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A quality comparison of protein crystals grown under containerless conditions generated by diamagnetic levitation, silicone oil and agarose gel

Hui-Ling Cao,[a]‡ Li-Hua Sun,[b]‡ Jian Li,[b]‡ Lin Tang,[b] Hui-Meng Lu,[a] Yun-Zhu Guo,[a] Jin He,[a] Yong-Ming Liu,[a] Xu-Zhuo Xie,[a] He-Fang Shen,[a] Chen-Yan Zhang,[a] Wei-Hong Guo,[a] Lin-Jun Huang,[a] Peng Shang,[a] Jian-Hua He[b]*§ and Da-Chuan Yin[a]*§

[a]School of Life Sciences, Northwestern Polytechnical University, 127 Youyi Road, Xi'an City, Shaanxi 710072, People's Republic of China, and [b]Shanghai Institute of Applied Physics, Chinese Academy of Sciences, No. 239 Zhangheng Road, Shanghai 201204, People's Republic of China

‡ These authors contributed equally to this work.
§ These authors are co-corresponding authors.

Correspondence e-mail: hejianhua@sinap.ac.cn, yindc@nwpu.edu.cn

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High-quality crystals are key to obtaining accurate three-dimensional structures of proteins using X-ray diffraction techniques. However, obtaining such protein crystals is often a challenge. Several containerless crystallization techniques have been reported to have the ability to improve crystal quality, but it is unknown which is the most favourable way to grow high-quality protein crystals. In this paper, a quality comparison of protein crystals which were grown under three containerless conditions provided by diamagnetic levitation, silicone oil and agarose gel was conducted. A control experiment on a vessel wall was also simultaneously carried out. Seven different proteins were crystallized under the four conditions, and the crystal quality was assessed in terms of the resolution limit, the mosaicity and the $R_{\rm merge}$. It was found that the crystals grown under the three containerless conditions demonstrated better morphology than those of the control. X-ray diffraction data indicated that the quality of the crystals grown under the three containerless conditions was better than that of the control. Of the three containerless crystallization techniques, the diamagnetic levitation technique exhibited the best performance in enhancing crystal quality. This paper is to our knowledge the first report of improvement of crystal quality using a diamagnetic levitation technique. Crystals obtained from agarose gel demonstrated the second best improvement in crystal quality. The study indicated that the diamagnetic levitation technique is indeed a favourable method for growing high-quality protein crystals, and its utilization is thus potentially useful in practical efforts to obtain well diffracting protein crystals.

1. Introduction

In the post-genomics era, it is essential to explore both the structure and the function of proteins. To date, more than 88% of the three-dimensional structures of proteins in the Protein Data Bank (PDB) have been determined by X-ray crystallography. Crystal quality is the key to obtaining accurate three-dimensional structures of proteins using this technique. However, obtaining well diffracting crystals continues to be the bottleneck for the technique (Chayen, 2002; Durbin & Feher, 1996).

There is typically a solid–liquid interface in a conventional crystallization technique, which often acts as a heterogeneous nucleation site for crystallization. Such sites are beneficial for initiating nucleation, but may not be beneficial for obtaining high-quality crystals (Chayen, 1996). A lattice mismatch between the crystal and the solid nucleation site may occur, which leads to internal stress in the crystal and subsequently deteriorates the crystal quality. Containerless crystallization

techniques, in which no contact between the growing crystals and the solid surface occurs, have thus been proposed as a solution to this problem (Yin *et al.*, 2008; Chayen, 1996). Various containerless crystallization techniques that are used in protein crystallization have been reported to improve crystal quality (Moreno *et al.*, 2002, 2007; Wolfova *et al.*, 2005; Chayen, 1996; Lorber & Giegé, 1996). For example, containerless crystallization of proteins can be achieved using oils (Bolanos-Garcia, 2005; Chayen, 1996, 1999) and gels (Tanabe *et al.*, 2009; García-Ruiz *et al.*, 2001; Lorber *et al.*, 1999). The diamagnetic levitation technique (Yin *et al.*, 2008, 2009) and the ultrasonic levitation technique (Cao *et al.*, 2012) are also suitable methods. The former is one of the best techniques for achieving containerless conditions and has been successfully applied to protein crystallization (Yin *et al.*, 2008). The technique has long been thought to enhance crystal quality judging from the reported positive effects exerted by the magnetic field and the simulated microgravity environment (Saijo *et al.*, 2005; Yin *et al.*, 2004; Kinoshita *et al.*, 2003; Sato *et al.*, 2000, 2001; Lin *et al.*, 2000). However, a systematic investigation of the diamagnetic levitation technique has not yet been reported. Therefore, it is of interest to explore whether or not this technique can help to promote crystal quality.

Because all of the abovementioned containerless crystallization techniques demonstrated potentially positive effects on protein crystal quality, it is of interest to compare them with one another and to determine how each technique affects the crystal quality in order to provide researchers with guidelines to choose the appropriate technique to obtain the desired crystals. However, there has not been a systematic investigation to compare the quality of protein crystals produced by different containerless techniques. In this paper, a study was conducted to compare the quality of protein crystals grown under different containerless conditions and those grown as a control in contact with a solid surface. The containerless techniques used were created using diamagnetic levitation, silicone oil and agarose gel. The ultrasonic levitation method was not examined in this study because the size of the levitated droplet changes rapidly during the crystallization process such that it is difficult to maintain identical crystallization conditions across the techniques. Seven different proteins were crystallized using the four techniques (the three containerless techniques and the control) and crystal quality was evaluated by X-ray diffraction analysis at the Shanghai Synchrotron Radiation Facility (SSRF). It was found that containerless crystallization conditions indeed improved the crystal quality, and that the different containerless techniques demonstrated different levels of enhancement: the diamagnetic levitation technique exhibited the best performance, followed by the use of agarose gel.

