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An attempt to increase the efficiency of protein crystal screening: a simplified screen and experiments

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Macromolecular crystallization remains a bottleneck in structure determination by X-ray diffraction. Based on the data reflecting success rates of crystallization conditions in different screens and the information derived from the BMCD and other related studies, a simplified screen has been designed to increase the success rate of traditional screening and to save samples, time and cost. The screen has been tested with six protein samples which had been crystallized before and its comparison with Crystal Screen (Hampton Research) was also performed with lysozyme crystallization. The experimental results show that for obtaining crystal leads, the success rate of the simplified screen is reasonably higher. In addition, it has been successful in crystallizing two new proteins from snake venom using the simplified screen that had failed with Crystal Screen. These results indicate that the simplified screen, which assembles and optimizes the efficient crystallization conditions from distinct screens, extends the region of crystallization and improves the success rate of screening. Based on information of newly published efficient crystallization conditions, the simplified screen could be developed and optimized continuously in the future.

1. Introduction

Protein crystallography has assumed an important role for the three-dimensional structure determination of biological macromolecules. High-quality crystals of macromolecules are a prerequisite for their structure determination by X-ray diffraction. However, it remains dependent on empirical approaches (McPherson, 1999). Crystallization of macromolecules is a major bottleneck for structure determination.

There are several main factors affecting crystallization of proteins, such as temperature, pH value, precipitant, buffer, additive, detergent *etc*. The assembly of the parameters makes up the protein crystallization conditions. There are three typical methods to produce crystallization kits: grid, footprint and random screening. It has been proved that random screening has the greatest average crystallization efficiency (Brent, 2001). Two theoretical innovations, the incomplete factorial experiment (Carter & Carter, 1979) and sparse-matrix sampling (Jancarik & Kim, 1991), introduced the idea of analyzing combinations of independent factors that affect crystallization. In the two approaches, matrices of crystallization conditions are commonly explored. Furthermore, the latter method selects efficient crystallization conditions from known or published ones. This suggested that in the crystallization region a proper combination of factors is more important than subtle variation of a single factor.

In recent years, many endeavours to develop protein crystallization have taken place and have resulted in several commercial and public domain crystallization screens (*e.g.* Jancarik & Kim, 1991; Abergel *et al.*, 1991; Stura *et al.*, 1992; Cudney *et al.*, 1994; Zeelen *et al.*, 1994; Bergfors, 1999; Brzozowski & Walton, 2001; Hampton Research, USA; Molecular Dimensions, UK; Emerald BioStructures Inc., USA *etc.*). The underlying principles of these screens were initially based on the two abovementioned innovations. All these screens have produced successful data or experience regarding protein crystal screening.

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Table 1
The simplified screen.

Abbreviations: PEG, polyethylene glycol; MME, monomethylether; MPD, 2-methyl-2,4-pemtanediol; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; Tris, Tris hydrochloride; CAD, Na cacodylate.

No.	Buffer	pH	Salts	Precipitant
1	0.1 *M* CAD	6.5	0.2 *M* $(NH_4)_2SO_4$	30% PEG 8K
2	0.1 *M* Tris	8.5	0.2 *M* Na citrate	30% PEG 400
3	0.1 *M* HEPES	7.5	0.2 *M* $CaCl_2$	28% PEG 400
4	0.1 *M* Tris	8.5	0.2 *M* $MgCl_2$	30% PEG 4K
5	0.1 *M* HEPES	7.5	0.2 *M* $MgCl_2$	30% 2-propanol
6	0.1 *M* CAD	6.5	0.2 *M* NaAc	30% PEG 8K
7	0.1 *M* Tris	8.5	None	2.0 *M* $(NH_4)_2SO_4$
8	0.1 *M* Tris	8.5	0.2 *M* NaAc	30% PEG 4K
9	0.1 *M* Tris	8.5	0.2 *M* NH_4Ac	30% 2-Propanol
10	None		None	0.4 *M* NaK tartrate
11	0.1 *M* HEPES	7.5	None	2.0 *M* NaAc
12	0.1 *M* HEPES	7.5	None	2.0 *M* $(NH_4)_2HPO_4$
13	0.1 *M* HEPES	7.5	None	2.0 *M* Na citrate
14	0.1 *M* HEPES	7.5	None	2.0 *M* Na formate
15	0.1 *M* HEPES	7.5	None	2.0 *M* $MgSO_4$
16	0.1 *M* HEPES	7.5	25 m*M* $MgCl_2$	2.0M KH_2PO_4
17	0.1 *M* CAD	6.5	0.2 *M* Na citrate	20% PEG 4K + $(NH_4)_2SO_4$
18	0.1 *M* HEPES	7.5	0.2 *M* Na citrate	20% MPD
19	0.1 *M* NaAc-Ac	4.5	0.2 *M* NaH_2PO_4	20% PEG 4K
20	0.1 *M* Na citrate	5.5	None	30% MPD
21	0.1 *M* HEPES	7.5	0.2 *M* NaCl	40% MPD
22	0.1 *M* Tris	8.5	2.5% *t*-butanol	30% MPD
23	0.1 *M* Tris	8.5	0.8 *M* Na formate	25% PEG 2K MME
24	0.1 *M* HEPES	7.5	0.2 *M* $MgCl_2$	15% PEG 4K
25	0.1 *M* HEPES	7.5	0.2 *M* $MgCl_2$	8% PEG 20K + 8% PEG 550 MME
26	0.1 *M* CAD	6.5	0.2 *M* KBr	15% PEG 4K
27	0.1 *M* HEPES	7.5	0.2 *M* KSCN	10% PEG 8K + 10% PEG 1K
28	0.1 *M* Tris	8.0	25 m*M* $MgCl_2$	18% PEG 5K MME
29	0.1 *M* CAD	6.5	0.2 *M* Li_2SO_4	15% PEG 4K
30	0.1 *M* HEPES	6.5	None	20% Jeffamine
31	0.1 *M* HEPES	6.5	0.8 *M* Na formate	25% PEG 4K
32	0.1 *M* HEPES	6.5	0.8 *M* Ca acetate	15% PEG 4K/PEG 2K MME
33	0.1 *M* Tris	8.0	0.2 *M* KSCN	20% PEG 5K MME
34	0.1 *M* HEPES	7.5	None	2.0 *M* Na malonate

