]. For protein crystal growers, the $B_{22}$ value is a useful parameter because it can indicate which solutions are not favorable for crystallization. A “crystallization slot” of $B_{22}$, which is in the range of about $-$1x10^{-4} to $-$8x10^{-4} mol·mL·g^{-2} [18], has been reported to be helpful. A $B_{22}$ value in this range does not guarantee successful crystallization, but a value outside of the slot will probably result in crystallization failure. By varying protein solution conditions (such as adding crystallization agents and additives or adjusting pH), $B_{22}$ values may be adjusted to fall well within the slot, which may favor crystallization [19,20].

It is now clear that the absolute values of $B_{22}$ are affected by many factors, such as the characteristics of the particles in the protein solution, pH, intermolecular surface potential and temperature [21]. Probing the relationship between $B_{22}$ and these factors may help to develop a rational strategy to determine the best crystallization parameters. For example, it has been reported that the temperature dependence of $B_{22}$ shows the same tendencies as the temperature dependence of the solubility [22,23]. This result...
implies that the temperature dependence of $B_{22}$ could be a clue to select a suitable crystallization temperature because the driving force of crystallization is strongly related to the solubility.

The driving force of protein crystallization is the difference in chemical potential between the supersaturated and equilibrium solutions, which is mainly determined by the supersaturation ($\delta$), i.e., the ratio of the solution concentration to the solubility ($s$). Therefore, the solubility is a crucial parameter for protein crystallization. Because the solubility usually depends on the temperature, it can be adjusted by changing the temperature. The driving force of the crystallization can thus be adjusted by selecting a suitable temperature. In routine protein crystallization screens, researchers usually choose the crystallization temperature arbitrarily because they don’t have a better method. For example, a typical temperature, like 277K, is often used. If we have information about how the solubility changes in relation to temperature, which can be inferred from the results of a $B_{22}$ temperature dependence measurement, we may rationally propose a suitable temperature for crystallization screens.

To demonstrate this idea, we measured the temperature dependence of $B_{22}$ for lysozyme, proteinase K, concanavalin A and $\alpha$-chymotrypsinogen A(II) in different solution conditions respectively (detailed solution conditions are listed in Table 1), and verified with reproducibility and crystallization screening studies that optimal temperatures can be selected according to the temperature dependence behavior of $B_{22}$.

### Materials and Methods

#### Materials and experimental instruments

Four proteins were used in this study. Hen egg white lysozyme (HEWL, Lot No. 100940, recrystallized six times) was purchased from Seikagaku Kogyo Co. (Japan), and proteinase K (Lot No. P6556), $\alpha$-chymotrypsinogen A(II) (Lot No. C4879) and concanavalin A (Lot No. L7647) were purchased from Sigma-Aldrich Co. (USA).

Sodium chloride (NaCl) was obtained from Tianjin Kermel Chemical Reagents Development Center (China). Sodium acetate and HEPES-Na [C$_6$H$_{12}$N$_2$O$_4$SNa] were obtained from Beijing Chemical Reagents Development Center (China). Both acetone and toluene were analytical reagents from Henan Mol Chemical Co., Ltd. (China).

Acetic acid (HPLC grade) was obtained from TEDIA Co. (USA). The crystallization screening kit was Index™ from Hampton Research Co. (USA). Sodium cacodylate trihydrate, Polyethylene glycol (PEG) 8000 and PEG 3350 were purchased from Sigma-Aldrich Co. (USA). Magnesium acetate was taken from Tianjin Chemical Reagent Co., Ltd. (China). Citric acid was obtained from Tianjin DQ Chemical Reagent Factory (China). Tris hydrochloride (Tris-HCl) was purchased from Shanghai Fanke Biotechnology Co., Ltd (China).

Measurement of pH was carried out using a digital pH meter (Sartorius PB-10, Sartorius Scientific Instruments Co., Ltd., Beijing, China). Water was prepared using a Nanopure Diamond Ultrapure Water System D11931 from Barnstead Co. (USA).

All prepared solutions were filtered through 0.1 µm low protein binding non-pyrogenic syringe filters (PN: 4611) from Pall China (Beijing, China).

