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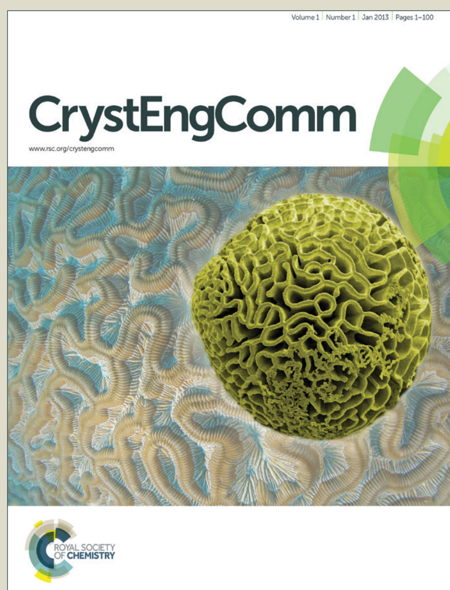

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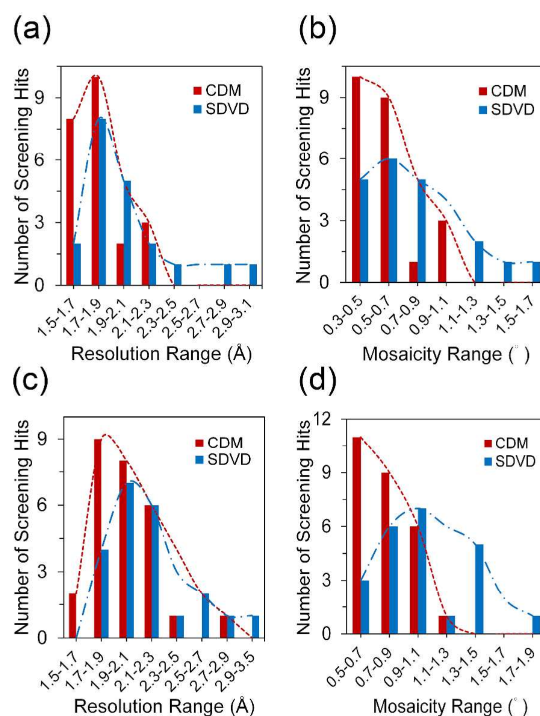
We present a systematic quality comparison of protein crystals grown using the cross-diffusion microbatch (CDM) method and the standard sitting-drop vapor-diffusion method. Crystallization conditions for eleven different proteins were screened using these two methods, and the crystals of all conditions were checked in terms of the resolution limit and mosaicity. It was found that crystals grown in the plate using the CDM method exhibit better morphology and higher crystal quality than crystals obtained using the standard sitting-drop vapor-diffusion method. X-ray diffraction tests show that the CDM method is indeed a practical and useful method for obtaining high-quality protein crystals to reduce the workload associated with both protein crystallization screening and optimization.



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A comparative study on the quality of protein crystals obtained using the cross-diffusion microbatch and sitting-drop vapor diffusion methods

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We presented a systematic quality comparison of protein crystals grown using the cross-diffusion microbatch (CDM) and standard sitting-drop vapor-diffusion methods. Eleven proteins were screened and found crystals grown using CDM exhibit better morphology. X-ray diffraction showed the CDM method is practical and useful for obtaining high-quality protein crystals.

In the research field of X-ray diffraction protein crystallography, one of the major challenges is obtaining high-quality protein crystals. Because higher-quality protein crystals can yield more detailed information regarding their three-dimensional structure, all structural biologists are eager to improve crystals to the required quality. However, obtaining high-quality crystals remains a technical challenge [1, 2]. Hence, the development of novel methods for improving crystal quality has been pursued. For example, container-less crystallization techniques, in which there is no contact between the growing crystals and any solid surface, have been reported to improve crystal quality [3, 4]. Additionally, magnetic fields and simulated microgravity environments [5-7] have also shown positive effects in enhancing crystal quality. Moreover, other chemical optimization approaches, such as pH value optimization [8], which affects crystal nucleation and growth processes [9, 10], precipitant concentration change, which causes a transition of the crystal growth mechanism from two-dimensional nucleation to dislocation growth [11], and protein engineering [12, 13], which uses mutagenesis or recombinant techniques to modify the protein in order to enhance its crystallizability, are widely used in structural biology. Overall, not only special environments that effect crystal quality but also chemical methods that change the crystal growth process can affect crystal quality. Apart from these methods, which are useful for obtaining crystals of the desired quality, additional equipment, experiments and methods are still needed for obtaining high-quality protein crystals.