The increasing information from the Biological Macromolecular Crystallization Database (BMCD; Gilliland *et al.*, 1994) has gradually evolved many compositions of crystallization conditions on the basis of currently published results and the intuitional experience of their creators. In addition, several salts were found to have higher efficiency for protein crystallization (McPherson, 2001).

In order to increase the efficiency of protein crystal screening, based on the relevant useful data or studies, we have designed a simplified screen for protein crystallization and tested it with different proteins.

2. Screen design

Based on the success rates of crystallization conditions, *i.e.* the percentage of proteins crystallized under the crystallization conditions in each of selected screens and the crystallization tests in published relevant studies, we have devised a simplified screen (Table 1). The screen combines the most efficient crystallization conditions selected from distinct screens and in each selected screen the selected conditions cover the major crystallization space (Rupp, 2003). The crystallization conditions of the simplified screen come mainly from incomplete factorial experiments (Carter & Carter, 1979), sparse-matrix sampling (Jancarik & Kim, 1991), clear strategy screens (Brzozowski & Walton, 2001) and the comparison of salts for the crystallization of macromolecules (McPherson, 2001) *etc*. The crystallization conditions were optimized based on the factors of successful crystallizations obtained from the BMCD (Gilliland *et al.*, 1994).

Table 2
Results of lysozyme crystallization with Crystal Screen and the simplified screen.

	Crystallization condition Nos. with crystals	Success rate of crystallization
Crystal Screen	7, 9, 13, 22, 32	5/50 = 10.0%
Simplified screen	2, 8, 11, 14, 16, 17, 25	7/34 = 20.6%

3. Experiments and methods

To compare the success rates with different screens, hen egg-white lysozyme was used for crystal screening. Then, to test the efficiency of protein crystal screening with the simplified screen, we have tested six protein samples crystallized previously: lysozyme, haemorrhagin I, acutothrombin C, nuclear phospholipase, acid phospholipase and trichosanthin. The first four protein samples had been crystallized during the Chinese mission SZ-3 (Cang *et al.*, 2003). The last two proteins were chosen just because they had been crystallized before in our institute (Pan *et al.*, 1993; Chen & Chen, 1989). Lastly, the initial crystallization conditions of two novel proteins vn5-3 and vn4-3 from Russian snake venom (work to be published), which had not been crystallized before, were investigated. The chemical agents used in the crystallization conditions were purchased from Sigma and Fluka Co.

In the experiments, we used two crystallization plates, a 16-well Linbro plate and a 96-well Corning plate, which were supplied by Corning Company. The former used the hanging-drop method and 0.4 ml reservoir solution and the latter the sitting-drop method and 0.2 ml reservoir solution. Each protein was prepared at a concentration of 20 mg ml^{-1} in distilled water and the experiments were performed at a temperature of 291 K. In the two kinds of experiments, drops were composed of 1.0 µl reservoir solution and 1.0 µl protein solution and tested in the same way for each protein. The plates were examined periodically under microscopes over one week.

According to the general rules for efficiency analysis of screens (*e.g.* Brent, 2001), the crystallization state was observed using a stereoscopic microscope with polarizer. The crystals were identified by the crystal morphology, polarization and density (Ducruix & Giegé, 1999) and in some cases the crystals were dyed with Izit Crystal Dye purchased from Hampton Research.

4. Results and discussion

To compare the success rates for the simplified screen and Crystal Screen, crystallization experiments of lysozyme were performed with

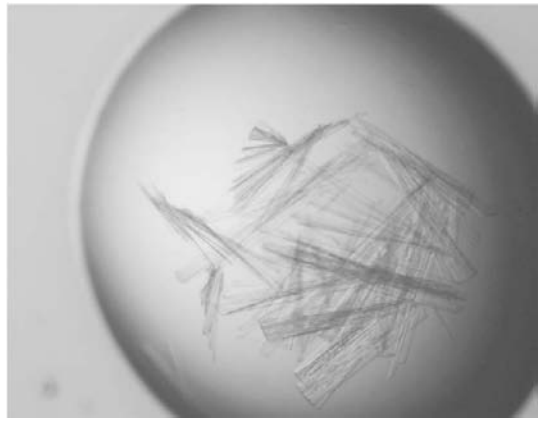

Figure 1
The initial crystals of the protein vn5-3 obtained with the simplified screen.

