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IIII Review IIII

Recent Advance in High Quality Protein Crystal Growth Experiment on the International Space Station by JAXA

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Abstract

JAXA has long been engaged in the High-Quality Protein Crystal Growth (PCG) experiment on the International Space Station (ISS). This project has undergone extensive development for the last few years. This review describes a general outline of the project and services provided by JAXA.

Keyword(s): Protein crystallization, Microgravity, Drug design, International Space Station
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1. Introduction

A common question about this project we often receive is: "Are you going to produce drugs in space?" The answer is "No", of course. The project is to grow high-quality protein crystals required for obtaining precise protein structures. The crystals grown in space are brought back to Earth and followed by X-ray diffraction experiments at synchrotron radiation facilities such as SPring-8, and the structure is calculated with the data. High-quality crystals can reveal protein structures in detail, on the other hand, insufficient quality crystals show only ambiguous molecular structures, and consequently do not allow us to design drug candidates that bind to the target protein and inhibit its function. An accurate molecular structure of protein is necessary to find the best compounds. Based on our experience, space experiments have often produced significantly higher quality of protein crystals than those on Earth. In some case, proteins crystallized poorly on the ground could be crystallized in high quality in space. In another case, a protein which tended to crystallize as a cluster on Earth, was crystallized as a single crystal in space and the structure was determined, **Fig. 1** shows some examples of protein crystals grown in space.

Readers of the IJMSA must be interested in the reason why proteins crystallize in high quality in space. Generally speaking, microgravity suppresses solution flow (convection) and allows protein crystals to grow in a stable environment and less impurities are delivered to the crystal surfaces. This is, however, a contentious matter [1–3] and not discussed here.

JAXA initiated PCG experiment in 2003. Before that, we believed that simply crystallizing proteins in space was enough to improve their crystal quality. In fact, high quality crystals were obtained in the early days, but results were not as good as we expected. We analyzed the results and found that a key to a successful experiment lied in pre-launch preparations on the ground. Thus, we have strengthened support for academic researchers through our contractor [4]. JAXA also defined a new policy on opening the door for private companies to its projects for making a visible contribution to society such as drug development. JAXA consulted various experts for their opinions. We received suggestions, such as increasing the experiment frequency and improving the services. Here we summarize the recent changes in the project, inventive efforts in hardware, services provided by JAXA staff, and its achievements.

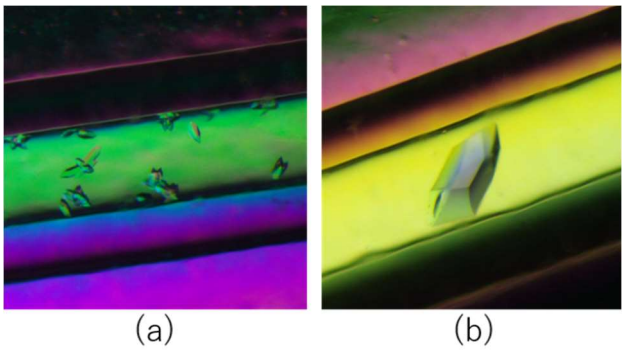

Fig. 1 Example of the crystals obtained on ground and microgravity. (a) Ground grown crystals diffracted up to 2.5Å, and (b) Space grown crystals diffracted up to 1.5Å which enabled the researchers to reveal the precise protein structure. Image credit: Meijo University/JAXA

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2. Outline of the Project

Between 2003 to 2013, one PCG experiment was conducted annually on average. This period was useful in terms of acquiring experimental skills and establishing space experiment protocols. However, the low experiment frequency did not match the pace of research on Earth. Since 2014, the frequency was gradually increased. Now four or five space experiments per year are available. The US and Russian rockets are used for transporting samples to the ISS.

Under the specific agreement on PCG experiments concluded with ROSCOSMOS (Russian Federal Space agency), JAXA uses Soyuz spacecraft for launching and recovering materials, and Progress spacecraft for launching. After 2003, the favorable and ongoing partnership with Russian officials has enabled JAXA to steadily carry out its project. Presently, Russian spacecrafts are used twice a year for launching experiment samples. One of the main advantages of using Russian vehicles is that their launch schedule is more stable.