To obtain protein crystals for X-ray diffraction analysis, two steps are usually necessary: (i) the screening of crystallization conditions to obtain more crystals and (ii) the optimization of the primary crystallization conditions to improve the quality of the crystals for X-ray diffraction [14]. In the first step, the vapor-diffusion screening method is widely used [15, 16], and many types of crystallization plates have been explored based on this method [17-19]. Additionally, the cross-diffusion microbatch (CDM) [20] method was recently reported to be potentially useful for increasing the probability of obtaining protein crystals. In this method, all of the crystallization droplets are dispensed onto one substrate and then sealed in the same space; as a result, the volatile components in the individual droplets can diffuse throughout the chamber. This technique is called the cross-diffusion microbatch method, due to the concentration evolution in the crystallization droplets is similar to that in a microbatch method and because of the cross-influence among droplets that share a same vapour space. However, as it is a new method, there has been no systematic study

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on its application in protein crystallization. In particular, there is no information regarding the quality of the crystals obtained using this method. In this paper, we conducted a comparative study on the quality of the protein crystals obtained by the CDM method and the conventional sitting-drop vapor diffusion (SDVD) method. Eleven different proteins were screened, and the obtained crystals were examined by X-ray diffraction analysis. The results show that the crystals obtained using the CDM method exhibited better crystal quality and better morphology, demonstrating that the CDM method can not only increase the probability of obtaining crystals but also improve the crystal quality.

In this study, 11 different proteins were subjected to the tests. Proteinase K, lysozyme, ribonuclease A I, concanavalin A VI, catalase, α-chymotrypsinogen A II, α-chymotrypsin II, myoglobin, cellulase, hemoglobin and ribonuclease A III were purchased from Sigma-Aldrich (USA). The crystallization plate used for the CDM method is shown in Supplementary Fig. S1. The crystallization plate material was polymethyl methacrylate (PMMA), and the plate thickness was 6 mm. The crystallization plate consists of 384 (96*4) pits and no reservoir. The size of this crystallization plate is compatible with that of the SBS standard plate [21].

It is a crystallization screening test using crystallization plates with the CDM method that we conducted to obtain the crystals for X-ray diffraction. SDVD crystallization plates were used as a control for comparison purposes.

The proteins were dissolved in 25 mM HEPES sodium buffer pH 7.0. For the CDM method, 11 proteins were prepared to an initial concentration of 20 mg mL^{-1} and were mixed with the IndexTM screening kit (Hampton Research) at a ratio of 1 μL:1 μL by a crystallization robot (Screenmaker; Innovadyne Technologies Inc., USA). The crystallization plate was then sealed with Crystal Clear Tape and placed in a temperature controller at 293 K. For the conventional SDVD method, standard Intelli-Plate 96-well crystallization plates (Hampton Research, Catalog No. HR3-143) were used and initially prepared by filling each well with 80 μL of the reservoir solution. The protein solution and the reservoir solution were then mixed at a volume ratio of 1 μL:1 μL.

To harvest the crystals, a syringe needle was used to slice the tape and open a small window above the droplet. Afterward, the small window was resealed with a small piece of Crystal Clear tape to avoid opening the entire plate and exposing all of the droplets to the open air. For each protein from each condition, the crystals with the best morphology and the most similar sizes were selected, and each crystal was picked up in a nylon CryoLoop (Hampton Research) for data collection at 100 K in a nitrogen stream on an IP (image plate) detector (MarResearch GmbH Norderstedt, Germany) using an X-ray single-crystal diffraction system (which is a home facility). Data processing and scaling were performed using the HKL2000 software package [22]. Once the X-ray diffraction data were collected, the resolution limit and mosaicity parameters were extracted and compared to describe the crystal quality. For detailed information on the diffraction data statistics, please refer to Supplementary Tables S1-S5 (some types of protein crystals lacked sufficient diffraction spots for data processing).

