

**Protein structure determination using magnetically oriented
microcrystals**

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Chapter 1

General Introduction

1.1 Background

The functions of biomacromolecules, such as proteins, polysaccharides, and nucleic acids, are closely related to their tertiary structures. Therefore, structural determination of biomacromolecules is of primary importance as a step towards their utilization. An example is the serotonin transporter; this protein regulates serotonin concentration in the synapse. It is reported that depression is related to a decrease in serotonin (Pezawas et al., 2005), and the structural basis for action of serotonin transporter has been studied for an understanding of the mechanism of antidepressants (Wang et al., 2013). The structure determination of proteins is important not only in pharmaceutical areas, as exemplified above, but also in various scientific areas such as structural biology, food science, and medical science. In addition, structure determination is highly in demand in energy-related areas whereby proteins such as those involved in photosynthesis and decomposition of celluloses, for example, are key players.

There are various methods for protein structure determination, which include X-ray diffraction (XRD), neutron diffraction (ND), nuclear magnetic resonance (NMR) spectroscopy, and cryo-electron microscopy (cryo-EM) (Kendrew et al., 1958; Kainosho et al., 2006; Tanaka et al., 2009; Bai et al., 2013; Yonekura et al., 2015). Among these, one of the most powerful techniques is the XRD method (Garman, 2014). In fact, among the 115,031 structures registered in Protein Data Bank (PDB) as of 14 January

2016, 89% were determined by XRD. There are no limitations of molecular weights in applying XRD if high-quality single crystals are available in sizes appropriate for measurement. Furthermore, owing to the high intensity of X-ray sources supplied by synchrotron facilities and to the technical developments for measurement, the crystal size required becomes smaller and smaller and the measurement time for collecting data is getting shorter and shorter (Smith et al., 2012).

However, there are some drawbacks in XRD technique as follows: (1) it is often difficult to grow crystals to required sizes; (2) crystals can incur radiation damage (Garman, 2010); and (3) difficulties in locating hydrogen atoms.

1.2 X-ray and neutron crystallography

The first step in XRD structure analysis is to prepare single crystals. It is difficult to grow large single crystals for many proteins even with intensive screening performed in an attempt to determine suitable crystallization conditions. In particular, it is difficult to obtain large and high-quality crystals of protein complexes and membrane proteins. It sometimes happens that the crystal quality is not sufficient for even a single crystal with sufficient size to be obtained by chance. Currently, the crystal sizes required are >50 μm for in-house diffractometers, >20 μm for synchrotron facilities, and > several micrometers if microfocus X-ray sources (Smith et al., 2012) and X-ray free-electron lasers (XFEL) (Schlichting, 2015) are used. Yet, there are many proteins that do not grow to these sizes.

High intensities of the X-ray source are sometimes required to collect high-resolution data from protein microcrystals. In particular, this holds true with

proteins having large unit cells such as protein complexes according to Darwin's formula (Holton & Frankel, 2010) that relates the diffraction intensity to the incident X-ray intensities. However, high X-ray doses could result in severe radiation damage to protein molecules (Zeldin et al., 2013). Photoelectrons are created with the impinging X-ray and they interact with water and protein molecules to produce radicals. These radicals could cause damage to protein molecules; scission of disulfide bonds and decarboxylation of carboxyl side chains can occur (Paithankar & Garman, 2010). In addition, the reduction of metal atoms in proteins can occur with X-ray radiation, which could affect the local structure. In fact, the distances between manganese and oxygen atoms present in the manganese-calcium cluster of Photosystem II (PSII) protein determined by XRD analysis at 1.9 Å resolution (Umena et al., 2011) do not correspond to those determined by calculations. This discrepancy could occur because of radiation damage to the manganese-calcium cluster. The issue of radiation damage can be partially reduced by flash-cooling protein crystals at the temperature of liquid nitrogen (Garman, 2003). However proteins are not perfectly damage-free.

Hydrogen bonds present in protein molecules play important roles in maintaining their tertiary structures. Additionally, they are important in view of their catalytic actions on substrates. However, it is difficult to determine the hydrogen atoms structure precisely in the protein molecule using XRD because the electron density around the hydrogen atoms is low, resulting in weak diffraction. On the other hand, neutrons are diffracted strongly by hydrogen atoms because they have large scattering cross-sections for neutrons (C. G. Shull & E. Wollan, 1948). Thus, neutron diffraction (ND) is powerful for determination of the position of hydrogen atoms. A

problem with ND is that the crystal size required is much larger than that required for XRD; in general, sizes as large as 10 mm^3 are required (Blakeley et al., 2015).

Because of this size limitation, only 49 structures that are determined by ND have been registered in PDB. Crystal size is therefore the main limitation to the ND technique.

1.3 Orientation control of microcrystalline proteins using magnetic fields

Magnetic susceptibility tensor χ of biaxial crystals (orthorhombic, monoclinic, and triclinic systems) has three different principal values χ_1 , χ_2 , and χ_3 (Nye, 1985), where we define $\chi_1 > \chi_2 > \chi_3$. Under a static magnetic field B , the χ_1 axis aligns parallel to B (**Figure 1.1**), while under a rotating magnetic field, the χ_3 axis aligns perpendicular to the plane of the magnetic field rotation (**Figure 1.2**). If a time-varying magnetic field shown in **Figure 1.3**, which is a sort of combination of the static and rotating magnetic fields, is applied to the crystal, then the magnetic axes are aligned three-dimensionally (Kimura & Yoshino, 2005). As the magnetic χ_1 , χ_2 , and χ_3 axes are embedded in the crystallographic unit cell, the three-dimensional (3D) alignment of these magnetic axes indicates the 3D alignment of crystallographic a , b ,

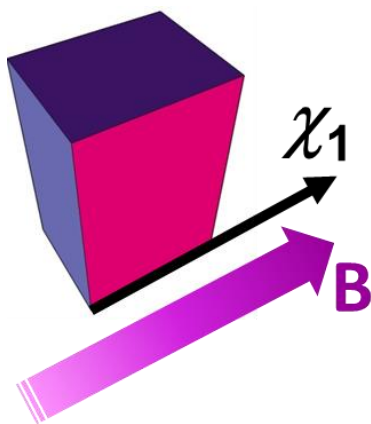


Figure 1.1. The χ_1 axis aligns parallel to the applied magnetic field B .

and c axes. This 3D alignment technique is applicable to biaxial crystals. If the time-varying magnetic field is applied to microcrystals suspended in a liquid medium, the individual microcrystals are all aligned three-dimensionally (**Figure 1.4**) (Kimura et al., 2006), which will give rise to the XRD images equivalent to those from a real single crystal.

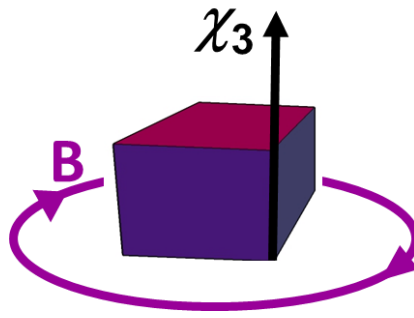


Figure 1.2. The χ_3 axis aligns perpendicular to the plane of the magnetic field rotation.

