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Comparison of the Quality of Protein Crystals Grown by CLPC Seeds Method

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Abstract: We present a systematic quality comparison of protein crystals obtained with and without cross-linked protein crystal (CLPC) seeds. Four proteins were used to conduct the experiments, and the results showed that crystals obtained in the presence of CLPC seeds exhibited a better morphology. In addition, the X-ray diffraction data showed that the CLPC seeds method is a powerful tool to obtain high-quality protein crystals. Therefore, we recommend the use of CLPC seeds in preparing high-quality diffracting protein crystals.

Keywords: CLPC seeds; crystal quality; resolution; mosaicity

1. Introduction

Proteins are important molecules with biological activities and they perform various functions, making them the most important biological macromolecules. To understand how proteins perform their complex functions, it is necessary to determine their three-dimensional structure. Currently, X-ray crystallography remains the most widely used technique for determining the three-dimensional molecular structures of proteins [1]. High-quality single crystals are vital for X-ray diffraction, and the need to obtain these crystals is the bottleneck of this technique [2,3].

Seeding in protein crystallization is a method of using protein crystals as the seeds for growing crystals, which can help to accelerate the crystallization process, and obtain high quality protein crystals. In this method, seed crystals are mainly inoculated into the pre-balanced protein crystal solution in metastable state, and the nucleation process is shortened by introducing foreign seed crystals to induce protein nucleation at low supersaturation, so as to obtain a highly ordered protein structure. To improve the quality of protein crystals by using tools such as electric fields [4,5], magnetic fields, ultrasound waves, light, mechanical vibration [6], microgravity [7,8], and nucleants [9], has always been studied. The addition of nucleants is one of the most commonly used methods for improving the quality of protein crystals. Many studies have been performed on using nucleants, and the results consistently show that their use has a positive effect on the crystal quality. For example, Quiocho [10] and others reported, for the first time in 1964, that cross-linked enzyme crystals were obtained through cross-linking carboxypeptidase-A crystals with bifunctional reagents such as glutaraldehyde. Diffraction analysis of three different proteins by Lusty [11] showed that cross-linking prevents, to a large extent, the lattice disorder normally observed in rapid cooling of these crystals. Weichsel studied the formation mechanism of lysozyme crystals induced by seeds [12]. Koizumi obtained strain-free lysozyme crystals by utilizing seed crystals [13]. Luo obtained ZnuA-domain protein crystals by using the initial crystals as the seeds, attaining a resolution of up to 2.03

Å [14]. This demonstrated the utility of heterogeneous nucleation, which can lead to the growth of diffraction-quality crystals. Zhou conducted a detailed review on the developments of nucleants and nucleation in protein crystal growth, which included the theoretical issues and the classification and application of nucleants [15]. O'dell and others used *Pichia pastoris* to obtain polysaccharide monooxygenase-2 protein from *Neurospora crassa* [16]. Using crystals as nucleating agents can improve the crystal morphology and obtain better diffraction resolution. Xu [17] and others used protein crystals as nucleating agents to successfully obtain antigen-binding fragments of mouse-derived monoclonal antibody 17B1.3, and protein crystals of the tumor cell surface protein B7-H6 complex, with a diffraction resolution of 2.5 Å [18]. They also elucidated the mechanism by which antibodies inhibit the expression of surface protein B7-H6 in cancer cells. Protein crystals, as nucleating agents for seed crystals, can also improve the crystallization of membrane proteins [19]. Abuhammad shows that the micro-cross-seeding matrix screening (MCMS) can shorten crystallization time [20]. D'arcy has set up a microseed matrix method that could be generally applicable to proteins where little or no nucleation is normally observed [21]. Rumpf proved that microseed matrix seeding (MMS) can be used to obtain crystals of human Sirt3 in its apo form, and of human Sirt2 in complex with ADP ribose (ADPR). Crystal formation using MMS was less error-prone, and yielded a higher number of crystals per drop than using conventional crystallization screening methods [22].

Cross-linked protein crystal (CLPC) seeds are protein crystals with a regular three-dimensional structure cross-linked by glutaraldehyde or other bifunctional reagents. CLPCs have superior performance characteristics such as high purity [23], high stability [24], and high activity in organic solvents [25,26]. CLPCs are insoluble in water and organic solvents [27], so they can be directly handled by mechanical contact. In addition, CLPCs have a very similar diffraction pattern to the native crystals [28]. All of these superior characteristics make CLPCs appropriate candidates for use as the seeds in protein crystallization.

In this work, we prepared CLPC seeds by cross-linking using glutaraldehyde. Then, the CLPCs were adapted to serve as the seeds in protein crystallization, and higher quality protein crystals were obtained by adding the CLPC seeds, as verified by X-ray diffraction analysis.

2. Materials and Methods

2.1. Materials

Proteins: Four different proteins were researched in the experiment. Hen egg white lysozyme (lysozyme; catalog No.100940) was purchased from Seikagaku Kogyo Corporation, Tokyo, Japan. Proteinase K (catalog No. P6556). Thaumatin (catalog No. T7638) were purchased from Sigma-Aldrich Corporation, St. Louis, MO, America. Glucose isomerase (catalog No. L112309) was purchased from Hampton Research, CA, America. All of the proteins were directly used without further purification.

