Efficient Method for Protein Crystallization

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Abstract: Strong magnetic fields have been used to improve protein crystal quality. To study the effect of strong magnetic field in obtaining first crystals, which is the pre-requisite for determining crystallographic structures, we screened the crystals of 15 proteins using Sparse Matrix Screen (SMS) in a strong and gradient magnetic field. Statistics showed that magnetic field can significantly increase the crystallization success (hits), and the average hits of the trial proteins from different positions in the magnet were improved by 41.3% compared to the control, whereas by 66.1% at "zero-gravity" position (0g/12T). Compared with 1g/0T, the hits of concanavalin A, chymotrypsinogen A, ribonuclease S and ribonuclease A at 0g/12T increased from 16.7%, 41.7%, 25.0% and 8.2%, to 37.5%, 62.5%, 37.5%, and 20.8% respectively. The hits can be further improved to 2.87 times combining magnetic field and temperature compared to the unfavorable temperature outside the magnet. Further investigations suggest that strong magnetic field can modify protein precipitation behaviour leading to the lift of the precipitate curve in phase diagrams, increasing their crystallizability by enlarging the nucleation zone. This novel method will provide a new means for improving the tried and tested protein crystallization, thus facilitating the development of structural biology.

Protein crystal growth (PCG) has provided the basis for crystallography, with which most structural entities currently found in the PDB bank have been determined [1,2]. PCG remains a major rate-limiting step and its study will be quite active for the coming years for the efficient development of structural biology. Although significant efforts have been devoted to study this trial and error process, the results of structural genomics projects (e.g., http://targetdb.pdb.org) demonstrate that crystallization is still a bottleneck that requires further attention. As it is essentially impossible to predict crystallization conditions for proteins, the process usually relies on extensive screening of hundreds to thousands of conditions, including different precipitants, salts, buffers, additives and etc[3,4]. Thus, development of high-throughput crystallization robots, able to prepare large number of samples while miniaturizing the volumes, is often an integral part of the structural genomics efforts[5,6]. While these robots undoubtedly make an important impact on high-throughput research, they do not necessarily enhance the crystallization efficiency of new proteins because the success of protein crystallization is not directly correlated with the number of conditions tested. The recent rapid development of structural genomics, which aims to determine the structures of thousands of proteins, has further encouraged the crystallography community to develop new methods for protein crystallization[7]. Few rational approaches to PCG have been established since decades' study (see below).

Although the principles of macromolecular crystallization are not fully understood, significant work that attempts to develop rational methods for PCG has been carried out recently[2,8-10]. Strong magnetic field, which can simulate a microgravity environment, inhibits convection and affects properties of some materials, is widely used in life

sciences and materials science[10-14]. In recent years, the utilization of strong magnetic fields (both inhomogeneous and homogenous) has been proposed as an alternative means of improving macromolecular crystal quality, and encouraging results have been obtained[15-17]. Furthermore, it was recently found that a large gradient magnetic field can suppress convection, which may affect PCG [18]. This can lead to the improvement of crystal quality with the control of the nucleation kinetics in the presence of a magnetic force. However, these reports focused on the improvement of crystal quality.

Can strong magnetic field improve the success rate of crystallization? This originated from an idea to verify the possible phase diagram modification of proteins in the magnetic field after defining the relative crystallizability, from observations that magnetic fields can simulate various gravity levels, strong magnetic field affects the dissolution process of crystals and that some protein crystals can interact with magnetic field. In this study, we used a strong magnetic field to facilitate the obtaining of the first crystals. Fifteen proteins were crystallized in this environment in order to see whether a strong magnetic field could increase protein crystallizability, via phase diagram (protein concentration versus precipitating agent concentration) modification. The number of "hits"- a hit being defined as finding one condition of crystallization, using polyethylene glycol (PEG) as precipitating agent within Sparse Matrix Screen experiments[19], to demonstrate the success rate of crystallization. Our screening was carried under four different positions ("upper", 0 g/12 T; "middle", 1 g/16 T; "lower", 2 g/12 T; and control, 1g/0T, see Materials an Methods) at varying temperatures. It was found that crystallizability was improved to different levels in various positions in the magnetic field.

We evaluated phase diagrams modifications at these positions in and out of the magnetic field for several proteins. We show here that a strong magnetic field modifies the precipitation behavior of proteins, resulting in a significantly larger nucleation zone (see Material and Method) in the phase diagram and hence a greater protein crystallizability.