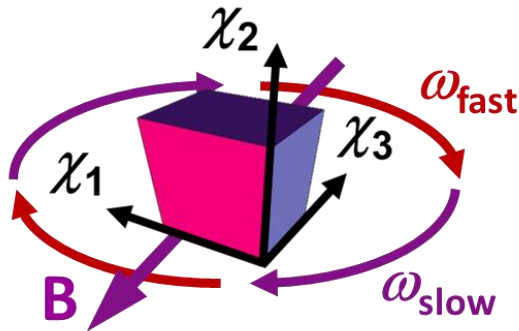


Figure 1.3. The χ_1 , χ_2 , and χ_3 axes undergo three-dimensional alignment under time-varying magnetic field characterized by two different rotation speeds, ω_{fast} and ω_{slow} .

In actual experiments, a suspension containing microcrystals is rotated in a time-varying manner under a static magnetic field, because it is difficult to rotate magnetic fields in a time-varying manner. After 3D alignment is achieved, the suspending medium is consolidated to fix the alignment and the obtained microcrystalline composite (referred to as a magnetically oriented microcrystal array (MOMA)) is subjected to XRD measurement (Kimura et al., 2006). So far, crystal structures of inorganic, organic, and protein microcrystals were determined using MOMAs (Kimura et al., 2009; Kimura et al., 2011; Kimura et al., 2014).

1.4 Objectives of this thesis

There are two questions regarding the improvement of the MOMA technique:

- (i) can 3D alignment be achieved using a simple uniform rotation? (ii) could other

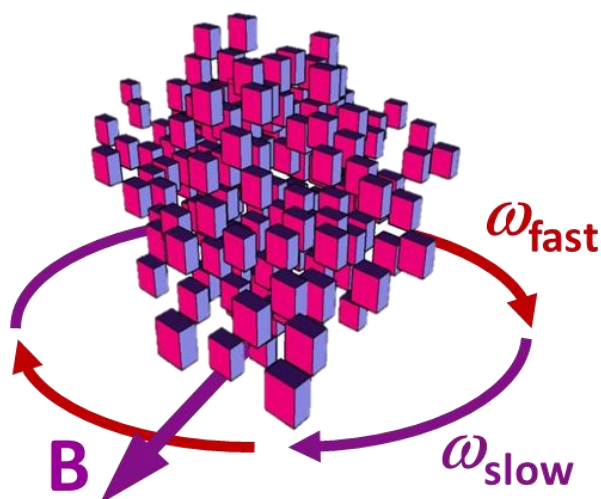


Figure 1.4. Microcrystals undergo three-dimensional alignment under time-varying magnetic field, resulting in MOMA.

consolidating media than UV curable monomer be suitable for preparing protein MOMAs?

Regarding (i), a simpler time-varying manner is in demand because the rotation control such as that shown in **Figure 1.4** is complicated. According to the classification of orientation dynamics, there are three kinetic regimes: synchronous rotation regime (SRR), asynchronous rotation regime (ARR), and rapid rotation regime (RRR). These regimes are characterized by the quantity $\tau\omega$, where ω is the rotation speed of a suspension, and τ is a time scale characteristic of the buildup of magnetic orientation. In SRR, $\tau\omega < 1/2$; in ARR, $1/2 < \tau\omega < \infty$ and in RRR, $\tau\omega \rightarrow \infty$. The schematic shown in **Figure 1.4** can only be applied to RRR in combination with two speeds of rotation, ω_{slow} and ω_{fast} . There is a possibility that 3D orientation is achieved in SRR with a single ω . If this is possible, the alignment procedure is greatly simplified. However, the basic study on the kinetics of crystal orientation under the SRR condition has not been explored.

Regarding (ii), consolidation has been conducted using ultra-violet (UV) curable monomers for suspending media. However, UV-curable monomers are not suitable for protein crystals because these synthetic monomers are mostly hydrophobic, while proteins are hydrophilic. The MOMA technique was first applied to lysozyme microcrystals (Kimura et al., 2011), where problems were that the microcrystals had to be partially dried in order to form a good suspension with UV curable monomers. As a result, the structure was solved at a resolution of only 3.0 Å with in-house diffractometer and the obtained structure was largely shrunk compared to that determined for the natural form. One way to overcome this situation is the use of

synchrotron sources in order to obtain high resolution. The other way is to use consolidating media other than UV-curable monomers in order to prevent the drying process. It might be necessary to explore aqueous consolidating media that have high affinity to protein crystals. In addition, D₂O-based consolidating media are in high demand for the preparation of MOMAs to be used for ND measurements.

The first objective of this thesis was to study the orientation kinetics under conditions of SRR in order to examine whether MOMAs could be prepared in a much simpler rotation manner. The second objective was to explore the MOMA technique in combination with synchrotron X-ray sources. The final objective was to explore consolidating media suitable for preparing protein MOMAs.

1.5 Outline of this thesis

In Chapter 2, the kinetics of magnetic orientation under conditions of SRR are studied in an attempt to explore novel and simple alignment. The experimental observation of the process of alignment of a single microcrystal is difficult because it is hard to determine the magnetic axes with respect to the shape of the crystal to be monitored. Therefore, in this work, biaxial rods of macroscopic size were prepared, for which the direction of the magnetic axes were precisely defined with respect to the rod long axis. The experimental observations are compared with the theoretical prediction.

In Chapter 3, MOMAs of lysozyme microcrystals prepared with UV-curable monomers are subjected to XRD measurements at SPring-8 to demonstrate that higher resolutions, such as 1.8 Å, can be obtained if measurements are performed at

synchrotron facilities. The crystal size of lysozyme is less than 10 μm , which is too small to be analyzed at conventional high-throughput beam lines of SPring-8 designed for protein crystal analyses (crystals $> 50 \mu\text{m}$ are required). In this chapter, it is demonstrated that crystals less than 10 μm in size can be analyzed at conventional high-throughput beamlines.

In Chapter 4, the use of aqueous gels for consolidating media is explored in order to expand the application area of the MOMA technique. With the use of aqueous gels, protein MOMAs can be prepared without a drying process. This enables the determination of protein structures in intact and natural states without the transformation of structures by drying crystals. In addition, gel MOMAs prepared with D_2O exhibit excellent neutron diffraction because the background from H_2O is greatly reduced. This is the first report on the observation of neutron diffraction images from microcrystalline protein crystals.

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Chapter 2

Magnetic alignment of magnetically biaxial diamagnetic rods under rotating magnetic fields

2.1 Introduction

Magnetic orientation is a promising means for aligning diamagnetic particles such as crystals and fibers, and has drawn attention as a novel method for material processing. A number of studies have been reported on magnetic orientation (Maret & Dransfeld et al., 1985; Kimura, 2003; Tanimoto, 2006). Uniaxial alignment of the easy magnetization axis (χ_1 axis) occurs under a static magnetic field, and that of the hard magnetization axis (χ_3 axis) occurs under a rotating magnetic field. (Singer et al., 1973; Kimura et al., 2004) Furthermore, it is known that the magnetically biaxial crystal can undergo three-dimensional alignment of the χ_1 and χ_3 axes (and χ_2 axis, accordingly) when time-varying magnetic fields are used (Staines, 1996; Kimura & Yoshino, 2005). Three-dimensional alignment of a microcrystalline powder of a biaxial crystal is useful not only for improving material properties of composites but also for preparing a sample for X-ray and neutron single crystal diffraction measurements (Kimura et al., 2006; Kimura et al., 2009; Kimura et al., 2010; Kimura et al., 2010; Kimura et al., 2011) because three-dimensionally aligned microcrystals give rise to diffractions equivalent to those from a corresponding single crystal.