The PCS6501-glass cuvette with round aperture, the container for $B_{22}$ measurement, was obtained from Malvern Company (Beijing, China). In the reproducibility study, 40-well plates (Keyu Co., Jiangsu, China) were used as crystallization plates, and 96-well crystallization plates (HR3-143, Hampton Research Co., USA) were used in crystallization screens.

The refractive index of protein solutions was measured by an Abbe Refractometer (Shanghai Changfang Optical Instruments Co., LTD, China). Weight measurements were carried out using a microbalance BS 224S (Sartorius AG Beijing, China). The sample concentration after light scattering measurement was detected by a UV Spectrophotometer U-3310 (Hitachi Technologies Co., Japan). $B_{22}$ was measured by a Nano Zetasizer (Nano-ZS, Malvern Instruments Ltd., UK).

The crystallographic trials were set up using an automated protein crystallization robot (Screenmaker 96+8, Innovadyne Technologies Inc. USA).

The resulting samples were examined by an automated crystal image reader (XtalFinder, XtalQuest Inc., China).

#### Table 1. Solution conditions for measuring $B_{22}$.

| A) | Proteins | Buffer | Crystallization agents |
|----|----------|--------|------------------------|
| Lys. | 25 mM HEPES-Na, pH 7.0 | | |
| Pro. K | 25 mM HEPES-Na, pH 7.0 | | |
| Chy. A | 25 mM HEPES-Na, pH 7.0 | | |
| Con. A | 25 mM HEPES-Na, pH 7.0 | | |
| B) | Pro. K | 20 mM NaAc, pH 4.6 | 12 mg/mL NaCl |
| Chy. A | 20 mM citric acid, pH 3.5 and 2.5%w/v PEG3350 | 10 mM sodium cacodylate trihydrate; 16 mM MgAc$_2$ and 5%w/v PEG8000, pH 6.5 |
| Con. A | 5 mM HEPES-Na, pH 7.0 | 5%w/v PEG3350 |

A): in the absence of crystallization agents; B): in the presence of crystallization agents. Lys.: lysozyme; Pro. K: proteinase K; Chy. A: $\alpha$-chymotrypsinogen A (II); Con. A: concanavalin A.

doi:10.1371/journal.pone.0017950.t001
using the static light scattering (SLS) technique. SLS data are analyzed using the classical Zimm equation [24,25]:

\[ \frac{KC}{R_{173}} = \frac{1}{M_W} + 2B_{22}C + \cdots, \]

where \( C \) is the concentration, \( R_{173} \) is the excess Rayleigh factor at a scattering angle of 173°, \( M_W \) is the molecular weight, and \( K \) is an optical constant given by

\[ K = \frac{4\pi^2n^2(dm/dC)^2}{N_A^2}, \]

where \( n \) is the refractive index, \( N_A \) is Avogadro’s number and \( \lambda \) is the wavelength of the detecting light.

Using the above equations, the Debye plot, which is the dependence of the solution’s scattering intensity on the concentration, can be plotted. Both \( B_{22} \) and \( M_W \) can then be derived simultaneously from the Debye plot. A detailed method can be found in the literature [26].

**Measurements.** Determination of protein concentration: an accurate protein concentration is important to calculate \( B_{22} \). The concentration levels of the proteins are preset to 0.5 mg/mL, 1.0 mg/mL, 3.0 mg/mL, 5.0 mg/mL, 7.0 mg/mL and 9.0 mg/mL. However, the exact concentration will normally deviate from the preset point. Therefore, to obtain reliable concentration data, we used the calculated concentration based on the actual values of solution volume and protein weight during the experiment.

Determination of \( dm/dC \): from Eq. (2), the \( dm/dC \) value should be determined prior to the SLS measurement. We used an Abbé Refractometer to measure the refractive index at different concentration levels. Then, the \( dm/dC \) value was derived from the data using a linear regression treatment [27].

Cleaning of quartz sample cells: to obtain highly precise results in DLS and SLS measurements, the sample solution should be free of dust. If there is dust in the quartz sample cells or dirt on the cell wall, the measurement results will be scattered. Therefore, cleaning the quartz sample cells is very important.