The results of this experiment were as follows.

(1) After the crystals grew, all of the crystals in the crystallization screening hits were checked by X-ray diffraction to exclude salt crystals. All results are summarized in Table 1. Supplementary Fig. S2(a) shows the crystallization screening hits which is defined as the number of crystallization conditions that yielded crystals using the CDM and SDVD methods for each of the 11 proteins. The results showed that in almost all cases, the CDM method produced more crystallization screening hits than the conventional SDVD method.

All of the numbers in the table correspond to the numbers of crystallization hits, which is defined as the number of crystallization conditions that yielded crystals. The number in the column titled "Sum over all protein crystallization hits" represents the numbers of crystallization conditions subtracted by the salt crystals for each protein.

Table 1. The number of hits in four categories of X-ray diffraction data for the 11 different proteins grown in the CDM and SDVD crystallization plates.

Proteins	Sum over all protein crystallization hits		Diffraction data[a]		Several diffraction spots[b]		No diffraction spots[c]		Salt[d]	
	CDM	SDVD	CDM	SDVD	CDM	SDVD	CDM	SDVD	CDM	SDVD
Proteinase K	47	39	23	20	18	14	6	5	7	10
Lysozyme	41	36	27	23	13	11	1	2	1	1
Ribonuclease A I	12	2	8	0	2	1	2	1	3	6
Concanavalin A VI	44	38	0	0	1	1	43	37	5	6
Catalase	48	35	0	0	1	0	47	35	3	0
α-Chymotrypsinogen A II	25	7	0	0	0	0	25	7	11	7
α-Chymotrypsin II	1	1	0	0	0	0	1	1	2	0
Myoglobin	4	2	0	0	0	0	4	2	8	0
Cellulase	4	2	0	0	0	0	4	2	1	2
Hemoglobin	3	2	0	0	0	0	3	2	2	0
Ribonuclease A III	4	3	0	0	0	0	4	3	16	0

[a] A diffraction pattern of the crystals that can be processed by the HKL2000 software package.

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[b] A diffraction pattern with only several (three, five or more) diffraction spots, which is not sufficient for data processing.

[c] A diffraction pattern with no diffraction spots.

[d] Diffraction pattern of salt crystals.

(2) It is known that not all of the tested crystals can produce favorable diffraction patterns under X-ray exposure due to the quality of the crystals. Therefore, we divided all of the diffraction patterns into four categories: (i) the category denoted "Diffraction data" corresponds to crystal diffraction patterns that can be processed by the HKL2000 software package; (ii) the category denoted "Several diffraction spots" represents diffraction patterns with only several (three, five or more) diffraction spots and that are thus insufficient for data processing; (iii) the category denoted "No diffraction spots" represents diffraction patterns with no diffraction spots; and the (iv) "Salt" category refers to the diffraction pattern of salt crystals. Table 1 shows the numbers of crystals in each of these four categories that were obtained for the 11 different proteins grown in the CDM and SDVD crystallization plates.
As shown in Table 1, ought to the limitation of the X-ray intensity, two proteins (proteinase K and lysozyme) yielded crystals using the SDVD method with X-ray diffraction patterns that can be indexed by HKL2000. However, three proteins (proteinase K, lysozyme and ribonuclease A I) were found to yield crystals using the CDM method that were classified into the "Diffraction data" category. Moreover, based on the numbers of crystals obtained using the CDM and SDVD methods that were classified into the "Diffraction data" category, i.e., proteinase K (23 by CDM vs. 20 by SDVD), lysozyme (27 vs. 23), and ribonuclease A I (8 vs. 0), it can be deduced that the crystals grown using the CDM method showed better quality than those obtained using the conventional method.
(3) The crystals grown in the CDM crystallization plates were larger and exhibited improved shapes compared with the crystals grown in the SDVD plates. Fig. 1 shows some typical images [(a)-(d) crystals grown in CDM plates and (a')-(d') crystals grown in SDVD plates]. The crystals grown in the CDM crystallization plate exhibited an improved appearance compare with those frown in the SDVD crystallization plates. Some defective crystals were obtained using the SDVD method.
The morphologies were compared based on the crystal size. In the crystallization conditions, we counted the numbers of conditions in the CDM plates in which the crystals were larger, smaller than and comparable to those grown in the SDVD plates. Supplementary Fig. S3 presents the percentages for the three categories. The results showed that 43% of the crystals grown in the CDM plates were larger than those grown in the SDVD plates, whereas only 27% of the crystals grown in the SDVD plates were larger than those grown in the CDM plates.
(4) The best data for two proteins (proteinase K and lysozyme) were selected for comparative analysis. The diffraction resolution analysis showed that proteinase K crystals grown by the CDM and SDVD methods reached 1.54 Å and 1.66 Å, respectively, and that lysozyme crystals reached 1.66 Å and 1.86 Å, respectively, and the mosaicity analysis showed that proteinase K and lysozyme reached values of