Understanding of the dynamics of magnetic alignment is vital for the control of alignment. Though a number of studies have been reported for the dynamics of magnetic alignment, (Brochard et al., 1975; Uyeda et al., 1991; Kimura et al., 2000; Hirota et al., 2008; Terada et al., 2008; Kimura et al., 2009; Yamaguchi et al., 2009;

Yamaguchi et al., 2010) very few have been reported on the dynamics for biaxial crystals. In this chapter, we prepared three types of magnetically biaxial rods ($1.1 \text{ mm} \phi \times 3 \text{ mm}$) whose rod axes are parallel to χ_1 , χ_2 , and χ_3 axes so that the behavior of the alignment dynamics is visualized and its analysis is facilitated. The results were compared with the simulation results based on a torque equation.

2.2 Experimental procedure

Rod samples were prepared following the method described previously. (Kimura et al., 2006) L-alanine crystals (Wako Pure Chemical Industries, Ltd.) were pulverized and mixed with an UV curable monomer (Arakawa Chemical Industries, Ltd., 550VC) at 30.1wt% and subjected to centrifuge (5,000 rpm, 20 min). A supernatant containing microcrystals ($<30 \text{ } \mu\text{m}$) was poured into a glass capillary (Fisher Scientific, $1.1 \text{ mm} \phi$) and placed in the center of an 8-T horizontal magnetic field (a Sumitomo cryogen-free superconducting magnet), followed by a frequency modulated rotation at angular velocities, $\omega_{slow} = 40 \text{ rpm}$ and $\omega_{fast} = 132 \text{ rpm}$, for 1 h with the rotation axis being vertical. Then, UV light was irradiated to photopolymerize the monomer and to consolidate the achieved alignment. The capillary was removed from the magnet and the rod-like sample was taken out from the capillary. The fabricated samples were subjected to the X-ray diffraction measurements to confirm that the χ_1 , χ_2 , or χ_3 axis was parallel to the long axis of the rod in each sample. The samples whose long axes were parallel to χ_1 , χ_2 , and χ_3 were named Rod I, Rod II, and Rod III as shown in **Figure 2.1**.

The experimental setup is shown in **Figure. 2.2**. Each rod was suspended in an isodensity liquid (a chloroform/toluene solution of polystyrene) in an optical cell. The cell was capped and rotated in the center of a magnetic field of 2 T generated by a Tamagawa electromagnet. The rotation axis and the field direction were horizontal and they were orthogonal. The motion of the rod was recorded by a CCD camera. From the recorded photos, the angle α (the angle between the χ_1 axis and the magnetic field) was determined for Rod I and the angle θ (the angle between the rod axis and the rotation axis) (Figure 2.3) was determined for Rod II and Rod III as a function of time.

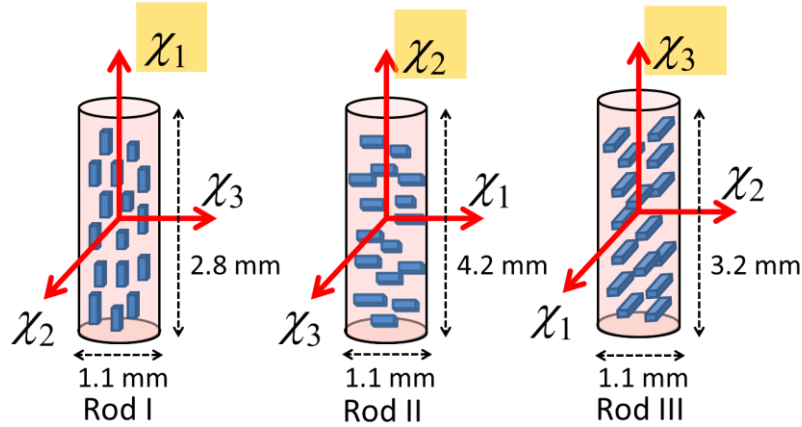


Figure 2.1. Three rod samples used in this chapter. In each rod, microcrystals of L-alanine are aligned three-dimensionally. The rod axis is parallel to the χ_1 -axis (Rod I), to the χ_2 -axis (Rod II), and to the χ_3 -axis (Rod III), where χ_1 , χ_2 , and χ_3 are magnetic susceptibility axes with $\chi_1 > \chi_2 > \chi_3$.

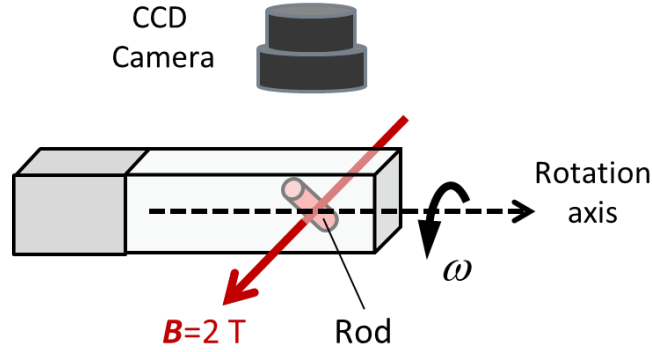


Figure 2.2. Experimental setup. A rod is suspended in an isodensity liquid in a glass cell, and the cell is rotated at angular velocity ω in a homogeneous magnetic field B and the motion of the rod is recorded using a CCD camera.

2.3 Simulation

Let us consider a prolate particle with diameter a and length l as an approximation of a rod used in the experiment. In the case of Rod III, for instance, the magnetic susceptibility in the direction parallel to the long axis is defined as χ_3 (hard magnetization axis) and those perpendicular to the long axis are defined as χ_2 and χ_1 (easy magnetization axis), where $0 > \chi_1 > \chi_2 > \chi_3$. This corresponds to Rod III. This particle is suspended in a liquid medium of the density same as the particle, and subjected to a magnetic field

$$\mathbf{B} = B(\cos \omega t, \sin \omega t, 0) \quad (2.1)$$

rotating on the xy plane at a rotation velocity of ω . In the theoretical analysis, the magnetic field is rotated, with the cell remaining fixed, but actual experiments were

performed by rotation of the cell under a static magnetic field. Both of them are equivalent. The magnetic torque that this particle is exerted by the applied field is expressed as

$$\mathbf{N}_{\text{mag}} = \mu_0^{-1} V (\boldsymbol{\chi} \mathbf{B}) \times \mathbf{B}, \quad (2.2)$$

where μ_0 is the permeability of vacuum and V is the total volume of the L-alanine crystallites contained in the particle. The susceptibility tensor $\boldsymbol{\chi}$ expressed in terms of the laboratory coordinates is related to the susceptibility tensor $\boldsymbol{\chi}_p$ expressed in terms of the coordinates embedded in the particle using equation, $\boldsymbol{\chi} = {}^t \mathbf{A} \boldsymbol{\chi}_p \mathbf{A}$, where the transformation matrix $\mathbf{A}$ is defined by the Eulerian angles (ϕ, θ, ψ) (Goldstein, 1950) that are described in **Figure 2.3**. Thus, the magnetic torque is expressed as a function of χ_1, χ_2, χ_3 and ϕ, θ, ψ as follows:

$$\mathbf{N}_{\text{mag}} = K_{\text{mag}} \mathbf{T}_{\text{mag}}(\phi, \theta, \psi, t; \omega, r_\chi), \quad (2.3)$$

where $K_{\text{mag}} = \mu_0^{-1} V B^2 (\chi_2 - \chi_3)$ and $r_\chi = (\chi_2 - \chi_3) / (\chi_1 - \chi_2)$.