Crystallization experiments: The crystallization experiments were conducted using the conventional batch method. The crystallization conditions of the four proteins used in the experiments are listed in Table 1. The proteins were dissolved in the corresponding buffers, and then centrifuged at 10,000 rpm/min for 3 min. The precipitant solutions were prepared by dissolving the chemical reagents in the corresponding buffers and filtering using 0.22 μm filters. Then, equal volumes of the protein and precipitant solutions were mixed to prepare the crystallization solution. Finally, 2 μl quantities of the crystallization solutions were dispersed in 96-well microbatch crystallization plates (No. HR3-267), and the volume of the reservoir was 60 μl. The crystallization temperature was maintained at 293 K. The preparation method of CLPC seeds (seeds 1, seeds 2, and seeds 3) was described in our previous report [29]. Three different CLPC seeds were used in this work. The CLPC concentration of each sample was 7.5 mg/ml. Seeds 1: cross-linked lysozyme crystals; seeds 2: cross-linked lysozyme and concanavalin A crystals, with a mass ratio of 1:1, and seeds 3: cross-linked lysozyme, concanavalin A and catalase crystals, with a mass ratio of 1:1:1.

According to our routine crystallization screening experiments, the reliable range of crystallization conditions for crystallization experiments [30–32] was screened out by us.

Table 1. Crystallization conditions of proteins researched in the crystallization experiments.

Protein	Initial C (mg ml^{-1})	T (K)	Crystallization Time (days)	Buffer	Precipitant
lysozyme	70	293	2	0.1 M sodium acetate pH 4.60	80 mg ml^{-1} NaCl
Proteinase K	30	293	2	0.05 M sodium cacodylate, 0.08 M magnesium acetate pH 6.50	20% (w/v) PEG 8000
Thaumatin	20	293	5	0.1 M HEPES-Na pH 7.00	0.2 M Potassium sodium tartrate tetrahydrate, 20% (w/v) PEG 3350
Glucose isomerase	7	293	3	0.1 M HEPES-Na pH 7.00	0.02 M magnesium chloride hexahydrate, 22% (w/v) poly (acrylic acid sodium salt) 5100 0.1 M HEPES, pH 7.5

2.2. Crystal Images

After the protein crystals were obtained, the crystal images were captured by a stereo microscope (Olympus SZX 16, Tokyo, Japan) to record their crystals' morphology. The same magnification was used for the crystals of one protein in order to analyze the influence of the CLPC seeds on the crystal morphology.

2.3. Crystal Diffraction

Crystals of similar size, grown in the presence or absence of CLPC seeds, were harvested by nylon CryoLoops (Hampton Research), and were then placed on an X-ray diffractometer (Mar μX, Mar Research, Norderstedt, Germany) to obtain the diffraction data. The wavelength is 1.54 Å, the diffraction energy is 8 kev, the diffraction exposure time of each diffraction pattern is 5 minutes, the angle of oscillation is 1 degree, the diffraction temperature is 100 K, and the antifreeze is composed of 20% glycerine and 80% precipitant solution. Three crystals were used, and the diffraction data were collected over the range of 100 degrees for each crystallization condition. Collecting a full data set for one crystal requires the use of the home facility for one or two days, meaning that completing the entire comparison experiment described in the paper takes a very long time. The environmental factors (temperature, humidity, pressure, etc.) do not stay exactly the same over such a long time period, introducing experimental errors. To minimize the errors, the number of diffraction shots was reduced to cover a range of 5 degrees, and three crystals were used, with 5 degrees of diffraction data also collected for each crystal. The HKL 2000 package and *BEST* in CCP4 software was used to analyze the diffraction data [33,34].

3. Results

3.1. Morphology of Protein Crystal

After the protein crystals were obtained, the images of the crystals were recorded using a stereomicroscope. Figure 1 shows some typical images of crystal morphology obtained in the

presence and absence of CLPC seeds. An inspection of Figure 1 shows that the crystals obtained in the CLPC seeds method exhibited larger size than that of the control crystals. It was observed that the crystals grown using the seeds technique exhibited an improved appearance, with comparatively few defects. In contrast, the crystals obtained without seeds had observable defects on the surface. The comparison of the crystals' morphology showed that the crystals obtained by the CLPC seeds method had a relatively better morphology than the control sample, as demonstrated by the crystal shape and size.