2. Materials and methods

2.1. Crystallization conditions

2.1.1. Diamagnetic levitation. The diamagnetic levitation technique was achieved using a large-gradient superconducting magnet (JMTA-16 T 50MF, Japan Superconductor Technology Co., Japan). Nearly all crystallization droplets are aqueous solutions that experience a repulsive force (magnetization force or Kelvin force) in the magnetic field. When the repulsive force is directed upwards and is large enough, a balance between the gravitational force and the magnetization

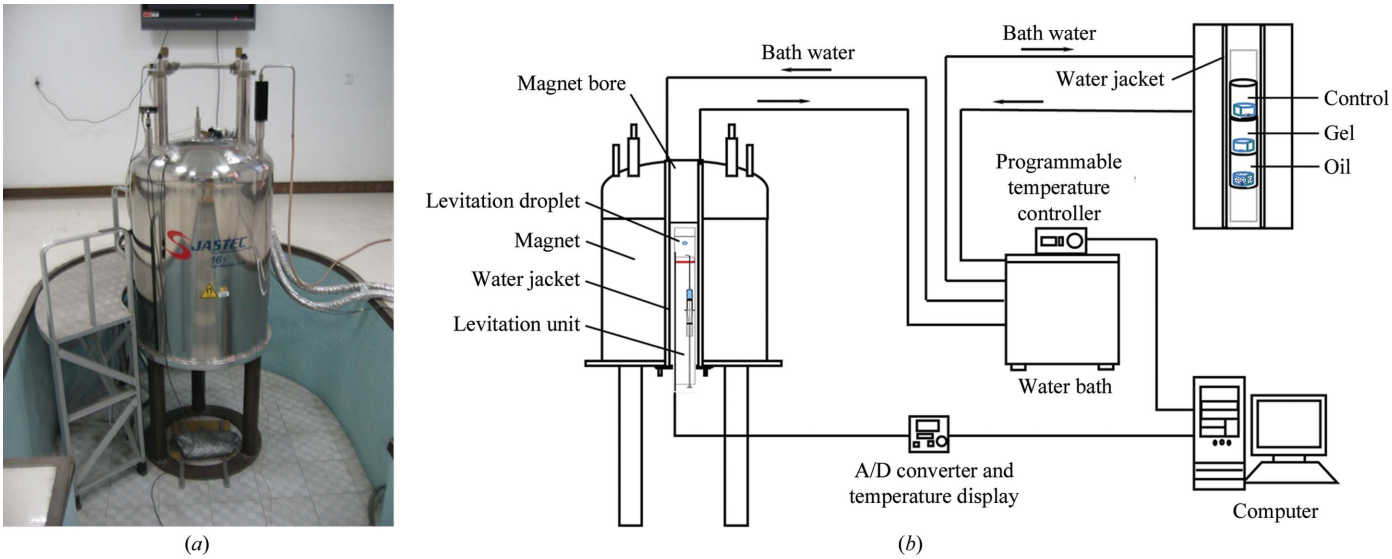

Figure 1
Experimental setup. (*a*) The superconducting magnet used in the experiment; (*b*) a schematic illustration of the experimental setup. Protein crystallization was performed in four different conditions: diamagnetic levitation (inside the superconducting magnet), the control, agarose gel and silicone oil. All crystallization experiments were conducted in sealed chambers with identical dimensions: 32 mm diameter × 66 mm. The chamber for the diamagnetic levitation technique was inserted into the temperature controller inside the magnet. The other three chambers for the control, agarose gel and silicone oil were placed in turn into a long cylinder that was inserted into the other temperature controller outside the magnet for crystallization experiments.

force can be reached and an aqueous droplet can be levitated stably in the air inside the magnet. Fig. 1 shows the superconducting magnet (Fig. 1*a*) and the configuration of the experimental setup (Fig. 1*b*) used in this experiment. A schematic illustration of the diamagnetic levitation technique is shown in Fig. 2(*a*).

For strict comparison of the four conditions, two temperature controllers (cylindrical water jackets) were built (Lu *et al.*, 2008) that were identical in shape and size and were controlled by the same bath circulator (PolyScience 9712, PolyScience, USA). One temperature controller was inserted into the magnet body for diamagnetic levitation experiments and the other was placed outside the magnet for the other three crystallization conditions (control, agarose gel and silicone oil, which will be described below) as shown in Fig. 1(*b*). The temperature of the setup could be controlled in the range 277–303 ± 0.1 K.

Inside the temperature controller placed within the magnet, a sealed levitation chamber was installed. The diamagnetic levitation experiment was performed in the levitation chamber; 200 µl crystallization solution was injected into the chamber (32 mm diameter × 66 mm) and was levitated by the magnetization force. The crystallization of the levitated crystallization droplet was then observed in real time and *in situ* by a CCD camera installed above the levitation position (Lu *et al.*, 2008). The levitation volume of drops in the magnet can range from 50 to 2000 µl. In the current research, the volume of the levitated droplet was 200 µl for convenient levitation operation and real-time observation. However, this volume may be too large for practical application. This problem could be solved by improving the levitation setup in order to minimize protein consumption.

2.1.2. Silicone oil. Containerless protein crystallization without contact with a solid surface can be achieved using oils (D'Arcy *et al.*, 2004; Chayen, 1996, 1999). Several silicone oils were tested and a high-density silicone oil with a density of 1.05 g cm^{-3} (Sigma–Aldrich catalogue No. 175633) was finally selected as the medium for the containerless crystallization experiment. The density of the crystallization solutions used in this study ranged from 1.02 to 1.06 g cm^{-3}, which was very close to the density of the silicone oil. The crystallization droplet was completely immersed in the oil and exhibited a spherical or elliptic shape, and there was only a small area of contact between the droplet and the bottom of the vessel. To begin the experiment, 3 ml silicone oil was injected into a small organic glass vessel (28 mm diameter × 12 mm). 200 µl of the crystallization droplet was then introduced into the silicone oil. Lastly, the small vessel was placed into a sealed chamber (32 mm diameter × 66 mm) as shown in Fig. 2(*b*).

2.1.3. Agarose gel. Protein crystallization can occur within agarose gel (Tanabe *et al.*, 2009; García-Ruiz *et al.*, 2001; Lorber *et al.*, 1999). The crystals grow in the gel without any contact with the solid surface and this can be considered to be the result of containerless crystallization. After tests, final concentrations of agarose gels were selected for the experiments as follows: 0.2%(*w*/*v*) agarose gel for lysozyme (hen egg-white lysozyme) and pK (proteinase K) and 0.1%(*w*/*v*) agarose gel for the other five proteins.