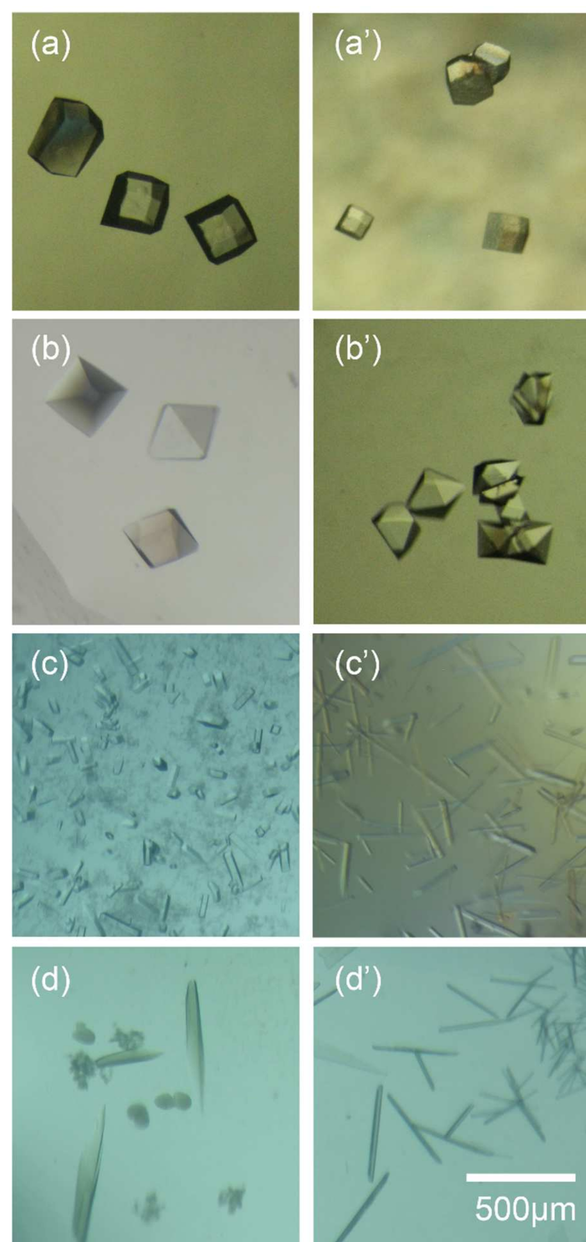

Fig. 1. Typical morphology of crystals grown in the CDM crystallization plate and the SDVD crystallization plate. (a)–(d) Images of crystals grown in the CDM plate. (a')–(d') Images of crystals grown in the SDVD plate. (a), (a') Lysozyme crystallization condition: H3. (b), (b') Proteinase K crystallization condition: F2. (c), (c') α-Chymotrypsinogen A II crystallization condition: F9. (d), (d') Catalase crystallization condition: D7. The crystals grown in the CDM crystallization plate usually exhibited better morphology (larger size and better defined faces) than the crystals grown in the control plates.