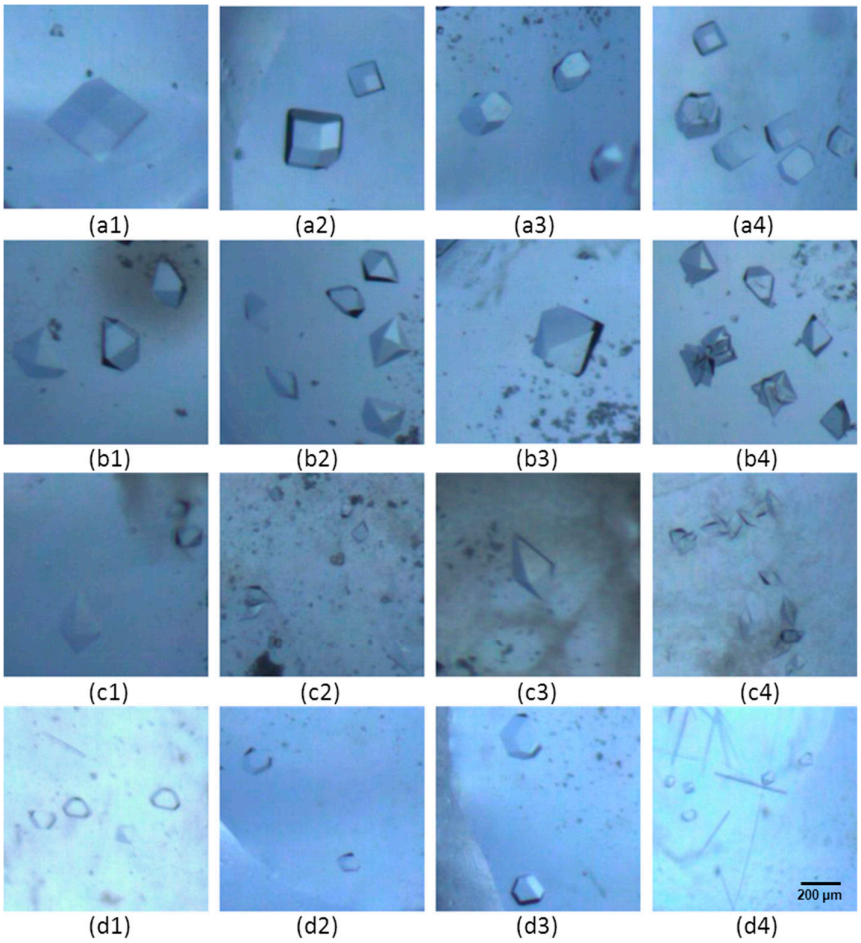

Figure 1. Some typical images of the crystal morphologies obtained in the presence and absence of CLPC seeds. (**a1–d1**) in the presence of seeds 1, (**a2–d2**) in the presence of seeds 2, (**a3–d3**) in the presence of seeds 3, (**a4–d4**) in the absence of seed, (**a1–a4**) Lysozyme, (**b1–b4**) proteinase K, (**c1–c4**) thaumatin, (**d1–d4**) glucose isomerase.

3.2. Diffraction Pattern of Protein Crystals

3.2.1. Comparisons of the Diffraction Data

The quality of protein crystals, grown in the presence or absence of CLPC seeds, was assessed using an X-ray diffractometer. To further identify the effect of CLPC seeds on the protein crystals, the resolution and mosaicity parameters were extracted and chosen as the analysis objects. Four kinds of protein (lysozyme, proteinase K, thaumatin, and glucose isomerase) crystals with the best appearance and similar sizes were selected, and the sizes of the crystals were 100–200 μm. All of the crystals were used for diffraction. Three groups of 100 degrees of diffraction data of the crystals grown with and without CLPC seeds from each crystallization condition were collected, and the best diffraction data sets (including the resolution, space group, cell parameters, Rmerge and I/Iσ) were compared. All of

the crystals of a given protein used for diffraction were simultaneously grown under the same environment with the same temperature, humidity, and air pressure.

Tables 2 and 3 lists is a summary of the diffraction quality data of the four proteins. It can be observed, from an examination of the data presented in the two tables, that the diffraction quality increased for all of the three proteins obtained using the CLPC seeds method, namely lysozyme (1.67 Å, 1.67 Å, 1.78 Å vs. 1.87 Å), proteinase K (1.71 Å, 1.89 Å, 1.76 Å vs. 1.91 Å), and thaumatin (2.10 Å, 2.18 Å, 1.96 Å, vs. 2.24 Å). In addition, the mosaicity of the crystals from the seeds showed an improved trend compared to the control. In addition, we obtained the diffraction data of glucose isomerase only when adding seeds 3, and the resolution and mosaicity reached up to 2.45 Å and 0.74, respectively. Meanwhile, diffraction data cannot be obtained when adding seeds 1, seeds 2 and control. Thus, in all cases, it was observed that the quality of the protein crystals grown from the seeds methods was better than that in the control.

Table 2. Optimal diffraction data of lysozyme and proteinase K crystals obtained from different conditions.

Crystallographic data	Lysozyme				Proteinase K			
	Control	Seeds1	Seeds 2	Seeds 3	Control	Seeds 1	Seeds 2	Seeds 3
Space group	$P4_32_12$	$P4_32_12$	$P4_32_12$	$P4_32_12$	$P4_32_12$	$P4_32_12$	$P4_32_12$	$P4_32_12$
Cell dimensions								
a (Å)	76.75	76.75	78.75	76.65	68	68.14	67.94	68.04
b (Å)	76.75	76.75	78.75	76.65	68	68.14	67.94	68.04
c (Å)	38.45	37.15	37.13	37.55	101.83	101.09	101.65	101.93
α (°)	90.00	90.00	90.00	90.00	90.00	90.00	90.00	90.00
β (°)	90.00	90.00	90.00	90.00	90.00	90.00	90.00	90.00
γ (°)	90.00	90.00	90.00	90.00	90.00	90.00	90.00	90.00
Resolution (Å)	50–1.87	50–1.67	50–1.67	50–1.78	50–1.91	50–1.71	50–1.89	50–1.76
I/σ I	25.53 (4.12)	48.91 (4.96)	44.24 (2.88)	24.98 (2.19)	14.65 (2.09)	22.54 (2.25)	17.67 (2.07)	18.07 (2.03)
Mosaicity	0.71	0.34	0.33	0.5	0.44	0.38	0.42	0.34
Redundancy	3.3 (2.1)	7.3 (6.1)	7.9 (5.6)	5.0 (2.2)	5.9 (3.2)	6.2 (3.5)	6.4 (3.4)	5.7 (3.4)
Completeness (%)	90.1 (71.2)	99.6 (99.1)	99.6 (98.1)	96.1 (72.2)	98.6 (85.8)	99.4 (92.6)	94.9 (85.7)	99.4(92.2)
No. reflections	30030 (9041)	102123 (14047)	109611 (13960)	53896 (10792)	112066 (19009)	163657 (26610)	119827 (18797)	141447 (24371)

Table 3. Optimal diffraction data of thaumatin and glucose isomerase crystals obtained from different conditions.