Results

Pre-screening of the appropriate temperatures for crystallization: We first carried out temperature screening using PEG as the precipitating agent in Sparse Matrix Screen (SMS) for the same batch of trial proteins to be later tested in the magnetic field, similarly to Zhu et al.[8] Firstly, the number of hits was measured in a normal gravitational field at three different temperatures, 285 K, 291 K, and 296 K, the same temperatures to be later used in the magnet. The results are illustrated in Fig. 1a. Three proteins, chymotrypsinogen A, concanavalin A, and ribonuclease A, present an increased crystallization probability at higher temperatures, while other proteins seem to be more easily crystallized at lower temperatures. To obtain the mean best hits, the best hits of all the fifteen trial proteins from their corresponding suitable temperature were averaged, for example, concavalin A, chymotrypsinogen A, lysozyme at lower temperature (285 K), whereas catalase, ribonuclease A, ribonuclease S, protinase K, and trypsin at higher temperaute (296 K), etc. In the same way, the mean unfavorable hits obtained from their

corrresponding unsuitable temperature. The result shows that optimal temperature improves the number of hits by about 78.6%, as compared to the unsuitable temperature for the given protein (Fig. 1b).

The magnetic field augments the number of hits of crystallization: To explore the effects of the magnetic field (expressed by B), the crystallization at the "upper" (0g/12T), "middle" (1g/16T), and "lower" (2g/12T) positions were compared with the "control" where B ≈ 0 T, all these being subject to three different temperatures, 285 K, 291 K, and 296 K (See Methods and ref. 11). Then a statistical analysis was performed on the set of 24 conditions using PEG as precipitating agent in SMS for this screen. The number of conditions producing protein crystals in the above screen, the "hits", was used to evaluate the success rate. The "hits" for different trial proteins at various positions in and out the magnetic field were shown in Fig. 2. Most proteins（i.e. concanavalin A, chymotrysinogen A, catalase, lysozyme, ribonuclease A, ribonuclease A, ribonuclease S, trypsin, epididymal-specific lipocalin (ELP), myoglobin）produced significantly higher crystallization success in the magnetic field especially at 0 g, as compared with the control. For example, hits for concanavalin A, chymotrypsinogen A, ribonuclease S and ribonuclease A were improved from 16.7%, 41.7%, 25.0% and 8.2% at 1 g/0 T, to 37.5% (Fig. 2a), 62.5% (Fig. 2a), 37.5% (Fig. 2c), and 20.8% (Fig. 2a) at 0 g/12 T. The improvement of hits means that more crystallization conditions were found.

To make sure that the crystals resulting from the screen are effectively protein crystals, we then verified their nature through X-ray diffraction and protein staining, as extensively as possible. (see blow)

Strong magnetic field effect on PCG: a statistical analysis was performed on the hits obtained in the screens to analyze the effect of the magnetic field under optimized temperature. Figure 3a, compiled from the same data as in Figure 2, displays hits obtained at each protein's optimal temperature. Most trial proteins, about 80% (twelve out of fifteen), demonstrated a magnetic field dependence for their crystallization success. One sample T test revealed that the number of hits for trial proteins was improved by 68.0% at 0 g position, 26.2% at 1 g, and 30.6% at 2 g (Fig. 3c) from the control. PCG can be increased by 187.4%±30.9% (mean±S.E.M.) by the best combination of magnetic field and temperature, or the success rate can reach 2.87 times that of the unfavourable condition (normal gravity environment and unsuitable temperature tested, Fig. 3b). The hits obtained at 0 g position were very significantly improved ($p < 0.01$), and the hits obtained at 2 g and 1 g positions were significantly improved ($p < 0.05$) compared with the control (Fig. 3c). There was a significant difference between 0 g and 2 g, but the difference between 0 g and 1 g was more significant (Fig. 3c). The average hits which were obtained from different conditions in the magnet as a whole showed an increase of 41.3%±5.1 (mean±S.E.M.) in the magnet (Fig. 3d) compared to control under the same temperature. Our results thus demonstrate that the magnetic field significantly improves protein crystallizability.

Phase diagrams modification in magnetic field: The diagrams plotting protein concentration versus precipitating agent concentration, in different magnetic field positions, have been evaluated experimentally for selected proteins in these conditions, procedure similar to the earlier phase diagram study in the absence of magnetic field[8]. The nucleation zone of chymotrypsinogen A has significantly increased in the magnetic field against control (Fig. 4a). It is worth notice that the precipitating curve moves more upward in 0g position in the magnet compared to other positions (Fig. 4a). However, the nucleation curve and solubility curve do not modify significantly (Fig. 4c insert). The ratio of the nucleation zone area (its area within the working area is hereby abbreviated as S_N) and the total phase area in our working zone were calculated as described in ref.11 and showed at Fig. 4d. At 0 g position the nucleation zone reaches the maximum among all the positions. The S_N at 2 g and 1 g are more similar, but they are also larger than that in the control. The phase diagrams of concanavalin A and catalase also suggest the similar change of nucleation zone under the effect of magnetic field (Fig. 4b, c, d). Thus, the nucleation zone modification in the magnet coincides well with the crystallization success shown by hits, similar to the results with the change of other crystallization parameters, while the 0 g condition demonstrates the most significant effect among all positions in the magnet and appears very significant compared to other parameter modifications. These results, in turn, clearly support the value of crystallizability study.