The agarose powder (catalogue No. 111760, Gene Tech Co. Ltd, People's Republic of China) used in this study has a gel

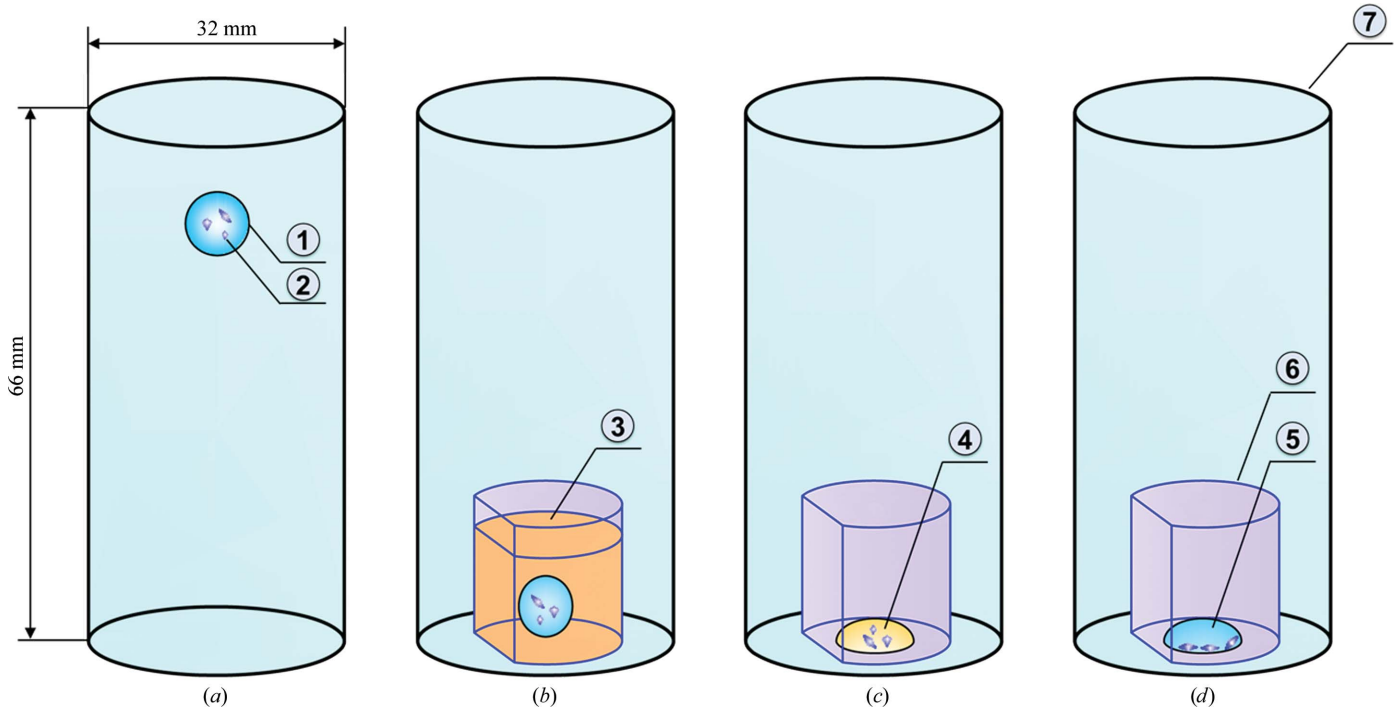

Figure 2
A schematic illustration of the crystallization experiments under the four conditions: (*a*) diamagnetic levitation; (*b*) silicone oil; (*c*) agarose gel; (*d*) the control. 1, Crystallization droplet; 2, crystals; 3, high-density silicone oil; 4, the mixture of gel stock solution and crystallization solution; 5, the control droplet; 6, the small organic glass vessel; 7, the sealed organic glass chamber.

Table 1
The initial experimental conditions used for protein crystallization.

Initial *C*, initial concentration of protein before being mixed with precipitant; *T*, temperature for protein crystallization; PEG 5000 MME, polyethylene glycol 5000 monomethyl ether; PEG 2000 MME, polyethylene glycol 2000 monomethyl ether.

Protein	Initial *C* (mg ml^{-1})	*T* (K)	Period (d)	Buffer	Precipitant	Reference
lys	50	293	3	0.1 *M* sodium acetate pH 4.60	60 mg ml^{-1} NaCl	Wang *et al.* (2010)
pK	20	293	3	0.05 *M* sodium cacodylate, 0.08 *M* magnesium acetate pH 6.50	20%(*w*/*v*) PEG 8000	Wang *et al.* (2010)
tcs	Saturated solution	293	7	0.075 *M* sodium citrate pH 5.40	14%(*w*/*v*) KCl	Sun *et al.* (2010)
con	8	293	5	0.1 *M* bis-tris pH 6.50	2%(*w*/*v*) PEG 5000 MME	Lu *et al.* (2010)
HSP90^{N}	13	277	5	0.1 *M* sodium cacodylate, 0.2 *M* magnesium chloride, 0.1 *M* sodium acetate pH 6.50	25%(*w*/*v*) PEG 2000 MME	Li *et al.* (2012)
thau	30	293	6	0.1 *M* potassium phosphate pH 7.00	1 *M* potassium sodium tartrate	Pietras *et al.* (2010)
cata	8	293	7	0.1 *M* succinic acid pH 7.00	15%(*w*/*v*) PEG 3350	Zhang *et al.* (2008)

strength [1%(*w*/*v*)] of 750 g cm^{-2} and a gelling temperature of 310 ± 1.5 K. Firstly, 0.2 and 0.4% gel stock solutions were prepared by heating the solutions in a microwave oven until transparent; they were then immediately filtered through 0.22 µm filters and stored at 277 K. Prior to crystallization, the prepared gel stock solutions were liquefied at 363 K and then maintained at 318 K.

Protein and precipitant solutions were prepared at double concentration, according to the conditions shown in Table 1. Equal volumes of protein solution and precipitant solution were mixed. Equal volumes (100 µl) of the mixtures and prepared gel stock solutions were then mixed in identical small vessels (28 mm diameter × 12 mm) and were placed in sealed chambers (32 mm diameter × 66 mm) as shown in Fig. 2(*c*). In this way, the final concentrations of proteins and precipitants were identical to those in the other crystallization conditions. During solution preparation, care must be taken to avoid bubble formation and protein denaturation owing to the transient high temperature of the agarose gel solutions.

2.1.4. The control. As a control, a 200 µl crystallization droplet was directly introduced into an identical small vessel (28 mm diameter × 12 mm) and this was then placed in a sealed chamber (32 mm diameter × 66 mm) as shown in Fig. 2(*d*). Lastly, the three identical sealed chambers for the control, agarose gel and silicone oil were placed in turn into a long cylinder that was inserted into the temperature controller outside the magnet for the crystallization experiments, as shown in Fig. 1(*b*). All other conditions for the control were identical to the containerless crystallization experiments.