Crystallographic Data	Thaumatin				Glucose Isomerase
	Control	Seeds 1	Seeds 2	Seeds 3	Seeds 3
Space group	$P4_32_12$	$P2_12_12$	$P4_32_12$	$P4_32_12$	$P2_12_12$
Cell dimensions					
a (Å)	58.24	58.15	57.72	57.6	84.59
b (Å)	58.24	58.15	57.72	57.6	92.57
c (Å)	150.38	150.33	149.75	150.01	98.95
α (°)	90.00	90.00	90.00	90.00	90.00
β (°)	90.00	90.00	90.00	90.00	90.00
γ (°)	90.00	90.00	90.00	90.00	90.00
Resolution (Å)	50–2.24	50–2.10	50–2.18	50–1.96	50–2.45
I/σ I	21.1 (3.24)	21.31 (2.01)	16.02 (2.11)	20.3 (2.1)	9.73 (2.08)
Mosaicity	0.65	0.66	0.56	0.49	0.74
Redundancy	5.4 (2.8)	5.1 (2.5)	6.4 (3.6)	6.8 (3.7)	3.4 (2.2)
Completeness (%)	95 (79.6)	96.5 (70)	99.2 (88.8)	98.6 (87)	94 (77.5)
No. reflections	67058 (12531)	77875 (15312)	89329 (13900)	127704 （18732）	47421 (13810)

3.2.2. Comparisons of the Diffraction Data for the 5 Degree Range

(1) Average analysis

Collecting the full data set of one crystal using the home facility requires one or two days, meaning that the completion of all of the comparison experiments in the work takes a very long time. The environmental factors (temperature, humidity, pressure, etc.) are not identical over such a long time period, which may introduce errors in the experiments. To minimize the errors, the range of the diffraction shots was reduced to 5 degrees. Ten crystals grown in the presence and absence of three CLPC seeds with equivalent shape and size were chosen, and the size of crystals were 100–200 μm. A statistical analysis of all diffraction data of the three proteins with the 5-degree range was conducted, as shown in Figure 1. The resolution and mosaicity of the crystals were chosen as the analysis objects. It can be observed from the figure 1 that the lysozyme crystals grown from the seeds 1, 2 and 3 had better resolutions than the control sample, and the crystals obtained from seeds 1 had the largest increased range, followed by those of seeds 2 and seeds 3. For proteinase K and thaumatin, the crystals obtained using the seeds 3 possessed the best quality. Therefore, the quality of protein crystals obtained by adding three types of seeds is better than that of control samples.

① Resolution

The resolution limits are the main indicator of crystal quality. Figure 2 shows a comparison between the resolution obtained for the crystals of the three proteins with and without CLPC seeds. As shown in the figure, the CLPC seeds improved the resolution of the crystals for all three proteins. Seeds 1 of lysozyme demonstrated the best resolution limit, while the crystals obtained from the control showed the worst resolution limit. The contents of the cross-linked lysozyme crystals in seeds 1, seeds 2, and seeds 3 were 100%, 50%, and 33.33%, respectively, indicating that the seeds with the same protein resulted in the greatest increase in resolution of the target protein. The normalized results for the resolution limit are shown in Figure 2b. An examination of Figure 2 shows that all of the three CLPC seeds can improve the diffraction resolution of lysozyme crystals, and the lysozyme crystals with the best quality were obtained when the seeds 1 was added, followed by seeds 3, seeds 2, and the control.

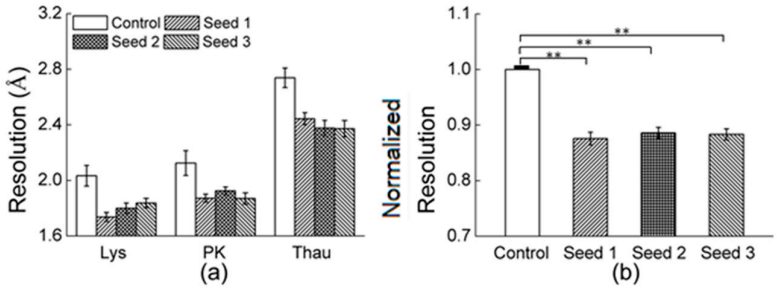

Figure 2. One-way ANOVA analysis on the resolution limit, the error bars showing the standard error of the mean. (**a**) A comparison of the resolution limits of the protein crystals obtained in the presence and absence of CLPC seed; (**b**). The averaged normalized resolution limits in the presence and absence of CLPC seed. (** represents saliency). In terms of the resolution limit of protein crystals, the results showed that there were significant differences between the control group and other groups. ($n = 3$, $P < 0.05$). Crystals with seeds showed better resolution limits.

② Mosaicity

Mosaicity is another measure of crystal quality. Figure 3 shows a resolution comparison of the three protein crystals with and without CLPC seeds. Compared to the control, all of the three CLPC seeds improved the mosaicity of the crystals. Here, seeds 1 exhibited greater improvement than the other two seeds. The normalized results for mosaicity are shown in Figure 3b. Overall, the CLPC seeds clearly demonstrated better mosaicities than the control.

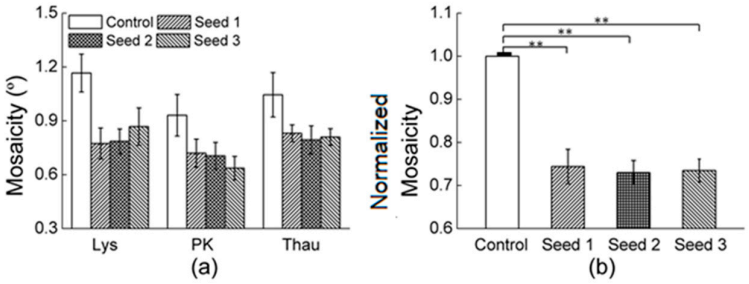

Figure 3. One-way ANOVA analysis on the mosaicity, with the error bars showing the standard error of the mean. (**a**) A comparison of the mosaicities of the protein crystals obtained in the presence and absence of CLPC seeds, (**b**). Averaged normalized mosaicities in the presence and absence of CLPC seeds. (** represents saliency). In terms of the mosaicity of the protein crystals, the results showed that there were significant differences between the control group and other groups. ($n = 3$, $P < 0.05$). Crystals with seeds showed a better mosaicity.