X-ray diffraction and crystal detection: To make sure that the newly found crystals are of protein nature, we then verified the crystals with x-ray diffraction and protein staining. The data has shown that the tested crystals of lysozyme, concanavalin A, ribonuclease S, and proteinase K yielded protein diffraction pattern. Other crystals which are too small to pick up with loop, were confirmed with selective dye. The result showed that most of these crystals could be stained with amido black (data not shown).

Discussion

The improvements of the relative crystallizability: to verify our initial idea of using strong magnetic field to increase protein crystallizability, we carried out systematic experiments for different trial proteins using PEG as the precipitating agent in the SMS in and out of the magnetic field. Not to be biased by a possible artificial temperature effect, the hits were parallely obtained at different positions with a water bath, water jacket, temperature sensor and a computer. So the change of "hits" at different positions can be used to evaluate the effect of magnetic field.

In our work, carrying out the screen in the magnet at different positions resulted in significantly more "hits" for trial proteins than the control. For example, hits for concanavalin A, chymotrypsinogen A and ribonuclease A improved to 37.5%, 62.5%, and 20.8% at 0 g/12 T/296 K (Fig. 2a) from 16.7%, 41.7%, and 8.2% at 1 g/0 T/285 K (Fig. 2c); ribonuclease S, proteinase K, and trypsin improved to 37.5%, 33.3%, and 75.0% at 0 g/12 T/285K (Fig. 2c) from 12.5%, 20.8%, and 20.8% at 1 g/0 T/296 K (Fig. 2a). The number of hits for trial proteins at different positions was improved by 187.4%

±30.9% (mean±S.E.M), resulting in a 2.87 fold success rate for the best combination of magnetic field and temperature over that of the unsuitable condition (Fig. 3b). The average hits obtained from different positions as a whole in the magnet were improved by 41.3%±5.1% (mean±S.E.M) (Fig. 3d) compared to control under the same temperature. Thus, these results clearly demonstrate that strong magnetic field improves crystallization success and the experimental results support the validity of our initial idea. More importantly the application of magnet can significantly broaden the crystallization conditions in PCG experiments. The proteins used in this study were selected arbitrarily, therefore some of them are diamagnetic (such as lysozyme, papain and proteinase K), and the rest are paramagnetic (such as catalase and myoglobin). The results shown in this paper indicated that high magnetic field can be used to facilitate crystallization of proteins regardless of their magnetism nature.

Strong magnetic field modifies protein precipitation behavior: our current study showed that magnetic field can affect the success rate of protein crystallization and such a novel method is useful to facilitate structural biology. Indeed, phase diagram study at different positions in the magnetic field has clearly shown that the precipitation curve is lifted significantly in the magnetic field as compared to outside the magnet, thus enlarging the nucleation zone and promoting higher success rate of crystallization, as revealed by the recent study of protein crystallizability.

The experimental data with phase diagram clearly advance our understanding though detailed physical mechanism awaits further study. Nevertheless, some previous results, such as the fact that magnetic field can simulate various gravity levels, its affection on the

dissolution process of crystals, and the interaction between proteins and magnetic field, etc. have provided us with some clues.

Firstly, strong magnetic field can affect the mass transfer in the solution by decreasing the diffusivity of the protein molecules[27] and suppressing the natural convection. At 0g position, the convection can be weaker than those at other positions (1g, 2g in the magnet and the control) due to the effect of magnetization force. The weaker mass transfer process may be advantageous to the crystallization process. Yin and colleagues reported that strong magnetic field decreases the dissolution of crystals, and may thus stabilize crystal nucleation. The latter nucleation is generally considered as a key limiting step of protein crystallization, its stabilization may thus facilitate the crystallization. Secondary, the interaction between protein and magnetic field is conducive to the uniform distribution of the protein, which decreases the disorder and random collision between protein molecules leading to their precipitation. It is widely accepted that protein crystals having anisotropic magnetic susceptibility will be oriented in a strong magnetic field. It is also reported that strong magnetic field can induce partial orientation of macromolecules. Protein molecules may be oriented to some degree by the magnetic field, limiting their disorder and the thermal motion. This restriction is conducive to the formation of protein crystals while reducing protein precipitation.