2.2. Materials

Seven different proteins were used in the tests. Hen egg-white lysozyme (HEWL; lysozyme; lys) was purchased from Seikagaku Kogyo Corporation, Japan (catalogue No. 100940). Proteinase K (pK; catalogue No. P6556), concanavalin (con; catalogue No. L7647), thaumatin (thau; catalogue No. T7638) and catalase (cata; catalogue No. C40) were purchased from Sigma–Aldrich. All of the above commercial proteins were used without further purification. Trichosanthin (TCS) was extracted from the root tuber of the perennial plant *Trichosanthes kirilowii* Maxim (Cucurbitaceae). The purity of lyophilized TCS powder can reach 99%. Because the solubility of TCS in water is low, saturated solutions were prepared for crystallization experiments. HSP90^{N} was expressed by the recombinant plasmid pET-28 in *Escherichia coli* strain BL21 (Invitrogen, Carlsbad, USA) and was purified by Ni Sepharose affinity chromatography and size-exclusion chromatography using a Superdex 75 column (GE Healthcare, Piscataway, USA). The high-density silicone oil with a density of 1.05 (catalogue No. 175633, Sigma–Aldrich) and the agarose powder (catalogue No. 111760, Gene Tech Co. Ltd, People's Republic of China) were used directly without further purification. All other chemicals were also purchased from Sigma–Aldrich and used without further purification.

2.3. Crystallization experiments

The crystallization solutions were prepared using the conventional batch method. The protein solutions were prepared by dissolving the proteins in the corresponding buffers as shown in Table 1, followed by centrifugation at 13 000*g* at 277 K for 15 min in a low-temperature centrifuge. The precipitant solutions were prepared by dissolving the chemicals in deionized water, adjustment to a suitable pH value and filtration through 0.22 µm filters. Equal volumes of protein and precipitant solutions were then mixed to yield the final crystallization solution. The initial concentrations and crystallization conditions for the seven proteins are listed in Table 1. The sample volume for all of the crystallization techniques was 200 µl. All of the samples were maintained at 277–293 K during crystallization over 3–7 d.

2.4. Image capture

A stereomicroscope (Olympus SZX 16, Japan) was used to capture images of the harvested crystals.

2.5. X-ray diffraction experiments

From each condition for each protein, 3–5 crystals with the best morphology were selected and each one was captured in a different nylon CryoLoop (Hampton Research). The crystals were soaked briefly in cryoprotectant solution, which consisted of 20–25% glycerol and the corresponding precipitants and buffers (as shown in Table 1), prior to cryo-cooling in order to prevent ice formation during cooling. The

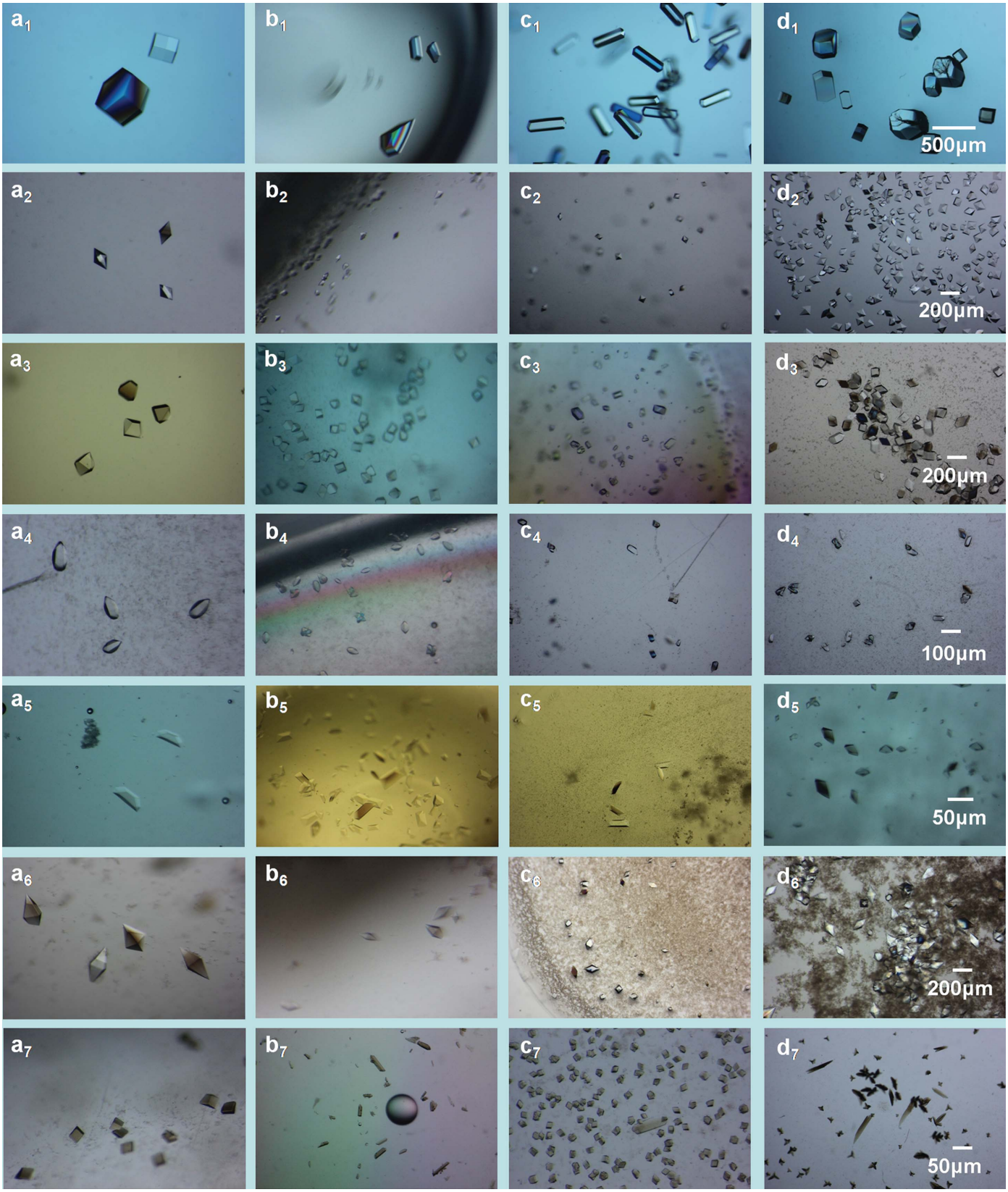

Figure 3
Images of crystals of the seven different proteins grown in the four crystallization conditions: a, diamagnetic levitation; b, silicone oil; c, agarose gel; d, the control. The subscripts 1–7 represent lysozyme, proteinase K, trichosanthin, concanavalin A, $HSP90^{N}$, thaumatin and catalase, respectively. Crystals grown under containerless conditions demonstrated better morphology than those of the control. Crystals grown using the diamagnetic levitation technique exhibited the best morphology among the three containerless conditions.