0.42° and 0.46° and of 0.57° and 0.61°, respectively. Thus, it can be easily observed that the quality of the best crystal obtained using the CDM method is better than that of the crystals obtained with the SDVD plate.

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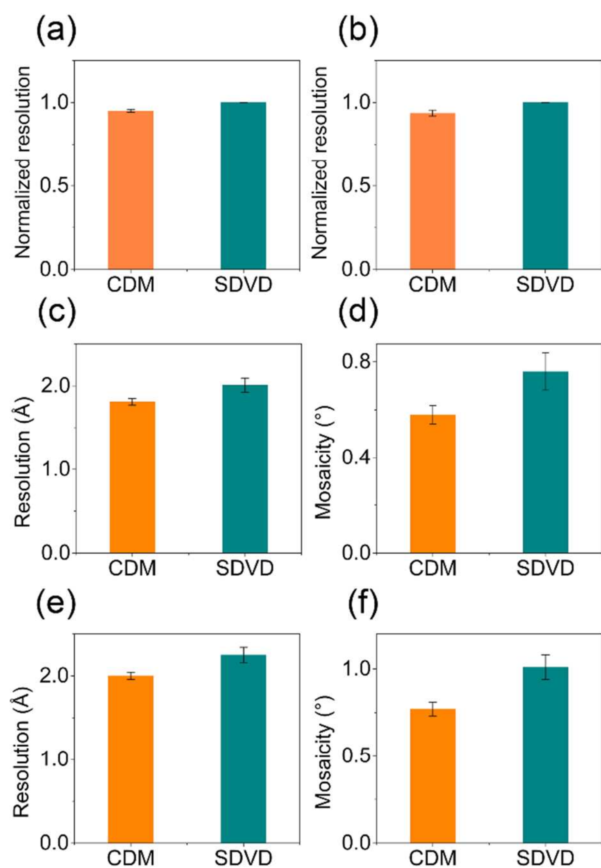

Fig. 2. (a) Statistical comparison of the resolution of the top five proteinase K crystals identified after normalization to the values found for the SDVD crystallization plate. The results demonstrated extremely significant difference between the two groups (n = 5, P = 0.00069, *i.e.,* < 0.01). (b) Statistical comparison of the resolution of the top five lysozyme crystals after normalization. The CDM method demonstrated improved resolution compared with the control. Moreover, the difference between the two groups was significant (n = 5, P = 0.0081, *i.e.,* <0.01). (c) Comparison of the resolution limits of all of the proteinase K crystals classified into the quality category named "Diffraction data". The results demonstrated a significant difference between the two groups (n_1 = 23, n_2 = 20, P = 0.0274, *i.e.,* <0.05). (d) Comparison of the mosaicity of all of the proteinase K crystals classified into the quality category named "Diffraction data". The results demonstrated a significant difference between the two groups (n_1 = 23, n_2 = 20, P = 0.0367, *i.e.,* <0.05). (e) Comparison of the resolution limit of all of the lysozyme crystals obtained in the CDM and the SDVD crystallization plates that were classified into the quality category named "Diffraction data". The difference between the two groups was significant (n_1 = 27, n_2 = 23, P = 0.0124, *i.e.,* <0.05). (f) Comparison of the mosaicity of all of the lysozyme crystals clarified into the quality category named "Diffraction data". The results showed a significant difference between the two groups (n_1 = 27, n_2 = 23, P = 0.00381, *i.e.,* <0.01).

(5) The five crystals of proteinase K and lysozyme with the best quality that were obtained using the CDM and SDVD methods were selected for comparative analysis. The diffraction data of top five crystals are shown as the first five crystals in Supplementary Tables S1-S4.