Table 3
The results of screening experiments with the simplified screen for six different proteins.

Proteins	Total of two plates		Linbro plate		Corning plate	
	Condition Nos. with crystals	Success (%)	Condition Nos. with crystals	Success (%)	Condition Nos. with crystals	Success (%)
Lysozyme	2, 5, 8, 11, 14, 16, 17, 25	23.5	2, 8, 11, 14, 16, 17, 25	20.6	5, 16, 25	8.8
Trichosanthin	1, 11, 16, 22, 30	14.7	1, 11, 16, 30	11.8	16, 22, 30	8.8
Haemorrhagin I	12, 13, 30, 33	11.8	12, 30, 33	8.8	13	2.9
Acutothrombin C	23, 25, 26, 27, 29, 31	17.6	26, 27, 29, 31	11.8	23, 25, 27, 29	11.8
Acid phospholipase	12, 21, 30, 31, 32	14.7	12, 21, 31, 32	11	21, 30	5.9
Nuclear phospholipase	3, 5, 20, 21, 23	14.7	3, 5, 20, 21	11.8	21, 23	5.9

the two kinds of screens. The results are listed in Table 2. The success rate of crystallization with the simplified screen is 20.6% and is 10% for Crystal Screen. This indicates that the success rate of the simplified screen is twice that of Crystal Screen for the lysozyme crystallization. In fact, crystallization conditions Nos. 2 and 8 in the simplified screen were chosen from Crystal Screen and were equivalent to its conditions Nos. 13 and 22, respectively. The crystallization conditions Nos. 11 and 14 in the simplified screen were equivalent to 'ammonium formate' and 'sodium acetate' conditions (McPherson, 2001), which produced crystals before in lysozyme crystallization. It is obvious that the simplified screen covers more crystallization space. This may be the reason why it has higher efficiency than Crystal Screen in lysozyme crystallization.

From the results of crystallization experiments with six protein samples (Table 3), we know that there is a diversity of initial crystallization efficiency among the six proteins. Furthermore, the total success rate of initial crystallization screening varies from 11.8 to 23.5%; that of the 16-well Linbro plate is from 8.8 to 20.6% and that of the 96-well Corning plate is from 5.9 to 11.8%. This may indicate that the screening effect of the simplified screen is satisfactory. In Table 3, the conditions producing crystal leads are distributed, to some extent, equally in the simplified screen, implying that all the important crystallization conditions from different screens selected could play a role in the screening and the simplified screen covers more extensive area of crystallization.

Comparing the efficiency of obtaining the crystal leads with both Linbro plate and Corning plate, as a whole, we can see that the Linbro plate has higher rate of crystallization than Corning plate. On the other hand, under some conditions of the simplified screen, crystal leads can be obtained using Corning plate, but precipitates were obtained with the Linbro plate. This kind of phenomena in the experiments partly illuminated the known effects of the experimental geometry (Luft & DeTitta, 1995), namely the crystallization chambers and distance between the drop and reservoir may result in the diversity of diffusion velocity, which leads to the difference in crystallization (Luft *et al.*, 1996; Forsythe *et al.*, 2002).

We have tried to screen crystal leads of two novel snake-venom protein vn5-3 and vn4-3 using Crystal Screen and Crystal Screen 2, but have failed. We then performed the experiments using the simplified screen and obtained good crystal leads for the two proteins (Fig. 1 for protein vn5-3). By optimizing the initial crystallization conditions of protein vn5-3, we obtained suitable crystals for X-ray diffraction data collection. The structure determination is under way. The primary crystallization conditions of protein vn4-3 have been optimized and higher resolution data have been collected with the improved crystals (Gao *et al.*, 2005). The crystallization results of the two novel proteins may indicate that the simplified screen covers more crystallization space than the two commercial screens and that this made the two proteins produce crystal leads.

Assembling the efficient crystallization conditions in a reasonable manner from selected screens, the combination of crystallization areas would improve the efficiency of protein crystallization. The results of test experiments with the simplified screen have demonstrated reasonableness of our design. Our idea is in agreement with the trends observed in other crystal screens. Kimber's research group (Kimber *et al.*, 2003) has tested 755 proteins and 45% of the proteins were crystallized with Crystal Screen. Of the proteins crystallized, 60% could be crystallized with as few as six conditions. This result has verified that the crystallization efficiency is different among the conditions in the screen and the minority of conditions covers the major area of crystallization space. Different screens focus on distinct accessible area of crystallization (Majeed *et al.*, 2003). Reasonable assembly of the efficient crystallization conditions from various screens should be a good direction towards higher efficient screens.

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