Using magnetic field to facilitate proteins crystallization: Zhu et al. has designed a pre-screening of precipitation curve modification as a function of temperature to assist the finding of new crystals or reproducing crystallization with higher success. When the best temperature has been chosen, we can carry out the screening at different positions in

the magnetic field. In several cases, significantly higher "hits" can be obtained in the magnet than out of it. The study on phase diagram modifications at different positions in and out the magnetic field demonstrated that precipitation behaviour of proteins can be modified, resulting in a significantly larger nucleation zone and hence a greater protein crystallizability. Such property can be used to detect the optimized position in the magnetic field before large-scale screening.

As we discuss earlier, the success of protein crystallization is not directly correlated with the number of conditions tested. This novel method detects the crystallization orientation by a first limited screening, and then a larger screen under focused conditions to improve the success rate. Several former studies have also suggested that magnetic field can improve the quality of protein crystal. Establishing conditions to promote a high crystallizability using strong magnetic field contributed to the success of arginyl-tRNA synthetase crystallization that was among the few proteins crystallized during the 1997-1998 Canadian Protein Crystallization Experiment on board the Russian MIR station when dramatic and unexpected condition changes occurred.

The novel approach of protein crystallization in magnetic field is able to enhance the probability of crystallization. It is worth to note that all the trial proteins chosen to do this work are normal model proteins, which don't contain any ligands that can interact with magnetic field. The interest results suggest that the secret, i.e. magnetic field affects protein crystallizability, seems to be hided inside or between molecules, for example, secondary bond. Further investigations will help to uncover the dynamic processes

during crystallizing, and understand the mechanism of crystallization of biological macromolecules.

METHODS

Protein Preparation. Most protein samples were purchased from Sigma: concanavalin A was from Canavalia ensiformis (C2272) and prepared as a 30mg/ml solution; chymotrypsinogen A (20 mg/ml) was from bovine pancreas (C4879); catalase (20 mg/ml) was from bovine liver (C9322); lysozyme (40 mg/ml) was from chicken egg white; ribonuclease A (70 mg/ml) was from bovine pancreas (R5125); ribonuclease S (40 mg/ml) was from bovine pancreas (R6000); rennin (50 mg/ml) was from *Mucor miehei* (83553); proteinase K (40 mg/ml) was from *Tritirachium album* (P6556); papain (40 mg/ml) was from papaya latex (P4762); albumin (30mg/ml) from bovine serum (A2153); trypsin (30 mg/ml) was from bovine pancreas (T1426); phosphoglucose isomerase (15 mg/ml) was from baker's yeast (P2338); α-amylase (10 mg/ml) was from *Bacillus licheniformis* (A4551); myoglobin (20 mg/ml) was from equine skeletal muscle (M0630).

Device Fabrication. A superconducting magnet located at the Key Laboratory of Space Bioscience & Biotechnology, Northwestern Polytechnic University, Xi'an, China, was used (see ref. 29). For protein crystal growth, it is important to be able to generate a stable magnetic field at indicated temperatures over a period of days to weeks. This became possible, due to the very recent developments of superconducting magnet technology.

At the center of the magnet where there is a strong but homogeneous field (the "middle" position), no force is exerted. So the "middle" position corresponds to a virtual environment of 1 g, with a B value of 16 T. On the other hand, at the "upper" position,

about 10 cm above the center along the vertical bore of the magnet, the upwards magnetization force balances the gravitational force of the diamagnetic substance (the magnetic susceptibility $\chi < 0$). Such a substance has a tendency to escape from a region of dense magnetic field toward that of a less dense field. As a result, at the "upper" position, a magnetic field of 12 T and a magnetization force corresponding to -1370 T^2/m exists simultaneously. Therefore, the magnetization force acting upwards on a unit mass of a protein solution corresponds therein to a virtual environment of 0 g. At the "lower" position, about 10 cm below the center of the magnet, a magnetization force of the same magnitude (1370 T^2/m) is exerted downward, with the same magnetic field of 12 T, therefore producing a hyper gravity environment of 2 g. Crystals were grown in three vessels situated simultaneously at these three positions and were compared. As a control, we used crystals grown outside the magnet, i.e. in the absence of both the magnetic field and the magnetization force.

The temperature is controlled through the combination of a water bath, a water jacket, a temperature sensor and a computer. The temperature sensor, installed in the magnet bore, measures the actual temperature of the environment where the crystallization plates are placed, and transfers the information to the computer. The computer compares the actual temperature and the expected temperature, and then determines how to adjust the water bath (Polyscience 9712, Polyscience Inc., US) which controls the temperature of the circulating water. The water is circulating in a copper water jacket, which is installed in the magnet bore. The resultant temperature can be controlled accurately up to within ± 0.1 K using the above system.