The particle rotating at the angular velocity of $\boldsymbol{\Omega}$ in a viscous medium receives a hydrodynamic torque exerted by the medium, which is expressed as

$$\mathbf{N}_{\text{hyd}} = -\eta \mathbf{L} \boldsymbol{\Omega}, \quad (2.4)$$

where η is the viscosity of the medium and $\mathbf{L}$ is the hydrodynamic tensor expressed in terms of the laboratory coordinates, which is related to the principal values (L_1, L_2, L_3) of $\mathbf{L}_p$, with L_3 corresponding to the long axis, by the equation $\mathbf{L} = \mathbf{A}^t \mathbf{L}_p \mathbf{A}$, where the transformation matrix $\mathbf{A}$ is the same as defined earlier. The expression for $\mathbf{L}_p$ is reported in the literature (Perrin, 1934). The hydrodynamic torque is then expressed as

$$\mathbf{N}_{\text{hyd}} = K_{\text{hyd}} \mathbf{T}_{\text{hyd}}(\phi, \theta, \psi, \dot{\phi}, \dot{\theta}, \dot{\psi}; R_L), \quad (2.5)$$

where $K_{\text{hyd}} = \eta L_1$ and $R_L = L_3 / L_1$.

In addition to the magnetic and hydrodynamic torques described above, we need to introduce a torque induced by the imbalance of the gravitation and buoyancy

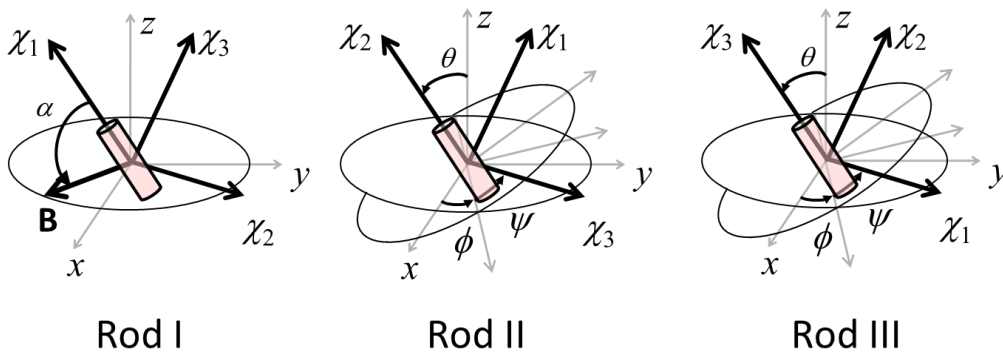


Figure 2.3. Angles α and θ determined by experiment for each rod. Angles ϕ , θ , and ψ are Eulerian angles used in simulation.

forces. If the centers of gravity and buoyancy are located at the same point, no torque arises. However, this condition is not satisfied in our sample because the spatial distribution of crystallites may not be perfect and/or small bubbles may be included inevitably during sample preparation. Therefore, we introduce a torque $\mathbf{N}_{\text{grv}}$ expressed empirically to account for this imbalance:

$$\begin{aligned}\mathbf{N}_{\text{grv}} &= K_{\text{grv}} \mathbf{T}_{\text{grv}}(\phi, \theta, \psi, t; \omega) \\ &= K_{\text{grv}} (\cos \omega t \cos \theta, \sin \omega t \cos \theta, \sin(\omega t - \phi) \sin \theta)\end{aligned}\quad (2.6)$$

where K_{grv} is an adjustable parameter. In this equation, the explicit time dependence reflects the fact that the cell (not the magnetic field) is rotating in the actual experiment. This torque does not appear when a single crystal is concerned because of the same center of the gravity and buoyancy.

In this study, the mass of the particle is small so that we ignore the inertia term in the equation of motion and thus we obtain

$$\mathbf{N}_{\text{mag}} + \mathbf{N}_{\text{hyd}} + \mathbf{N}_{\text{grv}} = 0. \quad (2.7)$$

Using Eqs. (2.3), (2.5), and (2.6), the above equation is rewritten as

$$\tau^{-1} \mathbf{T}_{\text{mag}}(\phi, \theta, \psi, t; r_{\chi}, \omega) + \mathbf{T}_{\text{hyd}}(\phi, \theta, \psi, \dot{\phi}, \dot{\theta}, \dot{\psi}; R_L) + k_{\text{grv}} \mathbf{T}_{\text{grv}}(\phi, \theta, \psi, t; \omega) = 0, \quad (2.8)$$

where unknown parameters are $\tau^{-1} = K_{\text{mag}} / K_{\text{hyd}}$, $k_{\text{grv}} = K_{\text{grv}} / K_{\text{hyd}}$, and

$r_\chi = (\chi_2 - \chi_3) / (\chi_1 - \chi_2)$. Using the expressions for L_1 and L_3 (Perrin, 1934), we estimated R_L for each rod using its aspect ratio. This differential equation was solved numerically using Mathematica whereby unknown parameters (τ , k_{grv} , and r_χ) were set such that the best fit to the experimental data was achieved. Calculations with $k_{\text{grv}} = 0$, i.e., $\mathbf{N}_{\text{grv}} = 0$, were also performed.

2.4 Results and discussion

The results for Rod I are shown in **Figure 2.4**. The angle α defined in **Figure 2.3** is plotted as a function of time. Experimental results show that χ_1 axis follows the rotation of magnetic field with a phase delay when the rotation speeds are slow like $\omega = 2$ and 3 rpm. Upon increase in the rotation speed, the χ_1 axis becomes unable to follow the rotation, and the magnetic field passes the χ_1 axis. This phenomenon is observed for the experiments with $\omega = 4$ and 5 rpm. The simulation results are also shown in the same figure. The values of the adjustable parameters, τ , k_{grv} , and r_χ , used for the simulation are summarized in **Table 2.1**.

Table 2.1. Parameters used for simulation

	τ/sec	k_{grv}	r_χ^*	R_L
Rod I	-0.68	1.83×10^{-2}	1.11	0.40
Rod II	39.0	1.43×10^{-3}	1.11	0.23
Rod III	65.0	5.00×10^{-3}	1.11	0.33

The results for Rod II are shown in **Figure 2.5**. The angle θ defined in **Figure 2.3** is plotted as a function of time. Experimental results show that the angle θ tends to decrease in time except for the measurement with $\omega = 0.5$ rpm. These experimental results are well reproduced by the simulation with adjustable parameters listed in **Table 2.1**. At lower rotation speeds such as $\omega = 0.1$ to 0.3 rpm, the angle θ cannot reach zero. This could happen because the torque due to the imbalance of the torques due to the gravity and the buoyancy exceeds the magnetic torque. However, if this imbalance does not exist ($k_{\text{gv}} = 0$), the simulation shows that the angle θ reaches

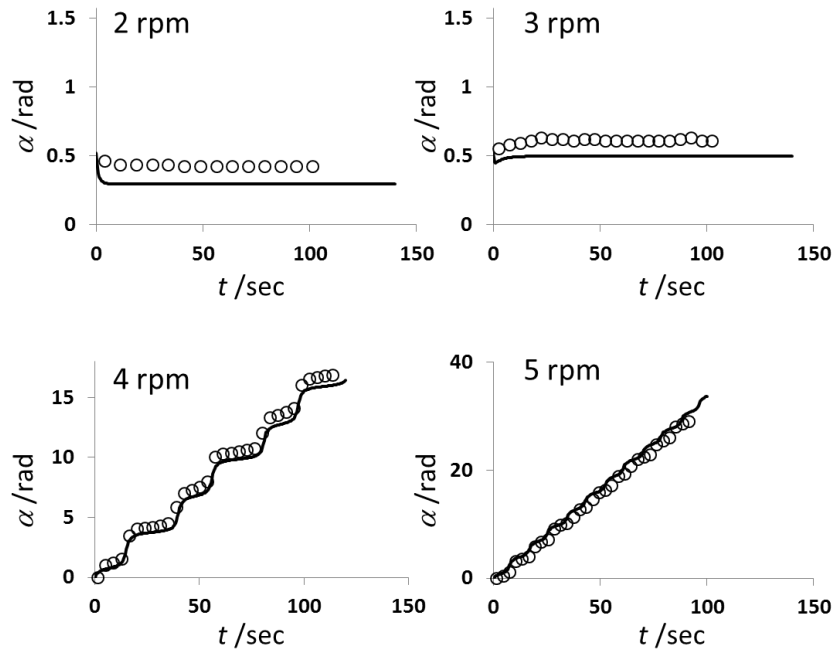


Figure 2.4. Experimental and simulation results for Rod I. Angle α (Figure 2.3.) is plotted against time at different rotation speeds (revolution per minute) indicated in the figure. Circle and solid line indicate experimental and simulation results, respectively. Parameters summarized in Table 2.1 are used for simulation.

zero in any cases except for the case with $\omega = 0.5$ rpm. This indicates that the χ_2 axis aligns parallel to the rotation axis.