The resolutions of the top five proteinase K and lysozyme crystals obtained using the CDM and SDVD methods were compared. In addition, one-way ANOVA was used to analyze the diffraction resolution, and the resolution limit was normalized based on the data from the SDVD crystallization plate. Fig. 2(a) and 2(b) show the results obtained after the normalization of proteinase K and lysozyme, respectively. Overall, the results demonstrated extremely significant differences between the CDM and SDVD crystallization plates (n = 5, P = 0.00069, *i.e.*, <0.01 for proteinase K and n = 5, P = 0.0081, *i.e.*, <0.01 for lysozyme). The CDM method clearly improved the crystal quality in terms of the resolution limit. Moreover, Supplementary Fig. S4 shows the statistical comparison of the mosaicity values obtained after normalization. The differences in the top five crystals obtained between the CDM and SDVD methods were not significant. This result showed that the mosaicity values of the top five crystals present a dispersed distribution in the different crystallization plates.

In addition to the resolution limit and mosaicity, the B factors were also calculated to further compare the crystal quality. Fig. 3(a) and 3(b) show the results of the statistical comparison of the B factors obtained after normalization to the values found for the SDVD method. Additionally, the results indicated the existence of significant differences between the CDM and SDVD methods (n = 5, P = 0.00242, *i.e.*, <0.01 for proteinase K and n = 5, P = 0.00029, *i.e.*, <0.01 for lysozyme). Compared with the SDVD method, the CDM method clearly improved the B factor of the crystals.

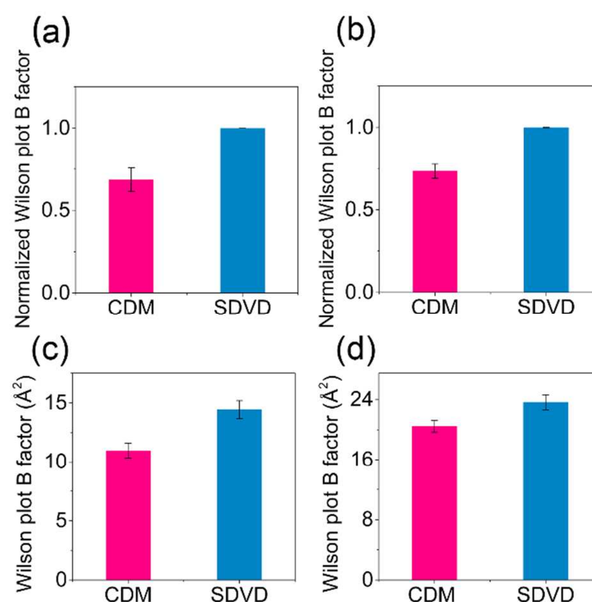

Fig. 3. (a) Statistical analysis of the B factor of the top five proteinase K crystals after normalization to the values found for the SDVD method. The B factor results demonstrated a significant difference between the two groups (n = 5, P = 0.00242, *i.e.,* <0.01). The crystals obtained by the CDM method exhibited improved B factors compared with the control. (b) Statistical comparison of the B factors of the top five lysozyme crystals obtained after normalization to the values found for the SDVD method. The results indicate that the crystals obtained by the CDM method exhibited improvements in the B factor. The results demonstrated a significant difference between the two groups (n = 5, P = 0.00029, *i.e.,* <0.01). (c) Comparison of the B factors of all of the proteinase K crystals classified into the quality category named "Diffraction data". Column height represents average values of Wilson B factor of all the crystals without normalization. The results demonstrated a significant difference between the two groups (n_1 = 23, n_2 = 20, P = 0.0011, *i.e.,* <0.01). (d) Comparison of the B factors of all of the lysozyme crystals obtained in the CDM and the SDVD crystallization plates that were classified into the quality category named "Diffraction data". Column height represents average values of Wilson B factor of all the crystals without normalization. The difference between the two groups was significant (n_1 = 27, n_2 = 23, P = 0.0127, *i.e.,* <0.05).

(6) The numbers of proteinase K crystals obtained using the CDM and SDVD methods that were categorized into the quality category named "Diffraction data" were 23 and 20, respectively, and the respective number of lysozyme crystals were 27 and 23, as shown in

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Table 1. As the most important comparison method, all of the "Diffraction data" of the crystals obtained using the CDM and SDVD methods were used for the comparative analysis. One-way ANOVA was used to analyze all of the "Diffraction data". Based on the resolution limit of the proteinase K crystals tested, the results indicated that the differences between the different crystallization plates were significant (shown in Fig. 2(c)). Moreover, Fig. 2(d) compares the mosaicity of the proteinase K crystals tested, and the differences between the different crystallization plates were also found to be significant.