Experiments involving the US Dragon spacecraft have started since 2015 (with full-scale operation since 2017). Launching samples by this spacecraft has the advantage of a high degree of freedom in replacing or modifying hardware. Therefore, the US flights can be used for testing new containers and crystallization methods such as the hardware described in Section 3. The US spacecraft launch is often delayed by several days. To avoid the proteins from crystallizing before reaching space, JAXA has introduced a new technique that enables crystallization to begin on orbit.

For the US spacecraft, an experiment temperature of 20°C or 4°C can be selected, and 20°C experiment can also be performed using Russian spacecraft. Unstable proteins such as kinase often aggregate in 20°C but one of our user yielded a good crystal of a kinase at 4°C [5], so experiments at 4°C have been in operation since 2017 to meet demands for experiments at low temperatures. In the past, the temperature could not be maintained during ground transportation or during observation of crystals after recovery, but now, the temperature has been maintained within approximately ±2°C from the desired temperature all the way including launch, recovery, ground transportation and observation.

3. Hardware

Maximizing the effect of microgravity, the counter diffusion method is mainly used in PCG experiment, while the vapour diffusion and the micro-batch methods are commonly used in ground research. The method was originally developed by a research group from the University of Granada, Spain [6].

One end of a glass capillary containing a protein solution is sealed with clay and a silicon tube filled with agarose gel is attached to the other end. The gel side is immersed in a crystallization solution in a sealed container. As both the crystallization and the protein solutions diffuse through the agarose gel, concentration gradient is gradually formed in the capillary. The gradient changes over time. Protein molecules move significantly slower than the precipitants molecules, since protein molecules are much larger than salt and organic compounds. By applying a dialysis membrane, protein erosion from the capillary can be prevented. In one to two months of the incubation period, crystals are grown at the right concentration area in the capillary (**Fig. 2**). In fact, some crystals can be improved by simply using this method before going to space. It takes about one month to modifying the condition from vapour diffusion or batch crystallization to counter diffusion, as the optimum condition usually changes.

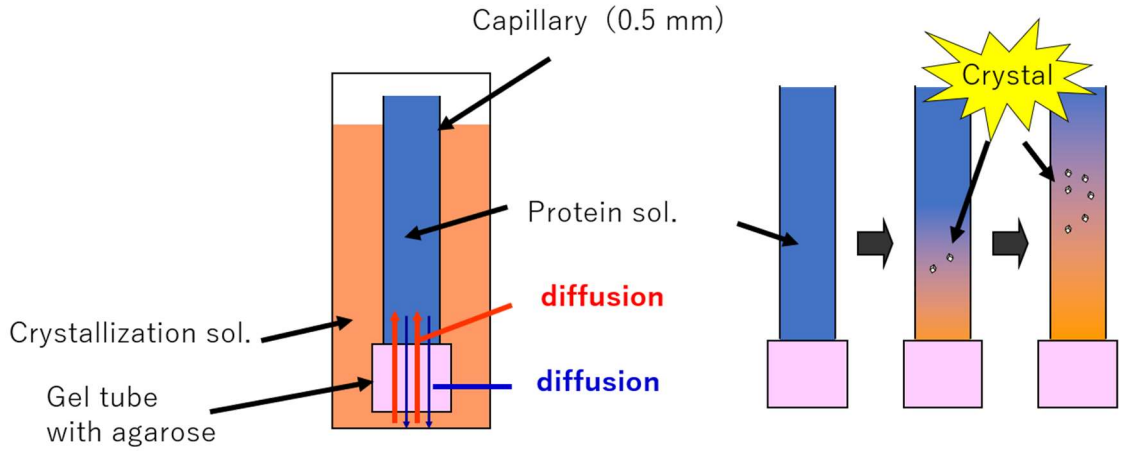

Fig. 2 Schematic figure of the counter diffusion method. Protein and crystallization solution gradually diffuses through the gel tube. Crystals grow at an appropriate concentration gradient.