Crystallization Experiments. Crystallization trials were performed using the hanging drop vapour diffusion, which were initiated by mixing one volume of protein with one volume of reservoir solution in 24-well cylindrical Plexiglass vessels of a diameter of 4 cm. ELP crystallization was carried out as described (ref. 11). The Hits of fifteen trial proteins were obtained by screening against 24 conditions using polyethylene glycol (PEG) as precipitating agent in Sparse Matrix Screen experiments at three different temperatures and four different magnetic field conditions when indicated. Protein phase diagrams were designed to plot the initial protein concentration versus the precipitating agent concentration under each crystallization condition. The multi-wells were plated in incubators under four different conditions (i.e. "upper", 0 g/12T; "middle", 1 g/16T; "lower", 2 g/12T; and control/1 g, without magnetic field) at 291 K. Concanvalin A phase diagrams were determined after crystallization at pH 6.5 in sodium cacodylate buffer, in the presence of 0.2 M $Zn(Ac)_2$ and 10–16% (w/v) PEG 4000, and 0.1-70 mg/ml protein. Chymotrypsinogen A phase diagrams were determined by crystallization at pH 4.6 (NaAc–HCl), in the presence of 0.2M ammonium sulfate and PEG 4000 from 25 to 35%, while the protein concentration varied from 0.1 to 100 mg/ml. Catalase phase diagrams were determined after crystallization at pH 6.5 in sodium cacodylate buffer, in the presence of 0.2 M ammonium sulfate and 22–26% (w/v) PEG 8000, and 0.1-70mg/ml protein.

Crystal Diffraction: The crystals were checked using X-ray diffraction at the Institute of Biophysics, Chinese Academy of Science, with Rigaku R-AXIS IV++ Image Plate and a Rigaku FR-E Cu-Kα rotating anode generator. The small crystals which could not be

picked up by loop were selectively dyed using amido black. All the drops were photographed using Nikon SMZ 1000.