Table 2
A summary of X-ray diffraction data statistics for the crystals of seven different proteins grown under the four crystallization conditions.

Values in parentheses are for the highest resolution shell.

		Diffraction data statistics					
Protein	Condition	Resolution range (Å)	Mosaicity (°)	$\langle I\rangle/\langle\sigma(I)\rangle$	$R_{\rm merge}$† (%)	Redundancy	Completeness (%)
lys	Magnetic levitation	50–0.95 (0.98–0.95)	0.17	5.6 (77.8)	73.9 (2.5)	24.9 (9.5)	98.3 (86.5)
	Silicone oil	50–1.20 (1.22–1.20)	0.39	7.4 (51.7)	88.5 (8.6)	27.2 (26.8)	99.9 (100)
	Agarose gel	50–1.10 (1.14–1.10)	0.57	6.2 (76.9)	59.6 (6.6)	24.4 (24.0)	99.4 (100)
	Control	50–1.20 (1.22–1.20)	0.27	9.3 (60.6)	54.0 (4.3)	14.3 (13.8)	99.9 (96.8)
pK	Magnetic levitation	50–0.95 (0.98–0.95)	0.13	10.9 (50.0)	67.5 (6.8)	23.8 (10.5)	98.2 (88.3)
	Silicone oil	50–1.12 (1.14–1.12)	0.40	7.8 (31.7)	59.9 (13.2)	26.8 (26.1)	100 (100)
	Agarose gel	50–1.02 (1.06–1.02)	0.25	11.5 (76.8)	46.7 (7.2)	25.2 (24.6)	98.8 (96.2)
	Control	50–1.14 (1.16–1.14)	0.19	15.3 (78.0)	24.9 (2.5)	26.4 (14.8)	99.9 (99.1)
TCS	Magnetic levitation	50–1.12 (1.14–1.12)	0.27	5.8 (43.5)	36.4 (4.1)	6.8 (6.6)	99.8 (99.6)
	Silicone oil	50–1.43 (1.45–1.43)	0.38	7.4 (47.7)	37.1 (4.0)	7.1 (7.0)	100 (100)
	Agarose gel	50–1.15 (1.17–1.15)	0.29	7.1 (42.9)	52.1 (7.3)	14.1 (13.8)	100 (100)
	Control	50–1.07 (1.09–1.07)	0.22	6.8 (42.9)	41.8 (5.0)	6.9 (6.6)	99.5 (98.8)
con	Magnetic levitation	50–1.23 (1.25–1.23)	0.34	6.6 (90.1)	58.0 (2.4)	14.0 (11.1)	99.1 (84.7)
	Silicone oil	50–1.76 (1.79–1.76)	0.53	7.6 (46.8)	51.6 (4.6)	7.1 (6.3)	99.1 (98.7)
	Agarose gel	50–1.79 (1.82–1.79)	0.67	4.9 (62.9)	60.7 (5.0)	14.0 (13.5)	99.7 (99.7)
	Control	50–1.78 (1.82–1.78)	0.77	6.6 (94.4)	54.2 (3.3)	14.2 (13.7)	99.9 (99.9)
$HSP90^N$	Magnetic levitation	50–1.61 (1.64–1.61)	0.14	11.3 (52.6)	63.3 (7.4)	14.6 (14.6)	100 (100)
	Silicone oil	50–2.13 (2.17–2.13)	0.91	11.9 (33.8)	77.8 (34.8)	14.2 (14.2)	99.5 (99.5)
	Agarose gel	50–2.15 (2.19–2.15)	1.86	8.8 (52.5)	42.3 (6.3)	14.2 (14.0)	100 (100)
	Control	50–2.89 (2.94–2.89)	2.26	13.5 (53.9)	10.5 (2.5)	3.1 (3.1)	88.7 (85.2)
thau	Magnetic levitation	50–1.35 (1.37–1.35)	0.21	7.9 (53.3)	76.0 (9.6)	28.2 (28.2)	100 (100)
	Silicone oil	50–1.60 (1.63–1.60)	0.70	9.3 (65.5)	78.6 (11.4)	27.6 (27.6)	99.0 (98.6)
	Agarose gel	50–1.50 (1.53–1.50)	0.38	6.8 (56.1)	57.6 (6.2)	14.8 (14.7)	100 (100)
	Control	50–2.70 (2.75–2.70)	1.18	16.6 (42.7)	91.6 (37.4)	24.8 (24.8)	99.9 (99.9)
cata	Magnetic levitation	50–2.28 (2.32–2.28)	0.77	15.3 (94.4)	31.5 (3.8)	10.3 (10.0)	100 (100)
	Silicone oil	50–3.59 (3.65–3.59)	0.73	16.8 (35.1)	29.2 (10.6)	9.5 (9.5)	91.5 (85.6)
	Agarose gel	50–2.70 (2.75–2.70)	0.43	12.8 (53.1)	10.4 (2.5)	10.4 (10.3)	85.5 (82.0)
	Control	50–4.64 (4.72–4.64)	1.35	59.7 (95.5)	16.1 (7.8)	3.9 (3.7)	85.5 (82.1)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle|/\sum_{hkl}\sum_i I_i(hkl)$, where $\langle I(hkl)\rangle$ is the mean intensity of the ith observation of reflection hkl.

crystals were then mounted and cryocooled in liquid nitrogen for X-ray diffraction data collection. All data sets were collected at 100 K in a nitrogen stream on the Macromolecular Crystallography Beamline (BL17U1) at the SSRF using an ADSC Quantum 315r CCD detector. After testing the harvested crystals, data sets were collected from one crystal of each protein crystallized using each of the four conditions (*i.e.* 28 data sets). All of the collected data were integrated and merged using the *HKL*-2000 software package (Otwinowski & Minor, 1997).