The results for Rod III are shown in **Figure 2.6**. The angle θ defined in **Figure 2.3** is plotted as a function of time. Experimental results show that the angle θ tends to decrease in time, indicating that the χ_3 axis aligns parallel to the rotation axis. The increase of the angle θ for the case with $\omega = 0.1$ rpm is attributed to the imbalance of the torques due the gravity and the buoyancy. Due to this torque, the complete alignment of the χ_3 axis is prohibited for all ω values studied here,

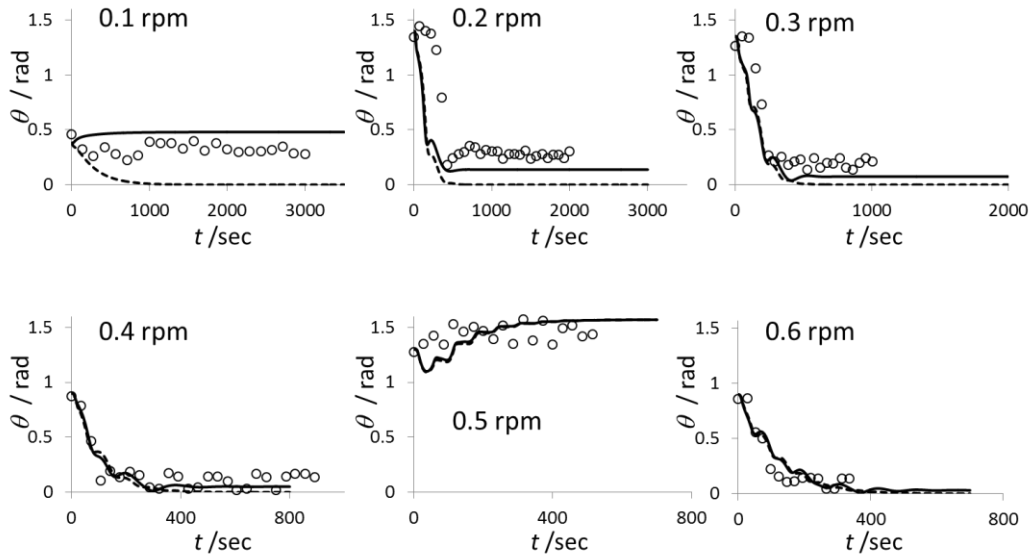


Figure 2.5. Experimental and simulation results for Rod II. Angle θ (Figure 2.3) is plotted against time at different rotation speeds (revolution per minute) indicated in the figure. Circle and solid line indicate experimental and simulation results, respectively. Parameters summarized in Table 2.1 are used for simulation.

Broken line indicates simulation results where $k_{grv} = 0$.

whereas the simulations with $k_{\text{grv}} = 0$ (no torque imbalance) shows that the χ_3 axis can align completely parallel to the rotation axis.

Now, we analyze the case with $\omega = 0.5$ rpm for Rod II. The magnetic energy of a magnetically biaxial particle, whatever shape it is, subjected to a magnetic field rotating on the xy plane (eq. (2.1)) is express as

$$E_{\text{R}} = -\frac{VB^2}{2\mu_0} \sum_{i=1}^3 \chi_i \sin^2 \beta_i \cos^2(\omega t + \delta_i), \quad (2.9)$$

where the angles β_1 , β_2 , and β_3 are angles that the z axis (parallel to the rotation axis) makes with respect to the χ_1 , χ_2 , and χ_3 , respectively (**Figure 2.7a**), and the angles δ_1 , δ_2 , and δ_3 are defined in **Figure 2.7b**. It might be assumed that the χ_1 axis lies approximately on the xy plane; then we put $\beta_1 \cong 1/2$. In this condition, $\beta_2 - \beta_3 \cong \pi/2$ should be satisfied. Thus, Eq. (2.9) is rewritten as

$$E_{\text{R}} \approx \frac{VB^2}{2\mu_0} K \sin^2 \beta_2, \quad (2.10)$$

where the isotropic term is neglected. The coefficient K that determines the location of the minimum of the energy is expressed as

$$K = -\chi_2 \cos^2(\omega t - \delta_2) + \chi_3 \cos^2(\omega t - \delta_3). \quad (2.11)$$

With a small deviation of the χ_1 axis from the xy plane, the value of $\delta_3 - \delta_2$ can vary between $\pi/2$ and π depending on the direction of the χ_2 axis. The sign of K could be positive or negative depending on the values δ_2 , δ_3 and ωt . Thus, the minimum of the energy changes interchangeably between $\beta_2 = 0$ and $\beta_2 = \pi/2$ in time. In other words, both χ_2 and χ_3 axes have chance to align in the z direction. The alignment $\chi_3 \parallel z$ has a lower energy than the alignment $\chi_2 \parallel z$. In addition to the energy consideration, we need to consider the dynamic aspect. Since the χ_2 axis in

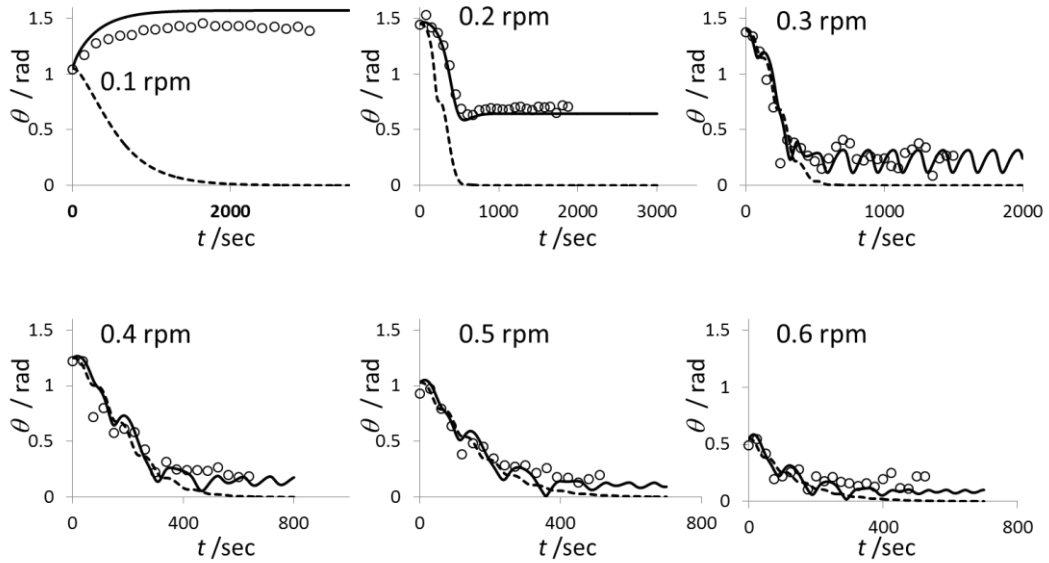


Figure 2.6 Experimental and simulation results for Rod III. Angle θ (Figure 2.3) is plotted against time at different rotation speeds (revolution per minute) indicated in the figure. Circle and solid line indicate experimental and simulation results, respectively. Parameters summarized in Table 2.1 are used for simulation.