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Figures

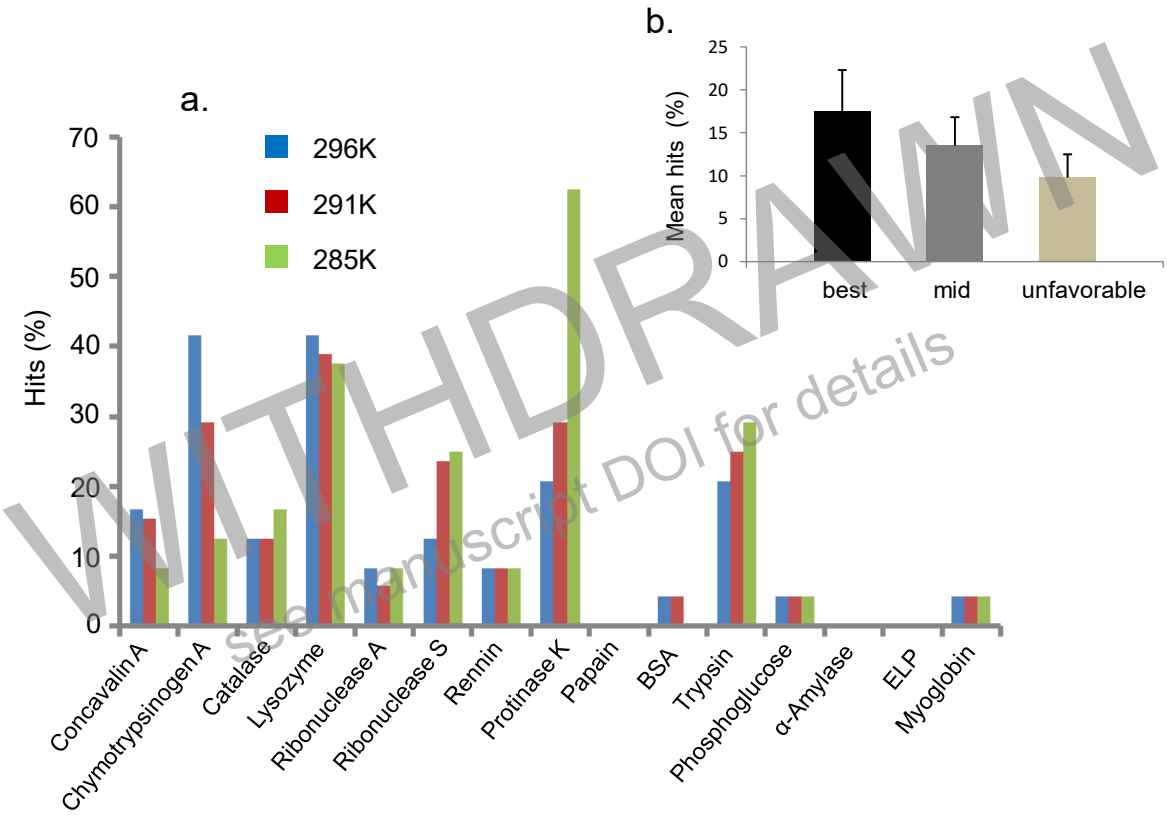

Figure 1 | An appropriate temperature enhances PCG success. (a) Hits distribution of trial proteins under different temperatures; (b) Mean hits which were calculated from different levels of the appropriate temperature, and represented as mean ± S.E.M. Best: mean hits which were calculated from all the fifteen trial proteins at their suitable temperature respectively; unfavourable: mean hits which were calculated from all the fifteen trial proteins at their unsuitable temperature respectively. To select a good temperature significantly improved the success rate of crystallization.

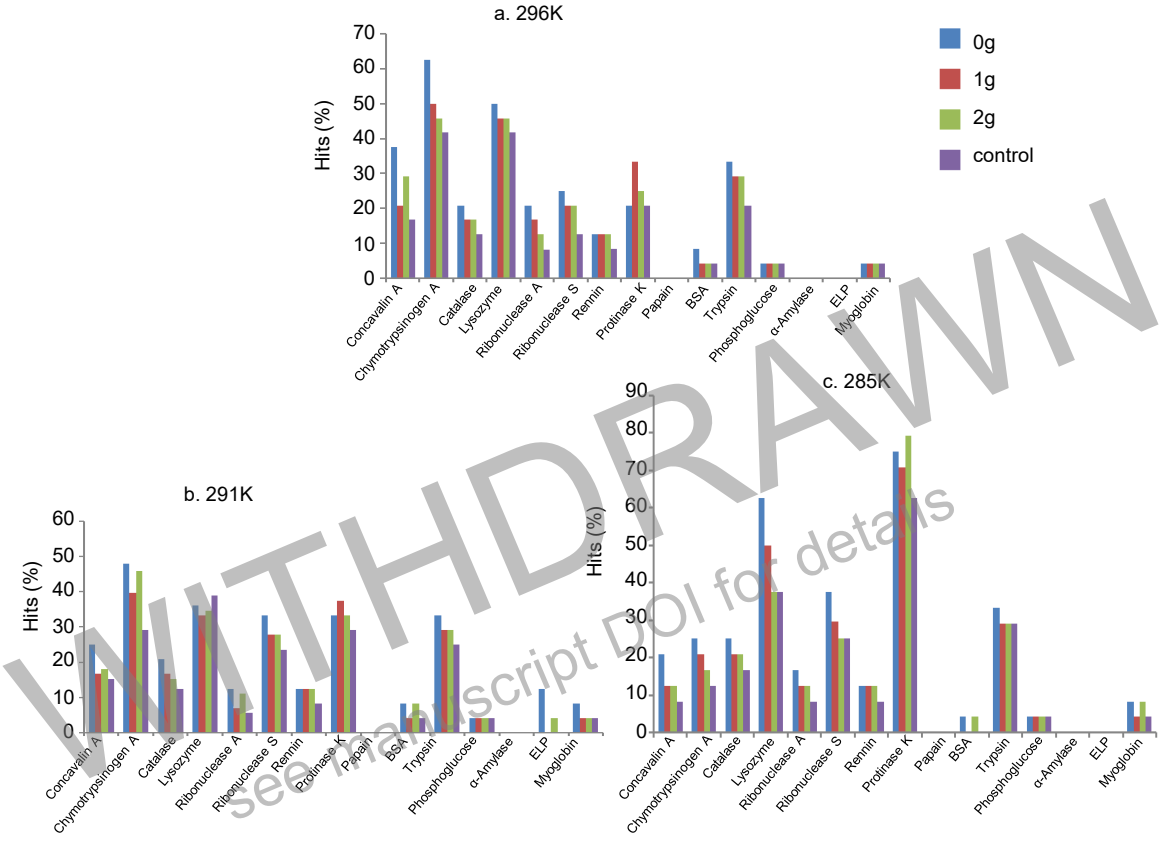

Figure 2 | Histogram of crystallization hits for Sparse Matrix screens of trial proteins crystallized at different positions in the magnetic field, i.e. “upper”, 0 g/12 T; “middle”, 1 g/16 T; “lower”, 2 g/12 T; and control, 1g/0T, at (a) 296K, (b) 291K, and (c) 285K.