We used the following cleaning procedure, which proved to be useful to enhance measurement reproducibility. First, wash the sample cell twice using distilled water and wipe with cotton swabs; then, rinse and dry the cells in a vacuum oven at 308K. Next, spray the sample cell with acetone or ethanol by using a syringe. Finally, wrap the sample cell with aluminum foil, which has itself been dunked in acetone to remove dust, to keep dust from entering the cell. Dry the cells in the ambient environment.

SLS & DLS measurements: we used SLS measurements to obtain \( B_{22} \) from the Debye Plot at different temperatures. Simultaneously, we performed DLS measurements using the same system. The DLS measurements were used to characterize the particle size distribution in the tested solution so as to get information on solution dispersity. To get a reliable Debye Plot, the solution should be monodisperse. If the DLS measurements show that the solution is not monodisperse, the SLS data must be discarded, and the experiments performed again.

**Crystallization screening and crystallization reproducibility tests**

According to the results of the temperature dependence of \( B_{22} \), we may postulate about the temperature dependence trends of the solubility. In low solubility conditions, a high probability of crystallization or precipitation might be achieved. To verify this postulate, we carried out both crystallization screening and crystallization reproducibility tests. In the crystallization screening tests, four proteins were tested at the following temperatures: 277K, 299K and 301K. All proteins are dissolved in 25 mM pH 7.0 HEPES-Na. Initial protein concentrations (before mixing) were 20 mg/mL for lysozyme, 20 mg/mL for \( \alpha \)-chymotrypsinogen A(II), 10 mg/mL for concanavalin A and 30 mg/mL for protease K [17,28]. In the crystallization reproducibility tests, one protein (\( \alpha \)-chymotrypsinogen A(II)) was tested at 277K and 293K. For comparison, crystallization reproducibility data for the other three proteins were extracted from previously published results.

**Results and Discussion**

**Measurement results of \( B_{22} \)**

Concentration dependence of refractive index (\( dm/dC \)). The refractive indices of the four proteins were measured at different concentration levels. Nearly the same measurement results were obtained for all tested proteins. Fig. 1 gives the measurement results for lysozyme. From the figure, it can be derived that the value of \( dm/dC \) was about 0.15 mL/g for proteins dissolved in a buffer of 25 mM pH 7.0 HEPES-Na. This value has been proven to be stable in the buffer and insensitive to the temperature change in the range between 277K and 303K. Therefore we used 0.15 mL/g as the value of \( dm/dC \) in the SLS measurement.

Concentration after SLS measurement. To make sure that the concentration data used in the SLS measurements are correct, we measured the concentration of the proteins using a UV Spectrophotometer after the SLS measurement. The results showed that there are only subtle differences between the measured results and the calculated results using the actual solution volume and weight of the proteins.

Obtaining a reliable Debye plot. A Debye plot is constructed by passing incident light through the protein solution and measuring scattered light intensity to see how the light interacts with the particles in the solution. Dust with diameters between 1 \( \mu \)m and 10 \( \mu \)m, which is about 100 times the size of the protein molecules, can ruin the experiment. By using a 0.1 \( \mu \)m filter, we successfully avoided introducing dust into the solution during solution preparation.

However, it is still very hard to completely prevent dust particles in the air from entering the solution when transferring the protein solution into the sample cell. Therefore, to make sure that the
measurement of $B_{22}$ is not affected by the dust particles in the solution, we performed a DLS measurement at the same time to check the monodispersity of the solution. Figs. 2(a) and (b) show examples of a DLS measurement of particle size distribution in the tested solution. From the figure, we can see that the solution presented in Fig. 2A was not monodisperse; therefore, we discarded the SLS measurement data for the corresponding solution. In Fig. 2B, the solution is monodisperse and thus the results of the SLS measurement were considered reliable and safe to use.