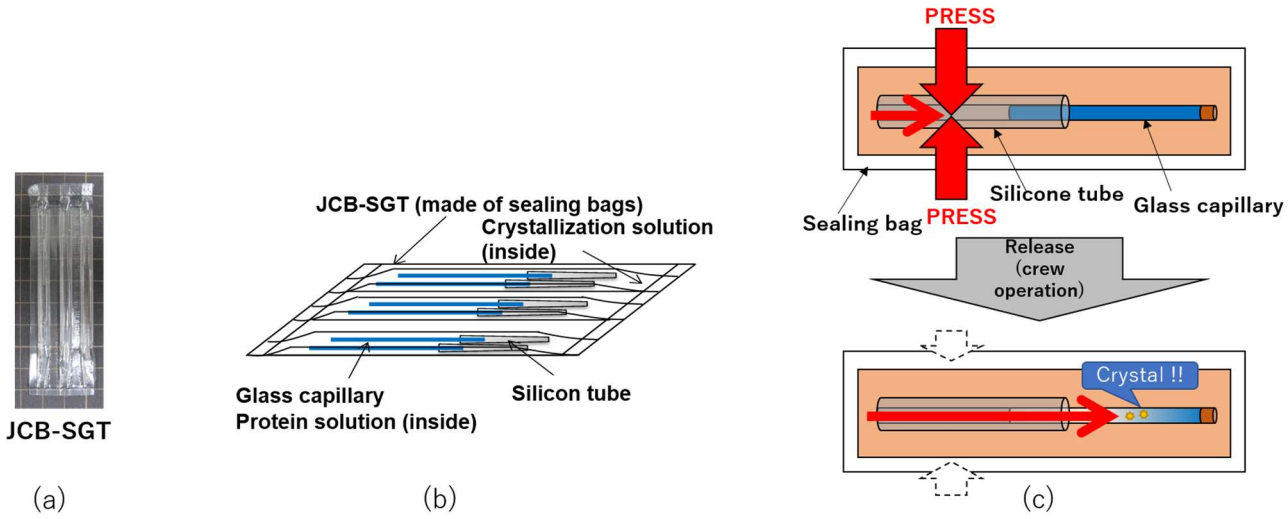

Fig. 3 Schematic figure of the basic hardware, JCB-SGT. Capillary with gel tube is inserted into the JCB-SGT(a,b). In Russian flights, the JCB-SGT is launched as is, and in US flights, the gel tube is mechanically compressed to prevent the solution from diffusing(c).

When we launch our crystallization container by a Russian spacecraft, the containers are layered and highly packed in a box with Peltier device called "Cell Unit". When the Cell Units arrive at *Kibo*, the Japanese Experimental Module of the ISS, they are installed in the Protein Crystallization Research Facility (PCRF) by an astronaut, where the temperature is tightly controlled.

We also have established the launch service using the US spacecrafts. Immediately after the containers are assembled, protein and precipitant solutions in a capillary are mechanically separated by pressing the agarose tube. This device named "Press" allows us to prepare the sample in Japan and send the containers to *Kibo* via the US. The crystallization can be initiated by releasing the pressing device by a crew at *Kibo*. This procedure has advantages that the preparation can be done in Japan and more adaptable to a launch schedule change.

Other than the "Press" device, we have developed the "Osmotic Tube (OT)" method, the "De-oxidisation (DX)" and the "Petit" bags to meet the demands from users. Using the "Petit" container, for example, protein and precipitant solutions filled in separate compartments are mixed by a crew's simple operation to initiate crystallization. These containers are shown in the **Figs. 3** and **4**.

4. Our Services on Earth

We have been constantly improving our hardware and increasing the number of launch opportunity. At the same time, we have worked hard to improve our in-house research facility and operational procedures. We are fully committed to support our users and determined to make a serious contribution to structural biology research by revealing protein structures useful for industries and academia. We support users who have no experience on protein crystallization or data collection.

As mentioned, preparation on the ground is crucial to growing high quality protein crystals in space, we give an example of our approach, when we receive a company's protein sample, whose crystallization condition was unknown.

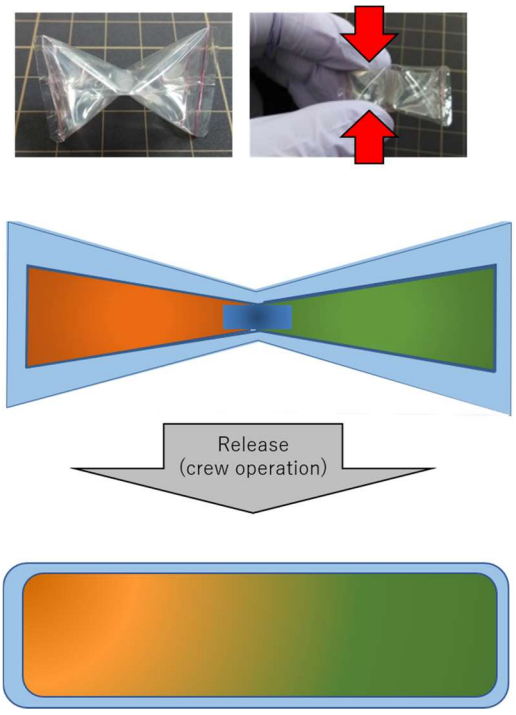

Fig. 4 Schematic figure of the Petit crystallization bag. Two liquids are mixed by breaking the seal separating both liquids by simply pressing the container.