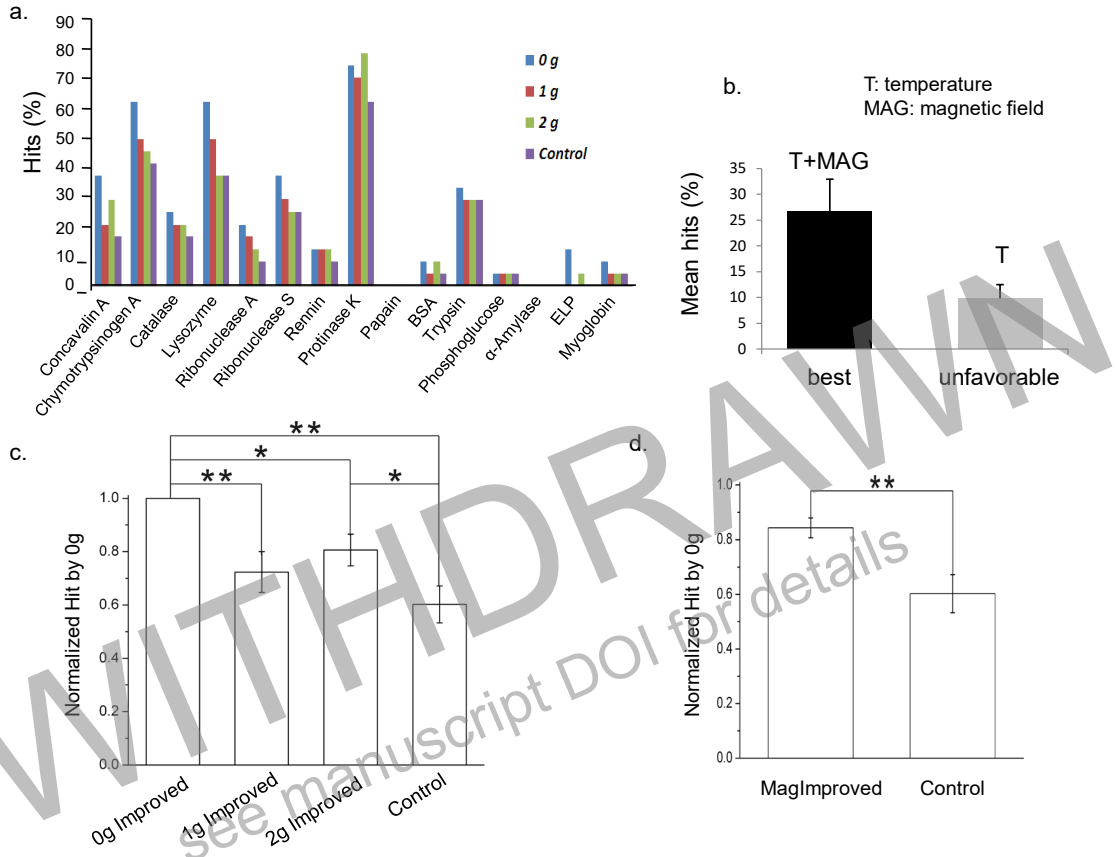

Figure 3 | (a) Histogram of crystallization hits for Sparse Matrix Screens of trial proteins under suitable temperature respectively. The hits displayed here of concanavalin A, chymotrypsinogen A, ribonuclease A were obtained at 296K, those of ELP at 291K, and those of other proteins at 285K. Data were taken from Figure 2. (b) Mean hits contributes by magnetic field under optimum temperature comparing to unsuitable temperature without magnetic field. T+MAG: magnetic field contributes to hits improvement, which were averaged from all fifteen trial proteins in the magnetic field, which were subject to suitable temperature; T: without magnetic field. Data were calculated from (a). Best: the best means hits, which were calculated from all trial proteins under condition which best hits were obtained; worst: data were taken from Figure 2b, worst (unsuitable temperature). (c) Comparison of different improved hits at 0 g, 1 g, and 2 g in the magnetic field against control. Histograms represent averaged hits improvement under the effect of magnetic field, which was calculated from (a). For normalization, the averaged hits improvement at 0 g was set to 1, and other data were expressed in relation to it. Normalized crystallization hits of every group was represented as mean ± SEM (n = 13). *, $p < 0.05$; **, $p < 0.01$; one-way ANOVA followed by Least Significant Difference (LSD) post-hoc analyses. (d) The mean improved hits under the effect of magnetic field as a whole against control. Data were calculated from (a). Data were expressed in relation to mean hits improvement at 0 g for normalization. The two-tailed independent samples t test was used to compare mean normalized crystallization hits in magnetic field with control, which showed that the difference between mean magnetic field and control normalized crystallization hits was significant ($p = 0.002$).