③ B-factor

The B-factor, which depends on structural heterogeneity, provides a wide spectrum of information on protein structure and dynamics. In different crystal structures of the same protein, the average B-factor tends to increase if resolution decreases [35,36]. The comparison in Figure 4 shows that the B-factor of the crystals obtained by the seeds method is always lower than that of the control. A one-way ANOVA test was also conducted to analyze the B-factor, as shown in Figure 4b. The three CLPC seeds all demonstrated a decreasement in the B-factor compared to the control sample.

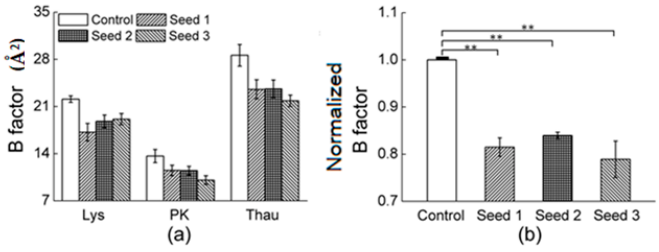

Figure 4. One-way ANOVA analysis on the B-factor, the error bars show the standard error of the mean. (**a**) A comparison of the B-factors of protein crystals obtained in the presence and absence of CLPC seeds, (**b**). Averaged normalized B-factor values in the presence and absence of CLPC seeds. (** represents saliency). In terms of the protein crystals, the results showed that there were significant differences between the control group and other groups. ($n = 3$, $P < 0.05$). Crystals with seeds showed a better B-factor values.

Based on the above comparisons, the protein crystals grown with CLPC seeds exhibited better resolution, mosaicity and B-factor, indicating that adding CLPC seeds in the protein crystallization process is beneficial for obtaining high-quality protein crystals.

(2) Discrete point analysis

In order to further study the difference in resolution and mosaicity between crystals with and without seed crystals, we compared the diffraction resolution and mosaicity of crystals in one image, as shown in Figures 5–7, which represent the squares of the crystals of three proteins (lysozyme, proteinase K and thaumatin) in the seed group. The closer the crystal resolution and tessellation points are to the origin of coordinates, the better the crystal quality is. The seed group is close to the coordinate origin, while the triangle representing the control group is far from the coordinate origin. In addition, the square distribution representing the seed group is compact, while the triangular distribution representing the control group is more dispersed, which indicates that it is more likely

to obtain crystals with better resolution and mosaicity after adding seeds, and that the quality of protein crystals grown in the seeds group is better than that in the control group.

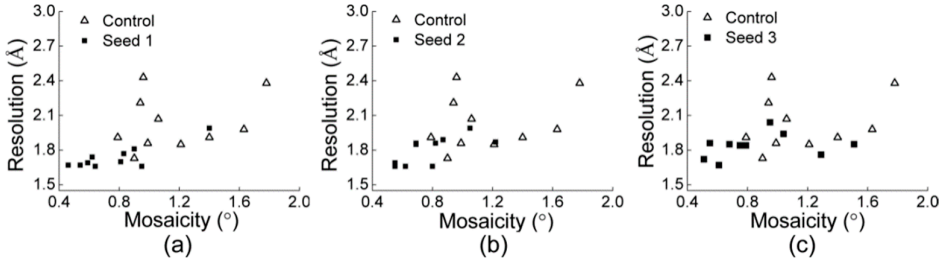

Figure 5. Combination of the resolution limit and mosaicity data for the lysozyme crystals in the same Figure. (**a**) in the presence of seeds 1, (**b**) in the presence of seeds 2, (**c**) in the presence of seeds 3.

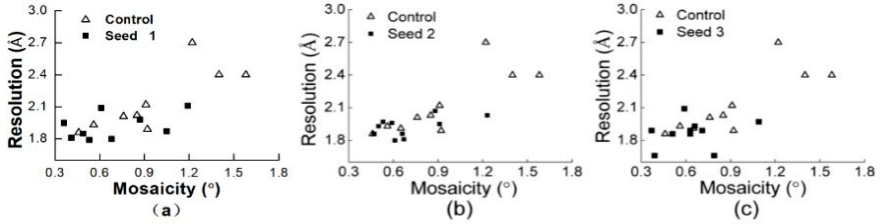

Figure 6. Combination of the resolution limit and mosaicity for the proteinase K crystals in the same Figure. (**a**) in the presence of seeds 1, (**b**) in the presence of seeds 2, (**c**) in the presence of seeds 3.

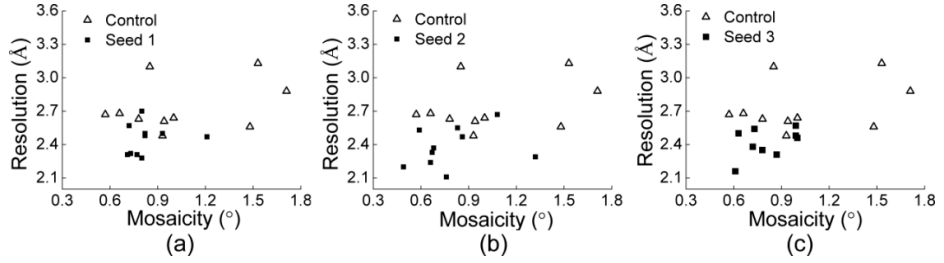

Figure 7. Combination of the resolution limit and mosaicity data for the thaumatin crystals in the same Figure. (**a**) in the presence of seeds 1, (**b**) in the presence of seeds 2, (**c**) in the presence of seeds 3.