The same comparison was conducted for the lysozyme crystals. Fig. 2(e) and 2(f) show the statistical comparison of the resolution limit and mosaicity. The results demonstrated extremely significant differences between the different crystallization plates in terms of resolution and mosaicity. Therefore, the CDM crystallization plate clearly demonstrated improvements in both resolution and mosaicity.

Additionally, all the crystals of proteinase K and lysozyme classified into the quality category named "Diffraction data" were performed one-way ANOVA without normalization to analyze the B factors. Overall, the results demonstrated significant differences between the samples obtained using the SDVD and CDM methods ($n_1 = 23$, $n_2 = 20$, $P = 0.0011$, *i.e.*, <0.01 for proteinase K and $n_1 = 27$, $n_2 = 23$, $P = 0.0127$, *i.e.*, <0.05 for lysozyme). The CDM method clearly improved the crystal quality in terms of the B factor.

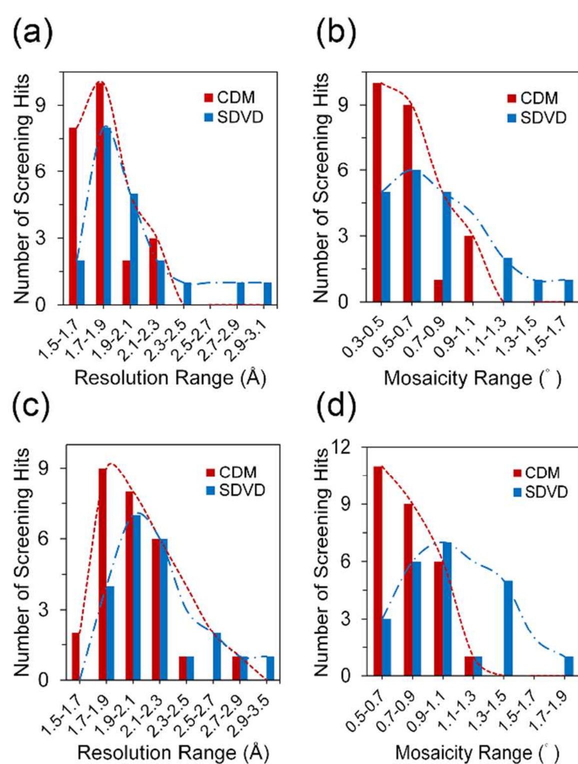

Fig. 4. The number of crystals classified into the category named "Diffraction data" was calculated at each 0.2-Å interval in the resolution range and at each 0.2° interval in the mosaicity range. The numbers of crystals in the CDM and SDVD crystallization plates are shown in the red and blue histograms, respectively. The tops of the histograms for each crystallization plate were connected with smooth curves, resulting in clear peaks. The peaks of the CDM curve for the resolution limit and mosaicity (shown in red) usually appeared at a lower value than that of the SDVD curve (shown in blue), indicating that the crystal quality obtained in the CDM crystallization plate was better than that obtained in the SDVD crystallization plate. (a) Resolution range of proteinase K. (b) Mosaicity range of proteinase K. (c) Resolution range of lysozyme. (d) Mosaicity range of lysozyme.

(7) Based on the diffraction data, we analyzed the numbers of crystals in more detail. For example, we divided the resolution range into 0.2-Å intervals from 1.5 Å to 2.9 Å and calculated the numbers of crystals in each resolution interval. The results are shown in Fig. 4(a) and 4(c). Subsequently, we linked the points that indicate the numbers of crystals in each 0.2-Å interval using smooth curves and found that the CDM curve usually peaked at a lower value than the SDVD curve, indicating that the crystals in the CDM crystallization plates exhibited better resolution than the crystals in the SDVD crystallization plates. Moreover, the same calculation method was applied to mosaicity. The mosaicity range was divided into 0.2° intervals from 0.3° to 1.9°, and the numbers of crystals in each 0.2° mosaicity interval were counted. A smooth curve was drawn to show that the CDM curve clearly peaked at a lower value than the SDVD curve (Figs. 4(b) and 4(d)). These results showed that the crystals in the CDM crystallization plate exhibited better mosaicity than the crystals in the SDVD crystallization plate.