3. Results and discussion

3.1. Morphology of the crystals grown under different crystallization conditions

Before harvesting the crystals, images of the crystals were taken using a stereomicroscope (Olympus SZX16, Japan). Fig. 3 shows pictures of crystals of the seven types of protein grown under the four crystallization conditions. The first visual impression is that the crystals grown using the diamagnetic levitation technique exhibited the largest sizes and were relatively few in number. Upon closer inspection, it was found that the crystals grown using the diamagnetic levitation technique typically demonstrated more well defined facets than those obtained under the other conditions. In contrast, some of the crystals obtained from the control exhibited observable defects on the surface (d_1 and d_3 in Fig. 3). Meanwhile, the crystals grown in silicone oil and agarose gel demonstrated better optical perfection than the control. The sizes of the crystals obtained using silicone oil were typically larger than those obtained using agarose gel. It was found that crystals formed more readily in silicone oil than in agarose gel. Nevertheless, the crystals obtained using agarose gel exhibited a more uniform morphology than those obtained using silicone oil.

Taking lysozyme crystals as an example, the diamagnetic levitation technique yielded square or rectangular-shaped single crystals, whereas agarose gel yielded uniformly long rod-shaped crystals (a_1 and c_1 in Fig. 3). For catalase, it was difficult to obtain single crystals with perfect morphology in the control: they were often thin plates or butterfly-shaped twin crystals. However, square-shaped single crystals readily formed in the diamagnetic levitated droplets (a_7 and d_7 in Fig. 3).

The morphological comparison showed that the crystals grown under containerless conditions demonstrated relatively better morphology than the control and that among the three containerless conditions the diamagnetic levitation technique exhibited the best improvement in the crystal morphology, as judged by the crystal size and shape. This was followed by the condition using agarose gel, in which the crystals appeared to have better morphology than crystals obtained using silicone oil, although crystallization in agarose gel was more difficult than in the other three conditions.

3.2. Diffraction analysis of crystals obtained under the different crystallization conditions

The quality of the protein crystals grown under the different conditions was assessed by X-ray diffraction analysis. Crystals with perfect morphology and similar size from each group (one protein crystallized under the four different conditions) were selected for diffraction analysis. The resolution limit, mosaicity and $R_{\rm merge}$ were extracted from the diffraction data for comparison of the crystal quality. Table 2 lists a summary of the diffraction data statistics for crystals of the seven proteins grown under the four conditions. Each protein was

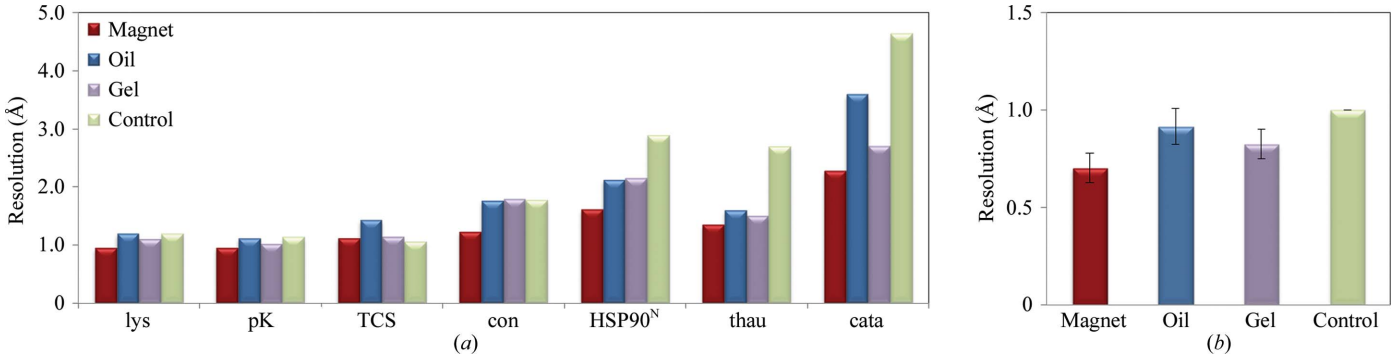

Figure 4
(*a*) A comparison of the resolution limits of the crystals of seven different proteins obtained from the four crystallization conditions; (*b*) a statistical comparison of the resolution limits after normalization to the values found for the controls (error bars show the standard error of the mean; $n = 7$). In terms of the resolution limit of the seven protein crystals, the results demonstrated a significant difference between different groups ($n = 7$, $P = 0.040$, *i.e.* <0.05). The crystals obtained using the diamagnetic levitation technique (labelled 'Magnet' in the image) exhibited the best improvement in resolution limit among the three containerless conditions compared with the control.

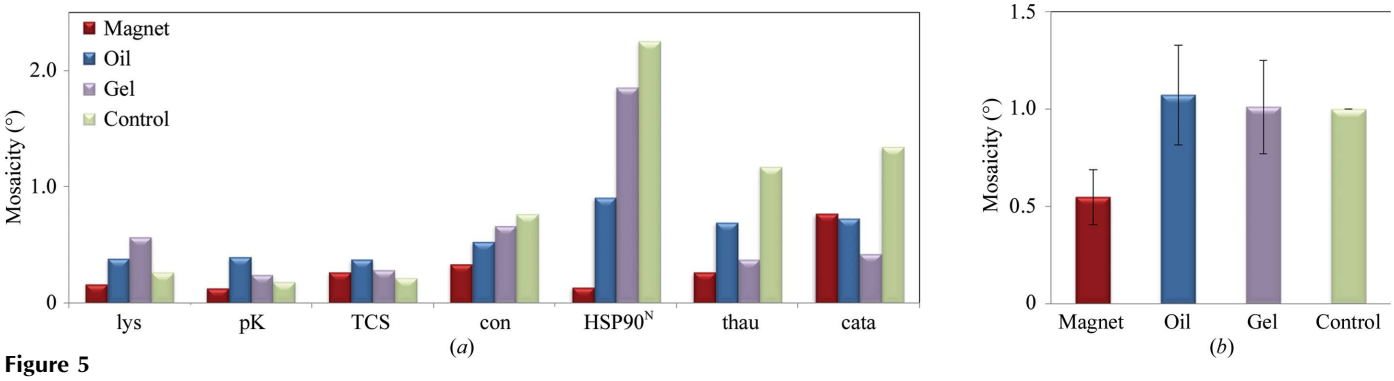

Figure 5
(*a*) A comparison of the mosaicity of the crystals of seven different proteins obtained from the four crystallization conditions; (*b*) a statistical comparison of the mosaicity after normalization to the values found for the controls (error bars show the standard error of the mean; $n = 7$). The results indicated that the crystals obtained using the diamagnetic levitation technique (labelled 'Magnet' in the image) exhibited the best improvement in mosaicity among the three containerless conditions compared with the control. Nevertheless, for the mosaicity, the difference between different groups was not significant ($n = 7$, $P = 0.208$, *i.e.* >0.05).

crystallized under the four conditions simultaneously, and diffraction data were then collected for comparison of the crystal quality. For more detailed information on the diffraction data statistics, please refer to Supplementary Tables S1–S7.[1]

The results of the diffraction analysis indicated that crystals from the three containerless conditions all demonstrated better diffraction properties than the control crystals in contact with the solid vessel wall. Of the three containerless conditions, the crystals obtained from the diamagnetic levitation technique typically exhibited the highest resolution limit, the lowest mosaicity and the lowest $R_{\rm merge}$, indicating that they were of the best quality among the four crystallization conditions. In contrast, the crystals obtained from the control demonstrated the worst crystal quality.