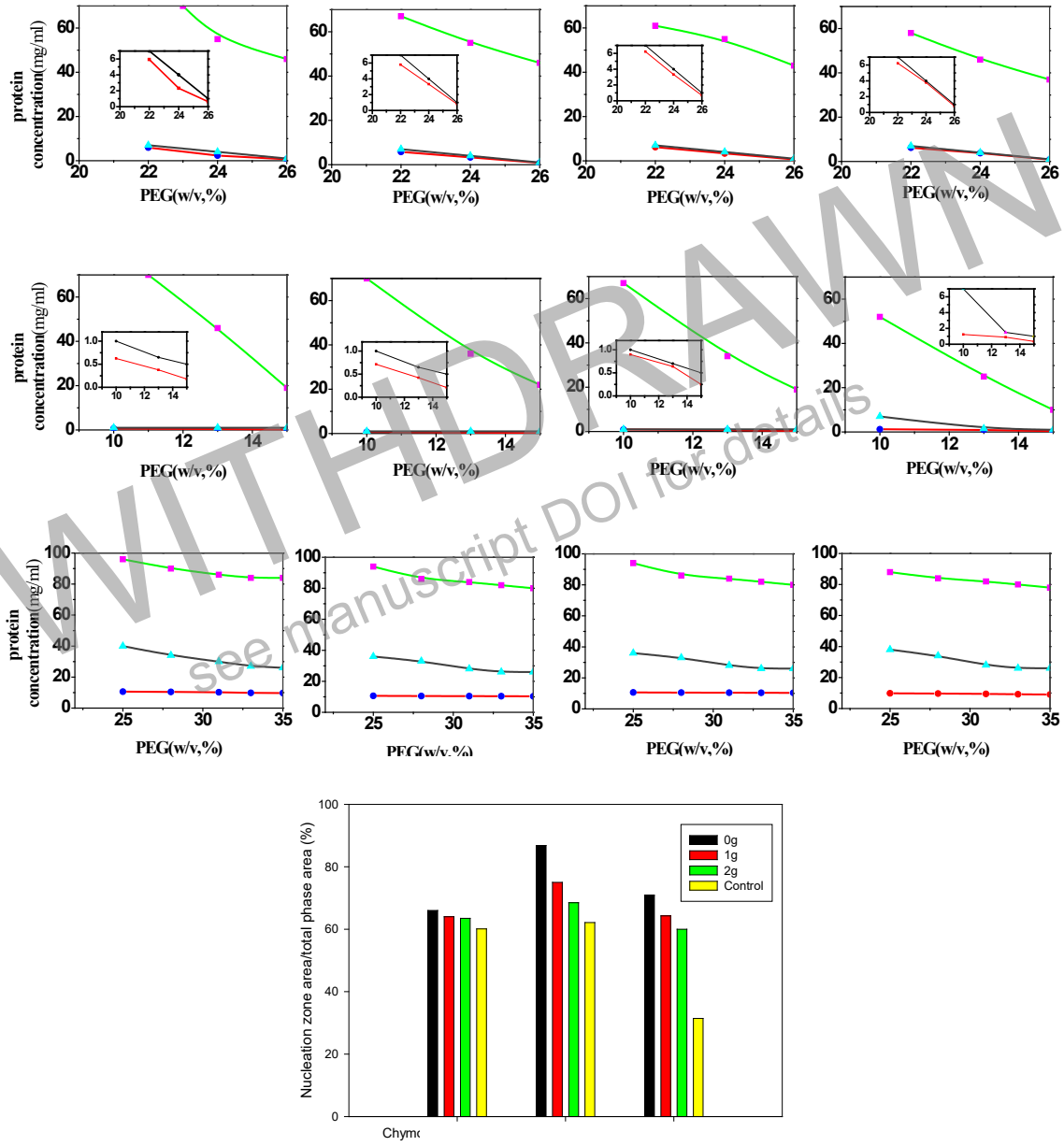

Figure 4 | Evaluation of two-dimensional phase diagram modification for catalase (a), concanvalin A (b), and chymotrypsinogen A (c) in a strong magnetic field at 291K. Solubility curve (in red) was obtained from the residual concentration in equilibrium with crystals 7-days after the initiation of crystallization. Nucleation and precipitation data are plotted in black and green, respectively. The precipitation zone is denoted by the region above the precipitation curve while the nucleation zone, where spontaneous nucleation occurs, lies between the nucleation and precipitation curves. The metastable zone is situated between the nucleation and solubility curves where crystals grow, while the area

below the solubility curve is the under-saturated zone. Phase diagrams were drawn using Origin 6.0. d. the percentage of the nucleation zone area in working phase area. Area of working phase and nucleation were calculated as described in ref. 11. Inset: the nucleation and solubility curves of catalase and concanvalin A at different position in the magnetic body, i.e. 0g (12T), 1g (16T), 2g (12T), and control.