The temperature dependence of $B_{22}$. The temperature dependence of $B_{22}$ in several proteins was obtained by measuring $B_{22}$ at different temperatures for each protein. Fig. 3A shows the measurement results when the proteins were dissolved in the buffer only (i.e., without crystallization agents). It can be seen that lysozyme showed a “normal” temperature dependence of $B_{22}$, i.e., $B_{22}$ increases with increasing temperature in the tested temperature range. Proteinase K, however, showed an opposite temperature dependence of $B_{22}$. The value of $B_{22}$ for the other two proteins, concanavalin A and $z$-chymotrypsinogen A(II), seemed not to be very sensitive to the temperature, though $B_{22}$ of the former decreased slightly with increasing temperature while that of the latter increased slightly with the temperature. Fig. 3B shows the measurement results when crystallization agents were used in the solutions. Although the absolute values of $B_{22}$ presented in Fig. 3B appears smaller than their counterparts in Fig. 3A, the temperature dependence of $B_{22}$ showed the same tendency against the temperature. Obviously, this result indicated that testing the temperature dependence of $B_{22}$ can be carried out without using crystallization agents.

Crystallization studies

As reported in the literature [15,21,23,29–31], the temperature dependence of $B_{22}$ can be an indication of the temperature dependence of the solubility. This rule may be used to guide the crystallization screens of proteins because the solubility is closely related to the supersaturation, which is the driving force of crystallization. A “normal” behavior of $B_{22}$ against temperature indicates a “normal” behavior of solubility versus temperature, i.e., the solubility increases with the temperature. In such cases, a solution at a certain concentration will exhibit a higher supersaturation level at lower temperature. The solution will thus exhibit a high driving force for the crystallization, which is beneficial for enhancing the success rate of crystallization. To examine this speculation, we carried out crystallization studies using the four proteins whose $B_{22}$ values had been measured. Both crystallization screening and crystallization reproducibility tests were used in this study.

**Crystallization screening tests.** For the screening tests, we used the two temperatures of 289K and 301K, which marked the ends of the tested temperature range in our $B_{22}$ measurement. To further check the crystallization outside the above temperature range, we also tested the crystallization screens at a lower but frequently used temperature of 277 K.

Fig. 4 shows the results of the crystallization screening tests. It can be seen that, in the case of crystallization of lysozyme, the
number of screening hits (defined as the number of droplets out of
the 96 crystallization conditions that yielded detectable crystals
under a stereomicroscope at 80× magnification) was higher at
lower temperature. The difference in screening hits at different
temperatures was clear: the screening hits were 24.8% greater at
277K than at 301K. In the case of crystallization of z-
chymotrypsinogen A(II), we also observed similar trends in
screening hits as seen in lysozyme crystallization, but the difference
in screening hits for this protein was not very obvious: the
screening hits were only 4.7% greater at 277K than at 301K.
Thus, the change was relatively insensitive to the temperature just
as the temperature variation of B22 for z-chymotrypsinogen A(II)
was small compared with that for lysozyme.

As expected, in the case of crystallization of proteinase K, which
exhibits opposite behavior of B22 versus temperature, the screening
hits were greater at higher temperature. In the case of
 crystallization of concanavalin A, we observed a similar trend to
proteinase K (screening hits were 15.5% greater at 301K than at
277K for proteinase K and 14.1% greater for concanavalin A),
though the B22 value of the latter only slightly decreased with
increasing temperature.

Fig. 5 shows a comparison of typical crystal images for each of
the tested proteins at the three screening temperatures. From the
figure it can be seen that the crystal number showed the tendency
against the crystallization temperature as predicted by B22
measurement.

**Crystallization reproducibility tests.** It is well known that
protein crystallization often suffers from the problem of bad
reproducibility [32,33], i.e., identical crystallization conditions
may not yield identical crystallization results. By setting up a
number of crystallization drops at identical crystallization
conditions, we can easily check the reproducibility of the
crystallization of a protein. The major application of a
reproducibility test in this study is to statistically clarify the trend
of the crystallization success rate versus the temperature.

In our recent publications, we have already presented some
reproducibility studies at two temperatures (277K and 293K) [34],
which can be used in the current study to show the trend of
crystallization success rate versus the temperature. We extracted
the data for reproducibility tests of three proteins (lysozyme,
proteinase K, and concanavalin A) from our previous publication [34] and carried out a new crystallization reproducibility test of α-
chymotrypsinogen A(II). Fig. 6 shows the results. From the figure, we can see that the crystallization success rate of these four proteins follows the same trends versus temperature as seen in the above crystallization screens section. The trend was especially clear in the cases of crystallization of lysozyme (Fig. 6A) and concanavalin A (Fig. 6D). In the case of α-chymotrypsinogen A(II) (Fig. 6C), the trend was not so clear; this result was similar to the results obtained in the screening tests (Fig. 4C). In conclusion, the crystallization reproducibility studies shown here confirm the results obtained in the screening tests.