3.2.1. Resolution limit. Fig. 4(*a*) shows a comparison of the resolution limits of the seven different protein crystals obtained from the four crystallization conditions. Three criteria were used to determine the diffraction resolution limit: $R_{\rm merge} < 50\%$, $\langle I\rangle/\langle\sigma(I)\rangle > 2.0$ and completeness > 85%. In most cases, the crystals obtained using the diamagnetic levitation technique demonstrated the highest resolution limit. The crystals obtained from the control exhibited the lowest resolution limit. For a more reliable comparison, a One-Way ANOVA test was applied to the data analysis. The resolution limit was normalized based on the data of the control. Fig. 4(*b*) shows the results after the normalization and statistical tests.

In terms of the resolution limits of the 28 protein crystals tested, the results indicated that the differences between the different crystallization conditions were significant ($n = 7$, $P = 0.040$, *i.e.* <0.05). Compared with the control, the three containerless techniques all clearly improved the resolution limit of the crystals. The diamagnetic levitation technique exhibited a greater improvement than the condition using silicone oil, and there was a similar improvement with the condition using agarose gel. The difference between the conditions using agarose gel and silicone oil was minor.

Further examining the resolution limits, it was found that the improvements were protein-specific. For proteins for which it was difficult to grow high-quality crystals, the

[1] Supplementary material has been deposited in the IUCr electronic archive (Reference: GM5023). Services for accessing this material are described at the back of the journal.

improvement was greater than for the less problematic proteins. For example, the improvement in the resolution limit for the HSP90^{N}, thaumatin and catalase crystals was dramatic, but it was minor for the remaining proteins.

3.2.2. Mosaicity. It is known that lower mosaicity typically indicates fewer defects in the crystal lattice and better crystal quality. Fig. 5(*a*) shows a comparison of the mosaicity of the 28 protein crystals tested. Similar to the previous analysis of resolution limit, a One-Way ANOVA test was applied to the analysis of mosaicity. Fig. 5(*b*) shows the normalized (to that of the control) mosaicity for the tested crystals. Compared with the control, the diamagnetic levitation technique clearly demonstrated a dramatic improvement in mosaicity among the three containerless conditions. However, the conditions using silicone oil and agarose gel exhibited no obvious improvement. According to the One-Way ANOVA test, the differences were between different crystallization conditions were not significant (n = 7, P = 0.208, *i.e.* >0.05). This result may be related to the dispersed distribution of the mosaicity of different protein crystals and in different conditions.

3.2.3. $R_{\rm merge}$. $R_{\rm merge}$ can be used as another indicator of crystal quality. Typically, a lower value of $R_{\rm merge}$ indicates better crystal quality. Fig. 6(*a*) shows a comparison of $R_{\rm merge}$ for the tested crystals. Notably, $R_{\rm merge}$ is always lower for the crystals obtained using diamagnetic levitation than for the control. Similar to the previous analysis, a One-Way ANOVA test was applied to the analysis of $R_{\rm merge}$. Fig. 6(*b*) shows a statistical comparison of $R_{\rm merge}$ after normalization (to that of the control). In terms of $R_{\rm merge}$ for the seven protein crystals, the results demonstrated significant differences between different groups (n = 7, P = 0.045, *i.e.* <0.05). The three containerless conditions all clearly demonstrated an improvement in $R_{\rm merge}$ compared with the control.

3.3. Mechanisms for effects on crystal quality

In §3.2, the results of evaluating the crystal quality in terms of diffraction resolution limit, mosaicity and $R_{\rm merge}$ were described. The results indicated that the diamagnetic levitation technique exhibited the best improvement in crystal quality. Compared with the control, silicone oil and agarose gel also demonstrated improvements in terms of resolution limit and $R_{\rm merge}$, in which agarose gel was a more favourable condition than silicone oil. The results indicated that containerless conditions are indeed beneficial to protein crystallization and that different containerless conditions exhibit different effects on the crystal quality. In this section, the possible mechanisms which are responsible for the phenomenon are discussed.

The crystal quality of proteins is affected by many factors (Chayen, 1999). These can be categorized into two groups: molecule-related and crystal-formation-related. The former includes the influence of the protein molecule itself. In other words, the crystal quality is correlated with the type of protein (Lu *et al.*, 2009). The latter includes the factors that affect the crystallization process, *i.e.* nucleation and growth processes (Newman *et al.*, 2007; Chayen, 1999). In the following, we will discuss how the physical environment affects the nucleation or growth process and further influences the crystal quality.

3.3.1. Effects from the container wall. When the crystallization solution directly contacts a solid surface, nucleation will be likely to occur on the solid surface because the energy barrier for heterogeneous nucleation is lower than that for homogeneous nucleation. The container wall plays an important role in crystal quality. To understand how the container wall affects the crystal quality, several possibilities should be considered: (i) the crystal in contact with the solid surface may introduce a lattice mismatch between the crystal and the solid surface, resulting in internal stress that may degrade the crystal quality; (ii) impurities on the container wall may introduce contamination into the crystals and thus may be unfavourable for growing high-quality protein crystals; and (iii) the heterogeneous nucleation that occurs on a container wall may be favourable for initiating nucleation, but not be beneficial to growing high-quality crystals, especially at the same supersaturation level (Otálora *et al.*, 2009; Snell & Helliwell, 2005; Chayen, 1996, 1999).