(3) Trend chart analysis

The resolution of lysozyme was divided into different intervals from 1.5 to 2.5, with intervals of 0.2, and the number of protein crystals in each interval was counted. The results are shown in Figure 8. The peak resolution of lysozyme crystal was 1.5–1.7 Å, 1.7–1.9 Å, and 1.7–1.9 Å, respectively, when cross-linked seed crystals were added, while the peak resolution of lysozyme crystals was 1.9 Å–2.1 Å when seed crystals were not added, indicating that the diffraction resolution value of the crystal added with seed crystals was smaller, and the diffraction quality was better. For mosaicity, the peak value of lysozyme crystal was 0.6–0.9°, while the peak value of the control group was 0.9–1.2°, that is to say, adding seed crystals can improve the mosaicity of crystals, and the distribution of B-factor had the same trend. In addition, the resolution, mosaicity and B-factor of proteinase K (Figure 9) and sweet protein (Figure 10) were analyzed by the same statistical method. The results also showed that the resolution, mosaicity and B-factor of protein crystals obtained by seed crystal technology were better than those of the control group.

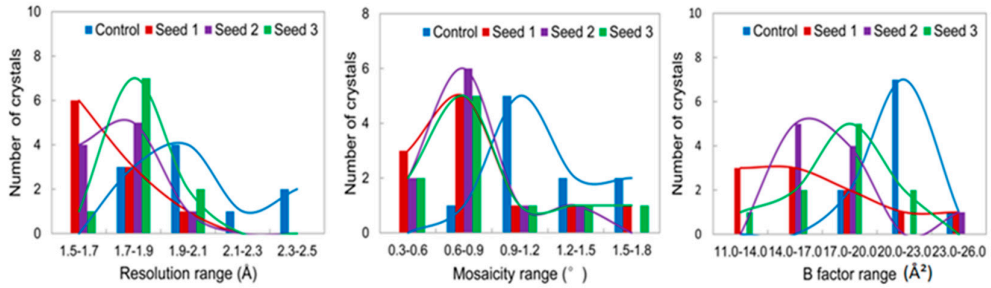

Figure 8. Comparison of the number of lysozyme crystals calculated by increases of (**a**) 0.2 Å in resolution range, (**b**) 0.3° in mosaicity range, and (**c**) 3 Å² in B-factor range.

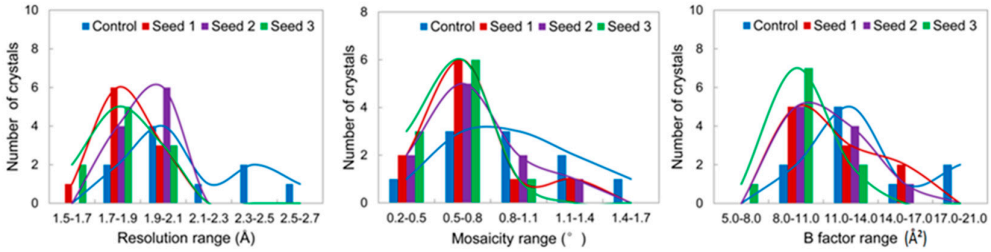

Figure 9. Comparisons of the number of proteinase K crystals calculated by increases of (**a**) 0.2 Å in resolution range, (**b**) 0.3° in mosaicity range, and (**c**) 3 Å² in B-factor range.

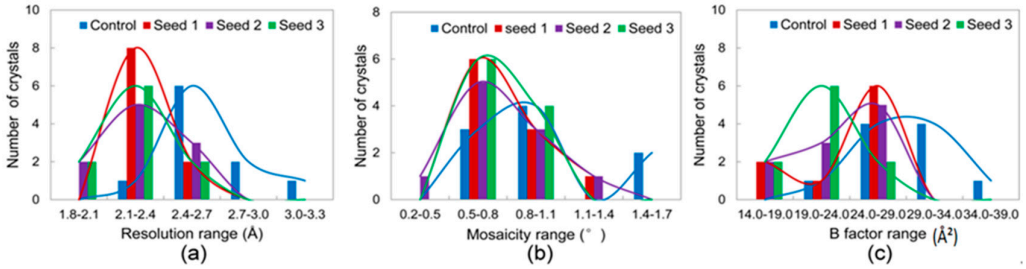

Figure 10. Comparison of the number of thaumatin crystals calculated by increases of (**a**) 0.3 Å in resolution range, (**b**) 0.3° in mosaicity range, and (**c**) 5 Å² in B-factor range.

4. Discussion

The results described above provided strong evidence that the crystals grown in the seeds method exhibited not only crystal appearance, but also better resolution limits, mosaicity, and B-factor than those of the crystals grown in the control, this showing that using CLPCs as the seeds for protein crystallization can improve the crystals' quality, especially in the X-ray diffraction parameter.

The mechanism of the improvement can be attributed to the characteristics of cross-linked protein crystals. Unlike most nucleants, CLPCs exhibit an ordered and porous structure. The porous structure can provide sites with nanopores which are suitable for capturing protein molecules, while the ordered structure can provide lattice points for protein molecules to pack into the crystal and grow directly. At the same time, crosslinked crystals can also act like normal nucleants, providing an interface for nucleation or growth, so as to reduce the energy barrier for nucleation.

The improvement in protein crystal quality is also related to the characteristics of CLPCs: firstly, the crystal of the same protein type as that of the CLPCs will have good lattice matching so that the internal stress can be effectively reduced, resulting in better crystal quality. In addition, the porous structure will enable the CLPCs to capture impurities, thus reducing the amount of impurities incorporated into the crystal, so as to improve the crystal quality. Furthermore, as a nucleant, CLPCs will have the capability to realize crystallization at low supersaturation level, which is also conducive to the improvement of crystal quality.