Broken line indicates simulation results where $k_{\text{grv}} = 0$.

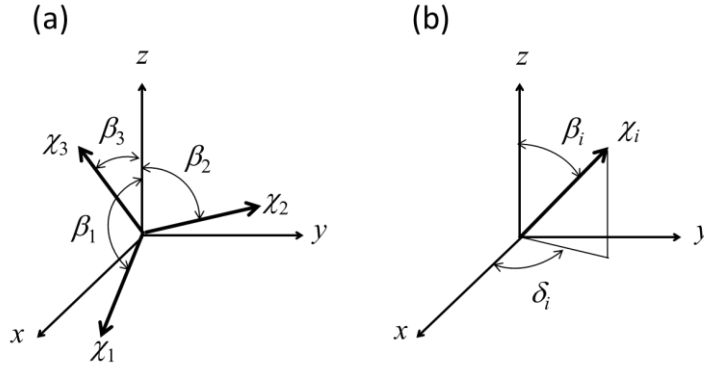


Figure 2.7. (a) Definition of angles β_1 , β_2 , and β_3 used to evaluate the magnetic energy (eq. (2.9)) under a magnetic field rotating in the xy plane, and (b) definition of angles δ_i ($i=1,2,3$).

Rod II is parallel to the rod axis, the hydrodynamic resistance is weaker when the rod rotates about the χ_2 axis (alignment $\chi_2 \parallel z$). As a result, the speed of the χ_2 axis directing to the z axis (going to the minimum at $\beta_2 = 0$) might be higher than directing onto the xy plane (going to the minimum at $\beta_2 = \pi/2$). In the case of Rod II, the hydrodynamic factor is dominant and hence the alignment $\chi_2 \parallel z$ is observed in most cases, with the exception of the alignment $\chi_3 \parallel z$ in the case with $\omega = 0.5$ rpm.

The above situation is in marked contrast to the case where the rotation velocity is infinitely high. At this condition, the coefficient K can be time-averaged over one cycle and the magnetic energy is expressed as

$$E_R \approx -\frac{VB^2}{4\mu_0}(\chi_2 - \chi_3)\sin^2 \beta_2. \quad (2.12)$$

This indicates that $\beta_2 = \pi/2$ is the minimum, i.e., the χ_3 axis aligns in the z direction. This result is consistent with our previous observation.(Kimura & Yoshino, 2005)

2.5 Conclusion

In conclusion, we have investigated the alignment behavior of magnetically biaxial rods under rotating magnetic fields, and the experimental results have been compared with simulation. In contrast to uniaxial rods where the hard magnetization axis always aligns parallel to the axis of magnetic rotation, the hard magnetization axis χ_3 does not align parallel to the axis of magnetic rotation depending on the alignment pathway. The rod whose rod axis coincides with the χ_2 axis aligns with the χ_2 axis (not χ_3 axis) parallel to the axis of magnetic rotation with few exceptions. Only under rotating fields with very high rotation speeds the χ_3 axis can align parallel to the axis of magnetic rotation irrespective of the particle shape.

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Chapter 3

X-ray crystal structure analysis of magnetically oriented microcrystals of lysozyme at 1.8 Å resolution

3.1 Introduction

Crystals of several tens of micrometers in size are commonly needed to perform single-crystal X-ray diffraction measurements. However, in many circumstances, protein crystals do not grow to these sizes. Smaller microcrystals can be analysed by using a high intensity X-ray beam focused on small crystals of only several micrometers in size to solve the crystal structure (Smith et al., 2012). Other techniques such as Serial Femtosecond Crystallography using X-ray free electron lasers (XFELs) (Chapman et al., 2011) or micro electron diffraction (Nannenga et al., 2014) have been successfully applied to micrometer- and nanometer-sized protein crystals (Kupitz et al., 2014; Sugahara et al., 2014; Yonekura et al., 2015).

We have previously proposed an alternative approach based on magnetic orientation of microcrystals (Kimura et al., 2009). By applying a time-varying magnetic field to a microcrystalline suspension, the individual microcrystals can be biaxially aligned (Staines, 1996; Kimura & Yoshino, 2005). The magnetic susceptibility of biaxial crystals (triclinic, monoclinic and orthorhombic crystal systems) has a susceptibility tensor with three different principal values, χ_1 , χ_2 , and χ_3 (here it is assumed that $\chi_1 > \chi_2 > \chi_3$). The principal axes corresponding to χ_1 and χ_3 are referred to as the easy and hard magnetization axes, respectively. The easy axis aligns parallel to the applied static field. The hard axis aligns parallel to the z axis when a rotating magnetic field in the xy plane is applied. The combination of these two magnetic fields (together referred to as a

modulated rotating magnetic field) causes the three-dimensional alignment of microcrystals. Consolidating the liquid suspension containing microcrystals by, for example, photopolymerization, a polymer composite is obtained in which the microcrystals are three-dimensionally aligned. This composite is called a magnetically oriented microcrystal array (MOMA) (Kimura et al., 2011).

The use of a MOMA for X-ray single crystal analysis has the characteristic feature that since the X-ray diffraction from a MOMA is a summation of the diffraction from the individual oriented microcrystals (say n in number), the total intensity of the diffraction, for a given incident X-ray intensity, is n times as large as that from a single microcrystal. Conversely, the incident X-ray intensity required is $1/n$ of that needed to obtain the same diffraction intensity from a single microcrystal.

In our previous paper, lysozyme was chosen as a model protein and its crystal structure was analysed using a MOMA mounted on an in-house X-ray diffractometer (Kimura et al., 2011). The crystal structure was obtained at 3.0 Å resolution. This low resolution was presumably attributable to the low intensity of the X-ray source. In this chapter here, a new sample was prepared and its diffraction was measured using the synchrotron X-ray source at SPring-8 to obtain higher resolution.

3.2 Materials and method

High-purity freeze-dried hen egg white lysozyme (HEWL) (Eisai Food Chemical, Inc.) was crystallized using a batch method at 313 K to obtain crystals of the orthorhombic form. Lysozyme was dissolved in a buffer (0.5 M sodium acetate/acetic acid (pH 4.2)) to a concentration of 200 mg/ml and filtered through a 0.20 µm membrane filter to remove insoluble residuals. A precipitant agent was prepared by dissolving

NaCl (concentration: 5% (wt/wt)) and polyethylene glycol (MW=4 000) (concentration: 16% (wt/wt)) in the same acetate buffer and filtering the mixture through a 0.20 μm membrane filter. 25 ml of precipitant agent was mixed with 25 ml of lysozyme solution using a magnetic stirrer. The batch was sealed and allowed to stand for 4 days at 313 K. The crystals obtained were separated by filtration, and dehydrated in air at 313 K, followed by pulverization with a mortar. They were then passed through a 45 μm -mesh sieve to remove larger crystals. The resulting microcrystals were dispersed in ultra violet (UV)-curable monomer (XVL-14 of Kyoritsu Chemical and Co., Ltd.; viscosity, 12.0 Pa s) at 20% (wt/wt). The suspension was allowed to stand for two days and poured into a plastic container (diameter, 3.5 mm; height, $\sim$ 5 mm).