In the present study, in the diamagnetic levitation technique the crystallization droplet can be levitated freely in the air without any contact with the vessel wall. The conditions using silicone oil and agarose gel make it possible to grow crystals without contact with a solid surface. As a result, the three

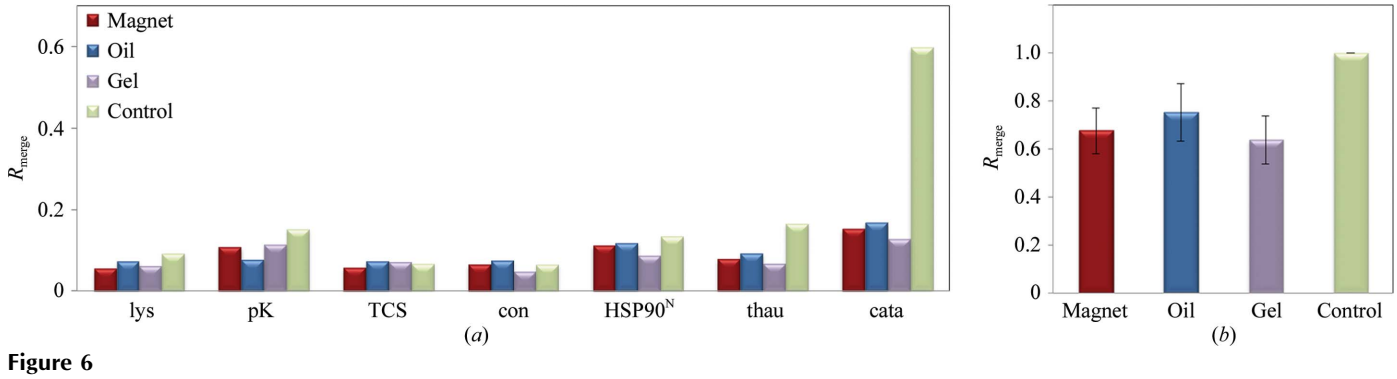

Figure 6
(*a*) A comparison of $R_{\rm merge}$ of the crystals of seven different proteins obtained from the four crystallization conditions; (*b*) a statistical comparison of $R_{\rm merge}$ after normalization to the values found for the controls (error bars show the standard error of the mean; n = 7). For $R_{\rm merge}$, the results demonstrated significant difference between different groups (n = 7, P = 0.045, *i.e.* <0.05). The three containerless conditions all clearly demonstrated an improvement in $R_{\rm merge}$ compared with the control.

containerless crystallization techniques all share the advantages mentioned above to varying degrees, which can thus result in an improvement in crystal quality.

3.3.2. Effects from the simulated microgravity environment. It has been widely reported that a microgravity environment can aid in growing high-quality protein crystals (Vergara *et al.*, 2005; Lorber, 2002; DeLucas *et al.*, 2002). The suppression of convection in microgravity is considered to be a key factor in this improvement. Transport by diffusion becomes the main route for depositing new crystal layers during crystallization because of the suppressed convection. On one hand, protein molecules will have sufficient time to locate correctly on the surface of the crystal lattice (Otálora *et al.*, 2009; Snell & Helliwell, 2005; Kundrot *et al.*, 2001), and on the other, a concentration-depletion zone (CDZ) may be formed around a crystal that is responsible for fewer nuclei and larger crystals (Otálora *et al.*, 2001). Restraint of sedimentation in a microgravity environment is thought to be another main factor for the improvement in crystal quality, which can contribute to reducing defects or twinning from collisions or merging between crystals during sedimentation (Vergara *et al.*, 2005; Lorber, 2002; Kundrot *et al.*, 2001). In addition, further ordered solvent molecules can be added into crystals in a microgravity environment, which can also lead to a more ordered crystal lattice (Vergara *et al.*, 2003, 2005; Dong *et al.*, 1999).

In the present investigation, crystal growth by the diamagnetic levitation technique or in agarose gel can be regarded as a simulated microgravity environment, because in both conditions buoyancy-driven convection and sedimentation are reduced or eliminated. Consequently, similar positive effects on the solute transport during crystallization and improvements in crystal quality can be exhibited by the two conditions.

3.3.3. Effects from the agarose gel. As well as the advantages mentioned above, using agarose gel as a crystallization medium can stabilize the crystal lattice because the gel can be trapped inside the channels of crystals during crystallization (Sauter *et al.*, 2009; Gavira & García-Ruiz, 2002). Furthermore, gel fibres can reinforce crystals, which can protect the crystals from damage during harvesting, cryocooling, transportation and irradiation (Sauter *et al.*, 2009; Sugiyama *et al.*, 2009; Zhu *et al.*, 2001).

3.3.4. Effects from the magnetic field. Magnetic fields have been investigated as a special physical environment for protein crystallization. Initially, an interesting phenomenon was that protein crystals appeared to be highly oriented along the direction of the magnetic field (Yin *et al.*, 2004; Ataka & Wakayama, 2002; Sato *et al.*, 2000). Later, magnetic fields were found to be beneficial for the growth of high-quality protein crystals (Nakamura *et al.*, 2012; Sazaki, 2009; Saijo *et al.*, 2005; Sato *et al.*, 2001; Lin *et al.*, 2000). The mechanisms of the improvement in crystal quality have been discussed extensively in the past few years, and the major points include the following: (i) the damping of convection in both homogeneous and inhomogeneous magnetic fields, especially in a large-gradient magnetic field (Leslie & Ramachandran, 2007; Wakayama, 2006; Qi *et al.*, 2001; Sazaki *et al.*, 1999), and (ii) an orientation effect arising from anisotropy in the magnetic susceptibility of crystals (Yin *et al.*, 2004). It has recently been found that in the same magnetic field crystals that are oriented demonstrated better quality than those that are not oriented (Nakamura *et al.*, 2012).

In the current study, the synergistic effects of both the containerless condition and the magnetic field resulted in the diamagnetic levitation technique exhibiting better improvements in crystal quality than the conditions using agarose gel and silicone oil.

4. Concluding remarks

In this paper, three containerless crystallization conditions have been investigated: diamagnetic levitation and the use of silicone oil and agarose gel. A solid vessel wall was used as the control. Seven different proteins were crystallized using the diamagnetic levitation technique to assess crystal quality and for comparison with crystals grown in agarose gel and silicone oil and as a control. The major conclusions are the following.

(i) Containerless crystallization using the diamagnetic levitation technique can aid in obtaining high-quality protein crystals.

(ii) Containerless crystallization using silicone oil or agarose gel was verified to be beneficial for the growth of high-quality protein crystals.

(iii) A comparison of crystal quality indicated that the diamagnetic levitation technique exhibited the best improvement in crystal quality, followed by the condition using agarose gel.

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