It is worth emphasizing again that CLPCs, as crystallization seeds, have obvious advantages in that they are highly stable, not only in the air, but also in solution. These merits make CLPCs readily available at any time and in any solution conditions. Compared with normal protein crystals which are kept in the mother liquid they are grown from, CLPCs can be independently placed outside the solution. Therefore, CLPCs can be used for commercial purposes. For example, their used can be explored for one type of optimization crystallization kit in a crystallization screen. So long as these CLPCs could adhere to the crystallization plate firmly, the commercial crystallization plate is likely to be constructed. Furthermore, normal protein crystal seeds added to the crystallization solution may be at risk of dissolution, if the solution is undersaturated. However, in the case of CLPCs, one need not worry about this risk. When using CLPCs, one can add them into the solution directly when the concentration is undersaturated, so as to provide more opportunities for crystallization to occur because the solution can experience a wide concentration range from low to high via vapor diffusion.

5. Conclusions

In this article we report a systematic study on the effect of CLPCs on the quality of protein crystals. Our results showed that the CLPCs could effectively improve the quality of protein crystals by X-ray diffraction. Due to the high stability, and the ordered and porous structure preserved in the protein seed crystals, the success rate of protein crystallization can be significantly increased, and the quality of protein crystals can be improved simultaneously, which is normally challenging in protein crystallization. Judging from the positive effects and the benefits that CLPCs can provide, we suggest that CLPCs be applicable as routine tools in protein crystallization screening and optimization.

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References

1. Kessel, A.; Ben-Tal, N. *Introduction to Proteins*; CRC Press; Taylor & Francis Group: Boca Raton, FL, USA; *Abingdon*-on-Thames, UK, 2018; pp. 209–253.
2. Chayen, N.E. Turning protein crystallisation from an art into a science. *Curr. Opin. Struct. Biol.* **2004**, *14*, 577–583.
3. Chayen, N.E. Tackling the bottleneck of protein crystallization in the post-genomic. *Trends Biotechnol.* **2002**, *20*, 98–98.
4. Koizumi, H.; Uda, S.; Fujiwara, K.; Tachibana, M.; Kojima, K.; Nozawa, J. Crystallization of high-quality protein crystals using an external electric field. *J. Appl. Crystallogr.* **2015**, *48*, 1507–1513.
5. Koizumi, H.; Uda, S.; Fujiwara, K.; Tachibana, M.; Kojima, K.; Nozawa, J. Technique for high-quality protein crystal growth by control of subgrain formation under an external electric field. *Crystals* **2016**, *6*, 95.
6. Lu, Q.Q.; Zhang, B.; Tao, L.; Xu, L.; Chen, D.; Zhu, J.; Yin, D.C. Improving protein crystal quality via mechanical vibration. *Cryst. Growth Des.* **2016**, *16*, 4869–4876.
7. McPherson, A.; Delucas, L.J. Microgravity protein crystallization. *NPJ Microgravity* **2015**, *1*, 15010.
8. Boyko, K.M.; Timofeev, V.I.; Samygina, V.R.; Kuranova, I.P.; Popov, V.O.; Koval'Chuk, M.V. Protein crystallization under microgravity conditions. Analysis of the results of Russian experiments performed on the International Space Station in 2005–2015. *Crystallogr. Rep.* **2016**, *61*, 718–729.
9. Guo, Y.Z.; Sun, L.H.; Oberthuer, D.; Zhang, C.Y.; Shi, J.Y.; Di, J.L.; Zhang, B.L.; Cao, H.L.; Liu, Y.M.; Li, J.; et al. Utilisation of adsorption and desorption for simultaneously improving protein crystallisation success rate and crystal quality. *Sci. Rep.* **2014**, *4*, 7308.