This container was then mounted on a sample-rotating unit placed at the bore centre of a cryogen-free superconducting magnet (Sumitomo Heavy Industry), generating a horizontal magnetic field of 8 T. The rotation axis was vertical, and the rotation speed was varied between $\omega_{\text{slow}}=30$ rpm and $\omega_{\text{fast}}=70$ rpm every 90° (**Figure 3.1**). The suspension was rotated for about 90 min, and to obtain a MOMA, the alignment of microcrystals which had thus been achieved was consolidated by UV-light irradiation for 15 min with the sample remaining in the time varying magnetic field.

A dataset for crystal structure analysis was collected at room temperature on BL38B1 at SPring-8 with scans from 0° to 360° using $\Delta\varphi = 0.5^\circ$. The full width at half maximum (FWHM) of the X-ray beam was 180 μm (vertical) by 87 μm (horizontal). The diffraction data were processed with the MOSFLM software and scaled with AIMLESS from the CCP4 suite (Leslie & Powell, 2007; Winn et al., 2011; Evans & Murshudov, 2013). The phases were determined with MOLREP, using PDB code 1VDQ as the starting model for molecular replacement (Vagin & Teplyakov, 2010). This was

followed by refinement of the atomic positions and isotropic thermal factors using REFMAC5 and Phenix.refine from the Phenix software suite (Vagin et al., 2004; Adams et al., 2010). Further cycles of manual iterative modeling in COOT and refinement were then performed (Emsley et al., 2010).

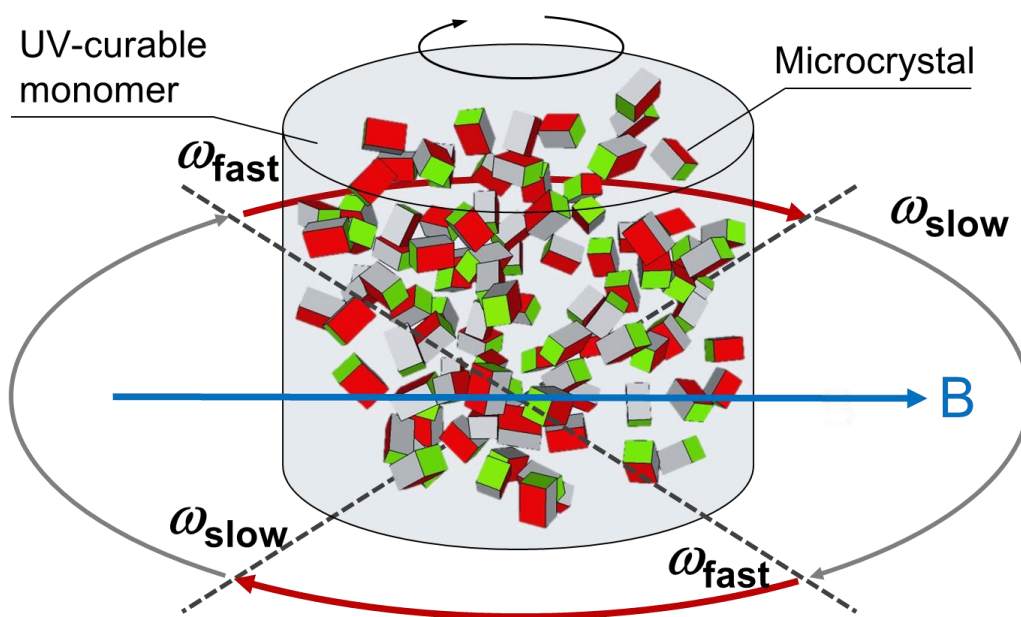


Figure 3.1. Preparation of a three-dimensionally oriented microcrystal array. A suspension of microcrystals is rotated about a vertical rotation axis at two different rotation speeds ω_{slow} and ω_{fast} ($> \omega_{slow}$) in a horizontal static magnetic field **B**. The rotation speed is switched every 90°.

3.3 Results and discussion

A polarized microphotograph of the suspension is shown in **Figure 3.2**. The size of microcrystals ranged from 5 to 10 μm . It is often difficult to acquire diffraction data from protein crystals of these sizes even at synchrotron facilities. In the experiment reported here, thousands of microcrystals were aligned three-dimensionally in the

diffraction volume, so that the diffraction intensity was considerably enhanced, and measurements could be performed at room temperature. In this chapter, a hydrophobic monomer was used as the suspending medium, and hence the microcrystalline powder had to be partially dehydrated in order to prepare a well-dispersed suspension. However, hydrophilic sols for the media can be used as an alternative, and the use of sols (solidified by gelation) is described in the chapter 4.

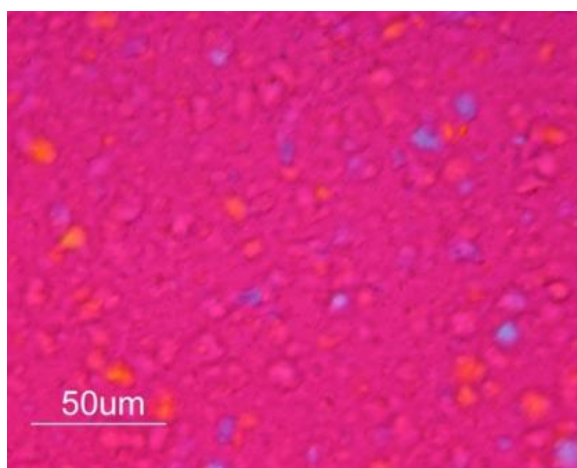


Figure 3.2. Polarized optical micrograph of microcrystal suspension of lysozyme.

The size of microcrystals is 5-10 μm .

Typical X-ray diffraction images are shown in **Figure 3.3** and the edge of the image corresponds to a resolution of 1.79 $\text{\AA}$. The diffraction peaks are broader compared with those normally measured for single crystals of HEWL. The mosaicity η of a MOMA is affected by the following factors: (i) η_{cry} , the intrinsic mosaicity of the individual microcrystals, (ii) η_{mag} , the variance of the angular distribution caused by thermal fluctuation of the microcrystals when they are aligned under a time-varying magnetic

field, and (iii) η_{sol} , the deterioration of orientation during the consolidation process.

The mosaicity η can be defined as

$$\eta = (\eta_{\text{cry}}^2 + \eta_{\text{mag}}^2 + \eta_{\text{sol}}^2)^{1/2} \quad (3.1)$$

Because dehydration is vital step in the preparation of a MOMA, it can be assumed that the mosaicity of the individual microcrystals is larger than the values usually observed for single crystals. In a separate measurement (data not shown), the mosaicity of a dehydrated 200 μm cubed HEWL crystal was found to be 0.43° (7.5 mrad), and this value will be used here for η_{cry} .