From different sources in the literature, we also found an example which supports our hypothesis. Wilson et al. [18] reported that $B_{22}$ of thaumatin I exhibits no temperature dependence, which implies an insensitive temperature dependence of its solubility. In our previous publication [33], we presented a variable temperature strategy to screen the crystallization conditions, which showed that the crystallization success rate of thaumatin is not sensitive to the temperature. This result implied that the solubility of thaumatin is insensitive to the temperature too. Apparently, the above two experimental results are in good agreement with each other and can be used as a good example to confirm our current research results.

Potential application in protein crystallization screens

As demonstrated above, the results of the crystallization studies were clearly in agreement with the theoretical speculation. In other words, knowing the temperature dependence of $B_{22}$ may be a good tool to select a suitable temperature for protein crystallization screens. The guideline could be as follows: when $B_{22}$ is lower at a lower temperature (indicating a lower solubility at lower temperature), a lower screening temperature, e.g., 277K, is preferred; when $B_{22}$ is higher at a lower temperature (indicating a lower solubility at higher temperature), a higher screening temperature is preferred, depending on the crystallization method and the protein. For example, only at 318K can crystal growers obtain the diffraction-quality crystals of an antifreeze protein [35]. In the rare case where $B_{22}$ is insensitive to temperature, any temperature in the range where the protein is stable can be used.

There is a practical consideration about the consumption of the protein to address before this method can be applied in protein crystallization screens. In our current research, we used a normal cuvette to measure $B_{22}$, which requires 1.2 mL of protein solution for each single measurement. Therefore, to obtain a complete temperature dependence of $B_{22}$ with this method, approximately 26 mg of protein is necessary. This amount is too large to use in actual crystallization because proteins are usually very precious and difficult to obtain in large amounts (to ensure the homogeneity of the sample). Fortunately, if we use the Low-volume quartz batch cuvette ZEN2112 (Malvern Instruments Ltd., UK), the total amount of protein consumed can be reduced to less than 2 mg. The amount of consumed protein can be further reduced to 30-300 µg by using a light scattering technique developed to analyze droplets because droplet volume can be reduced to less than 1 µL [10]. In such cases, it would be easy to apply the method without any large sample consumption.

Conclusions

In this paper, we presented an alternative method to help crystal growers determine a favorable temperature for protein crystallization screens by using knowledge of the temperature dependence of the second virial coefficient $B_{22}$. This information is a good indicator of the temperature dependence of the solubility of the protein. By using this method, we examined the crystallization success rate of four proteins in both crystallization screening and crystallization reproducibility studies. We verified that the temperature dependence of $B_{22}$ may be used as an indicator to choose a favorable crystallization temperature, which can help to increase the crystallization success rate.
The temperature for protein crystallization screens is usually chosen arbitrarily in most labs. When a crystallization screening experiment yields no hits, it is hard to know if the cause was an unsuitable temperature or unsuitable solution conditions. If the chosen temperature is shown to be unsuitable after a couple of trials, it must be changed to other temperatures using only trial and error tests, which will waste time, money, and protein before a suitable temperature is found. Therefore, determining a more favorable temperature in advance will be very useful for carrying out the crystallization screens more easily and efficiently. The favorable temperature in advance will be very useful for carrying out the crystallization screens more easily and efficiently.

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Acknowledgments

We are grateful to Hui-Meng Lu for assistance in data analysis, Wei-Hong Guo and Wei-Ju Zhang for technical support on utilization of the crystallization robot and the Nano Zetasizer.

Author Contributions

Conceived and designed the experiments: D-CY. Performed the experiments: JL Y-ZG X-KW S-XX Y-ML. Analyzed the data: JL Q-QL D-CY. Wrote the paper: JL D-CY.