10. Quiocho, F.A.; Richards, F.M. Intermolecular Cross Linking of a Protein in the Crystalline State: Carboxypeptidase-A. *Proc. Natl. Acad. Sci. USA* **1964**, *52*, 833–839.
11. Lusty, C.J. A gentle vapor-diffusion technique for cross-linking of protein crystals for cryocrystallography. *J. Appl. Crystallogr.* **1999**, *32*, 106–112.
12. Weichsel, U.; Segets, D.; Thajudeen, T.; Maier, E.M.; Peukert, W. Enhanced Crystallization of Lysozyme Mediated by the Aggregaion of Inorganic Seed Particle. *Cryst. Growth Des.* **2016**, *17*, 967–981.
13. Koizumi, H.; Uda, S.; Tachibana, M.; Tsukamoto, K.; Kojima, K.; Nozawa, J. Crystallization. *Cryst. Growth Des.* **2016**, *16*, 6089–6094.
14. Luo, Z.; Morey, J.R.; McDevitt, C.A.; Kobe, B. Heterogeneous nucleation is required for crystallizaion of the ZnuA domain of pneumococcal AdcA. *Acta Crystallogr. Sect. F Struct. Biol. Commun.* **2015**, *71*, 1459–1464.
15. Zhou, R.B.; Cao, H.L.; Zhang, C.Y.; Yin, D.C. A review on recent advances for nucleants and nucleation in protein crystallization. *CrystEngComm* **2017**, *19*, 1143–1155.
16. O'dell, W.B.; Swartz, P.D.; Weiss, K.L.; Meilleur, F. Crystallization of fungal lytic polysaccharide monooxygenase exprssed from glycoengineered Pichia pastoris for X-ray and neutron diffraction. *Acta Crystallogr. Sect. F Struct. Biol. Commun.* **2017**, *73*, 70–78.
17. Xu, X.; Narni-Mancinelli, E.; Cantoni, C.; Li, Y.; Guia, S.; Gauthier, L.; Chen, Q.; Moretta, A.; Vély, F.; Eisenstein, E.; et al. Structural Insights into the Inhibitory Mechanism of an Antibody against B7-H6,a Stress-Induced Cellular Ligand for the Natural Killer Cell Receptor NKp30. *J. Mol. Biol.* **2016**, *428*, 4457–4466.
18. Xu, X.; Li, Y.; Gauthier, L.; Chen, Q.; Vivier, E.; Mariuzza, R.A. Expression, crystallization and X-ray diffraction analysis of a complex between B7-H6, a tumor cell ligand for the natural cytotoxicity receptor Nkp30, and an inhibitory antibody. *Acta Crystallogr. Sect. F Struct. Biol. Commun.* **2015**, *71*, 697–701.
19. Kolek, S.A.; Brauning, B.; Stewart, P.D. A novel microseeding method for the crystallization of membrane proteins in lipidic cubic phase. *Acta Crystallogr. Sect. F Struct. Biol. Commun.* **2016**, *72*, 307–312.
20. Abuhammad, A.; McDonough, M.A.; Brem, J.; Makena, A.; Johnson, S.; Schofield, C.J.; Garman, E.F. "To cross-seed or not to cross-seed": A pilot study using metallo-β-lactamases. *Crys. Growth Des.* **2017**, *17*, 913–924.
21. D'Arcy, A.; Villard, F.; Marsh, M. An automated microseed matrix-screening method for protein crystallization. *Acta Crystallogr.* **2007**, *63*, 550–554.
22. Rumpf, T.; Gerhardt, S.; Einsle, O.; Jung, M. Seeding for sirtuins: Microseed matrix seeding to obtain crystals of human Sirt 3 and Sirt 2 suitable for soaking. *Acta Crystallogr. Sect. F Struct. Boil. Commun.* **2015**, *71*, 1498–1510.
23. Yan, E.K.; Cao, H.L.; Zhang, C.Y.; Lu, Q.Q.; Ye, Y.J.; He, J.; Huang, L.J.; Yin, D.C. Cross-linked protein crystals by glutaraldehyde and their applications. *RSC Adv.* **2015**, *5*, 26163–26174.
24. Lee, T.S.; Turner, M.K.; Lye, G.J. Mechanical stability of immobilized biocatalysts (CLECs) in dilute agitated suspensions. *Biotechnol. Prog.* **2002**, *18*, 43–50.
25. Noritomi, H.; Sasanuma, A.; Kato, S.; Nagahama, K. Catalytic properties of cross-linked enzyme crystals in organic media. *Biochem. Eng. J.* **2007**, *33*, 228–231.
26. Roy, J.J.; Abraham, T. Continuous biotransformation of pyrogallol to purpurogallin using cross-linked enzyme crystals of laccase as catalyst in a packed-bed reactor. *J. Chem. Technol. Biotechnol.* **2006**, *81*, 1836–1839.
27. Khalaf, N.; Govardhan, C.P.; Lalonde, J.J.; Persichetti, R.A.; Wang, Y.F.; Margolin, A.L. Cross-linked enzyme crystals as highly active catalysts in organic solvents. *J. Am. Chem. Soc.* **1996**, *118*, 5494–5495.
28. Iimura, Y.; Yoshizaki, I.; Rong, L.; Adachi, S.; Yoda, S.; Komatsu, H. Development of a reusable protein seed crystal processed by chemical cross-linking. *J. Cryst. Growth* **2005**, *275*, 554–560.
29. Yan, E.K.; Zhao, F.Z.; Zhang, C.Y.; Yang, X.Z.; Shi, M.; He, J.; Liu, Y.L.; Liu, Y.; Hou, H.; Yin, D.C. Seeding Protein Crystallization with Cross-Linked Protein Crystals. *Cryst. Growth Des.* **2018**, *18*, 1090–1100.
30. Liu, Y.; Zhang, X.F.; Zhang, C.Y.; Guo, Y.Z.; Xie, S.X.; Zhou, R.B.; Cheng, Q.D.; Yan, E.K.; Liu, Y.L.; Lu, X.L.; et al. A protein crystallisation screening kit designed using polyethylene glycol as major precipitant. *CrystEngComm* **2015**, *17*, 5488–5495.
31. Hou, H.; Liu, Y.; Wang, B.; Jiang, F.; Tao, H.R.; Hu, S.Y.; Yin, D.C. Recrystallization: A method to improve the quality of protein crystals. *J. Appl. Cryst.* **2015**, *48*, 758–762.

32. Hou, H.; Shi, M.; Chen, Z.H.; Ahmad, F.; Liu, Y.; Deng, X.D.; Yin, D.C. A high-performance protein crystallization plate pre-embedded with crosslinked protein microcrystals as seeds. *CrystEngComm.* **2018**, *20*, 4713–4718.
33. Otwinowski, Z.; Minor, W. Processing of X-ray diffraction data collected in oscillaion mode. *Methods Enzymol.* **1997**, *276*, 307–326.
34. Bourenkov, G.P.; Popov, A.N. Optimization of data collection taking radiation damage into account. *Acta Crystallogr. D Biol. Crystallogr.* **2010**, *66*, 409–419.
35. Carugo, O. Atomic displacement parameters in structural biology. *Amino Acids.* **2018**, *50*, 775–786.
36. Sun, Z.; Liu, Q.; Qu, G.; Feng, Y.; Reetz, M.T. Utility of B−Factors in Protein Science: Interpreting Rigidity, Flexibility, and Internal Motion and Engineering Thermostability. *Chem. Rev.* **2019**, *3*, 1626–1665.