Firstly, we thoroughly characterize the protein sample and improve its quality. Not only standard SDS-PAGE, Native-PAGE, isoelectric focusing PAGE (IEF-PAGE) and two-dimensional (2D) PAGE can be used to detect impurities and contaminations. Molecular dispersity is evaluated by Dynamic light scattering (DLS), protein purity by analytical chromatography, accurate molecular weight by mass spectroscopy and oligomeric state and its molecular weight by SEC-MALS (size exclusion chromatography with multiple detectors).

Improving the purity and homogeneity of the sample and removing contaminations, additional liquid chromatography preparation is followed. Furthermore, we search protein stabilizing conditions such as pH and additives by the thermal shift assay (TSA). If necessary, chemical modification can be used to facilitate crystallization.

When protein quality reaches the sufficient level, crystallization screening is performed. The information from TSA helps the crystal optimization. Preliminary diffraction data can be obtained by our in-house X-ray diffractometer. You know the feeling when your crystal turns out to be a salt crystal, it is still better to get disappointed at your lab rather than at a synchrotron facility. JAXA now has four staff members who hold a Ph.D. degree in protein crystallography and six technicians. Our laboratory equips chromatography systems, crystallization robots, crystallization observation systems, liquid handling robots, DLS SEC-MALS, anaerobic chamber, mass spectrometer, X-ray diffractometer. We have temperature controlled (20°C and 4 °C) lab spaces for crystallization and crystal handling.

Considerable amount of efforts and sample is consumed at this point, but they are really rewording. This is one of our success stories -- we received a protein sample with unknown crystallization condition. Although we managed to crystallize it, which diffracted to only 20 Å. Due to our massive efforts of characterization, quality control and crystal optimization, we could improve the resolution to 3.5 Å on the ground. It was even higher for a space grown crystal as high as 2.1 Å. The diffraction quality such as spot profiles were better, and the structure was well determined. This resolution is sufficient to design drug candidates based on the structural information.

5. Utilization of the ISS

5.1 Promotion for Utilization by the Private Sector

JAXA's services presented in Section 4 are offered to private companies and foreign researchers. PeptiDream Inc., a top-class pharmaceutical venture in Japan, has placed a high valuation on JAXA's technologies and signed a strategic partnership agreement for multiple years. With alliance contracts with pharmaceutical companies of the world under its belt, the firm designs drug candidates for the target proteins supplied by such companies. PeptiDream provides the target proteins and drug candidates to us to co-crystallize them in space. The high-quality co-crystals obtained in *Kibo* are subjected to X-ray analysis, and their structure and binding modes are determined by JAXA. All the data acquired are fed back to the firm. PeptiDream will use the data to improve the drug candidates. In June 2017, high quality crystals of HER2, a protein promoting the growth of breast cancer cells, and the drug candidate complex were obtained. The structure was successfully determined at 2.5Å resolution including a cyclic peptide (drug candidate) bound to HER2. A joint press release reporting this result was published. (http://global.jaxa.jp/press/2017/06/20170609_protein.html). We are very pleased that we could contribute to drug development, even indirectly.

User requests and opinions were considered, and JAXA's services related to private company utilization of the ISS include consultation on protein mass production and the purification process. T JAXA's services covers a series of activities in a package that includes further purification, sample quality check, crystallization screening, optimization for a space experiment, space experiment, X-ray data collection at SPring-8, and structure analyses (by molecular replacement method). Of course, required service items can be chosen à la carte in the contract. Every progress made by the operations is fed back to the client. JAXA is bound by an obligation of confidentiality and wavers all rights. The only exception to confidentiality is that the company's name is disclosed at the commencement of a space experiment. Domestic companies can choose a free "Trial Use Course" available one time only. JAXA believes this makes companies more accessible to the space experiments. We hope our services deserves your attention and consideration.