All of the above-mentioned results showed that crystallization using the CDM method can not only increase the probability of obtaining protein crystals but also improve the quality of the protein crystals obtained compared with those produced using the SDVD method. This observed phenomena can be attributed to the different growth environments used in the two methods. Cross-diffusion creates a new vapor-diffusion environment. In the CDM method, all of the volatile reagents can diffuse throughout the plate because all of the droplets are found in a common space without any barrier.

(i) The CDM method can achieve a higher concentration level than the SDVD method. The CDM crystallization plate used in this study is a permeable plate. The CDM crystallization plate is composed of PMMA [23], which is water-permeable. If water and some volatile chemicals can penetrate through the CDM plate, the droplets in the CDM plate can reach higher supersaturation than the droplets in the SDVD plate. To verify that the CDM reach a higher level of supersaturation, we examined the crystallization plates after three weeks, and found that the number of drops containing amorphous precipitate in CDM crystallization plate was greater than that in the SDVD plate in 10 proteins (shown in Supplementary Table S6). In addition, we have demonstrated in the previous paper [20] that the crystallization screening hits in the CDM method increased more slowly over time compared with in the SDVD method, but the hits in CDM method also always increase to a higher level than in SDVD method, showing that the supersaturation level increased more slowly, but due to the permeable nature of the CDM crystallization plate, the final supersaturation in the CDM method is higher, resulting in a larger number of screening hits in CDM method than in SDVD method.

Another mechanism is also responsible for the increased crystal quality. Some volatile gas or small organic molecules that can diffuse from one droplet to another are found in the crystallization droplets. This process is equivalent to adding some volatile additives to all of the crystallization conditions, thereby increasing new crystal optimization conditions.

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It is necessary to note that the CDM crystallization plates have more opportunities to produce salt crystals. For example, as shown in Table 1, five proteins (catalase, α-chymotrypsin II, myoglobin, hemoglobin and ribonuclease A III) did not yield salt crystals in the SDVD method but produced many salt crystals in the CDM crystallization plate. This result is probably because the CDM crystallization plate is a permeable plate, which can result in a wider concentration range in the crystallization drop over time. At higher concentrations in the crystallization drop, salt crystals could be produced in the CDM crystallization plate, and once the protein cannot crystallize in a given crystallization condition, salt crystals will have greater competitive ability.

(ii) Supersaturation in CDM method varies more slowly than in the SDVD method. Again, as described above, the supersaturation level increased more slowly in the CDM method than in the SDVD method. It is easy to understand this phenomenon. As all the droplets are in the same space, there is a common equilibrium vapor pressure in the space. In the initial stage, all of the droplets are different from each other, and hence their vapor pressures are different from each other. When the vapor pressure of one droplet is lower than the common equilibrium vapor pressure, the droplet will absorb solvent from other droplets, decreasing the supersaturation of this droplet. When the vapor pressure of one droplet is higher than the common equilibrium vapor pressure, the solvent of this droplet will diffuse from it to other droplets with lower vapor pressure, and in this case the supersaturation of this droplet will increase. As the CDM plate is permeable, the transfer of the solvent and some volatile chemicals from inside the plate to outside will occur, finally resulting in an increased supersaturation level of all of the droplets in the CDM method. From the above postulation and the observed experimental facts, we can understand that the supersaturation in the CDM method varied more slowly than in the SDVD method, which is the major reason for the improved crystal quality in the CDM method.

Conclusions

In conclusion, 11 different proteins were screened in the CDM crystallization plate, and it was found that the CDM method can not only increase the number of crystallization screening hits but also improve the quality of protein crystals compared with the conventional SDVD method. This crystallization screening method may be suitable for routine protein crystallization.

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Notes and references

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Abbreviations: CDM, cross-diffusion microbatch; SDVD, sitting-drop vapor-diffusion.

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