5.2 Basic Research Use Course

The main users of our PCG experiment are researchers from universities and institutes in Japan. They can launch their sample free of charge on the condition that their research results will be published (fee-based for foreign academic users). JAXA calls for proposals twice a year. Some users from universities have proposed research on vital mechanisms and research on an attempt to identify unknown enzyme reactions. Other types of research have led to actual drug developments [7]. JAXA as a national research and development agency, hopes that space experiment results will contribute to further progress in such research activities.

Many academic researchers involved in protein structural studies have skills for protein crystallization and structure analyses. Such researchers do not need JAXA's full services. JAXA's role then is to optimize the crystallization conditions to space experiments and the retrieval of crystals from the glass tube [4]. These supports are mainly provided through out contractors. Other researchers having an interest in molecular structures but

who have no experience, may feel it difficult to do by themselves. Thus, JAXA has recently inaugurated a new service called "Quick Crystallization Diagnosis" for domestic researchers. The researchers send a sample to JAXA, then we perform preliminary property check and initial crystallization screening. If the sample is successfully crystallized, they are requested to submit an application to shift to space experiment. If you have a sample you wish to know the structure, please do not hesitate to contact us.

6. Results

Ever since we started our PCG experiment in 2003, a cumulative number of the protein sample launched is 538 (as of September 2018). In the early days of our project, we compared the resolution of space grown crystals and ground control crystals which used the same sample lot, and same hardware set-up to show the microgravity effect. However, there are cases where we obtain the first crystal through our services, so a simple comparison of space *vs* ground is not so important anymore. **Fig. 5** shows the resolution of space and ground grown crystals, where the vertical axis shows the ground grown crystal resolution obtained before the collaboration with JAXA, and the horizontal axis shows the space grown crystal resolution. There are 12 proteins not plotted in this figure; these are proteins which were not crystallized before the collaboration with JAXA, but crystallized and were able to obtain diffraction data after the collaboration and space experiment. 10 protein structure was unknown before the project, but the molecular structure was solved after the project. Moreover, the binding mode of target proteins and drug candidates are frequently revealed from space grown crystals, but those are not plotted either. This issue of IJMSA features such research results.

7. Conclusion

This is a brief introduction to the high-quality protein crystal growth experiment project underway in *Kibo* of the ISS. By making space experiments more accessible to researchers, JAXA intends to help revealing high-quality structures of medically important proteins, and accelerate the initial stages of structure based drug design. You have just read about some actual results from research on *Kibo*. We are always striving to improve experiments following our policy, "More easy and convenient to use from a user point of view". Any readers interested in the matter feel free to drop us a line at Z-KIBO-PROMOTION@ml.jaxa.jp

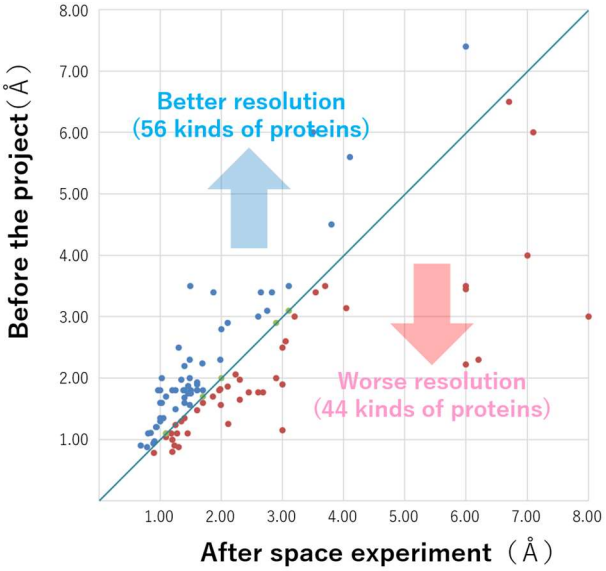

Fig. 5 Resolution of space and ground grown crystals. The vertical axis shows the ground grown crystal resolution obtained before the collaboration with JAXA, and the horizontal axis shows the space grown crystal resolution. There are 12 proteins not plotted in this figure; these are proteins which were not crystallized before the collaboration with JAXA, but crystallized and were able to obtain diffraction data after the collaboration and space experiment.

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