The fluctuations in orientation of microcrystals in magnetic fields are anisotropic

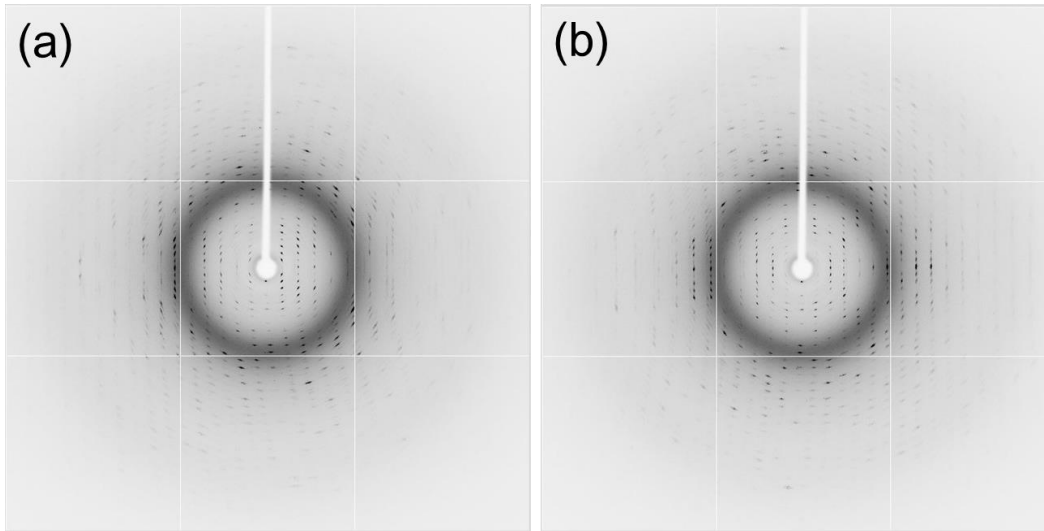


Figure 3.3. X-ray diffraction images, (a) and (b), taken at two different angles (0° and 90° respectively). The edge of the images corresponds to a resolution of $1.79 \text{ \AA}$. The average half width of the diffraction spots is 2.14° and 2.77° for (a) and (b), respectively. The diffuse rings are due to the diffraction from the polymer matrix.

and expressed by the relationship

$$\eta_{\text{mag}}^2 = K_a \frac{2k_B T \mu_0}{|\chi_a| V B^2} \quad (3.2)$$

where k_B : Boltzmann's constant; T : absolute temperature; μ_0 : magnetic permeability of vacuum; V : volume of a microcrystal; B : applied magnetic field and K_a is a proportionality constant. Lastly, $|\chi_a|$ is the anisotropic magnetic susceptibility, representing $\chi_1 - \chi_2$, $\chi_2 - \chi_3$, or $\chi_1 - \chi_3$, where the χ_i are the principal values of magnetic susceptibility tensor of the crystal. Although it depends on which χ_a is involved and on the condition of the rotating magnetic field, the proportionality factor K_a is typically of order unity. By using the values: $T=300$ K, $B = 8$ T, $V = (10 \times 10^{-6})^3$ m³ estimated from polarized optical microscope observations, and $K_a = 1$, the value of η_{mag} can be estimated. If we use the anisotropic magnetic susceptibility of tetragonal HEWL crystals; 76×10^{-6} emu/mol (cgs units) (Yanagiya et al., 1999) to estimate χ_a of orthorhombic HEWL crystals, we obtain $|\chi_a| = 10^{-8} - 10^{-7}$ (SI units). Thus, using eq (3.2), a value of $\eta_{\text{mag}} = 0.07^\circ - 0.23^\circ$ (1.3 - 4.0 mrad) is obtained.

The full width at half maximum of the reflection profile, φ_R , is affected by the mosaicity of the crystal as well as by the geometric and spectral parameters of the X-ray source, and is expressed by the formula (Bellamy et al., 2000)

$$|\varphi_R| = (L^2 \zeta^2 \gamma_h^2 + \gamma_v^2)^{1/2} + L d^* \cos \theta_{hkl} \left[\eta + \left(\frac{\delta \lambda}{\lambda} \right) \tan \theta_{hkl} \right] \quad (3.3)$$

Thus, η can be expressed as

$$\eta = \frac{|\varphi_R| - (L^2 \zeta^2 \gamma_h^2 + \gamma_v^2)^{1/2}}{L d^* \cos \theta_{hkl}} - \left(\frac{\delta \lambda}{\lambda} \right) \tan \theta_{hkl} \quad (3.4)$$

where all the parameters apart from η are determined by the properties of the X-ray source used (**Table 3.1**). L is the Lorentz correction factor

$$L = \frac{1}{[\sin^2(2\theta_{hkl}) - \zeta^2]^{1/2}} \quad (3.5)$$

and ζ is the position of the corresponding reciprocal-lattice point projected onto the rotation axis

$$\zeta^2 = \frac{h^2}{h^2 + v^2} \sin^2 2\theta_{hkl} \quad (3.6)$$

where h and v are the horizontal (along the rotation axis) and vertical distance of the observed reflection from the direct beam position. In addition, the reflection angle θ_{hkl} and d^* are expressed as follows

$$2\theta_{hkl} = \tan^{-1} \left[\frac{(h^2 + v^2)^{1/2}}{D} \right] \quad (3.7)$$

$$d^* = \lambda/d \quad (3.8)$$

where $d = \lambda/2 \sin \theta_{hkl}$ and D is the MOMA to detector distance.

Table 3.1

Geometric and spectral parameters		
γ_h	Horizontal crossfire angle at sample (mrad)	0.134
γ_v	Vertical crossfire angle at sample (mrad)	0.829
$\frac{\delta\lambda}{\lambda}$	Wavelength dispersion	10^{-4}
D	Sample to detector distance (mm)	250

In this chapter, an experimental value of $|\varphi_R| = 2.29^\circ$ (40 mrad) was obtained from the integration program MOSFLM, which gives the average value of all measured (hkl) reflections. Using this φ_R in eq. (3.4), the mosaicity was determined to be $\eta = 1.00^\circ$ (17 mrad). Then, from eq. (3.1), using $\eta_{cry} = 0.43^\circ$ (7.5 mrad) and $\eta_{mag} = 0.07^\circ$ - 0.23° (1.3 - 4.0 mrad), η_{sol} was calculated to be 0.88° - 0.91° (15.3 - 15.8 mrad). From this result, it can be concluded that the large mosaicity observed here is attributable to the subsequent consolidation processes. It should be noted, however, that the contribution of η_{mag} and η_{sol} to the total mosaicity depends on the experimental conditions such as the intensity of the magnetic field used, the choice of suspending media, the quality of the

suspension, consolidation techniques, etc, and the exact value of $|\chi_a|$ of the crystal under consideration.

In **Table 3.2**, the present analysis is compared with that previously obtained from the in-house source (Kimura et al., 2011). The unit cell parameters a , b , and c are slightly different in the two cases. This can be attributed to the difference in water content of the crystals. The degree of dehydration was not exactly the same as in our earlier published study, which could explain the difference in the unit cell dimensions.

Table 3.2. Data collection, structure solution and refinement statistics

Data collection		
Diffraction source	in-house [10]	synchrotron
Wavelength (Å)	1.5418	1.0000
Temperature	Room Temperature	Room Temperature
Crystal-detector distance (mm)	150	250
Space group	$P2_12_12_1$	$P2_12_12_1$
a , b , c (Å)	51.26, 59.79, 29.95	49.40, 60.11, 30.11
Resolution range (Å)	26.77-3.00 (3.16-3.00)	49.39-1.79 (1.83-1.79)
Total No. of reflections	10604	90550 (3958)
No. of unique reflections	1828 (250)	7494 (365)
Completeness (%)	89.6 (88.6)	85.1 (72.7)
Multiplicity	5.8 (5.5)	12.1 (10.8)
$mean\ I/\sigma(I)$	9.3 (2.7)	19.7 (5.0)
R_{sym}	0.194 (0.487)	0.076 (0.366)
$CC_{1/2}$	n. a.	0.999 (0.954)
Structure solution and refinement		
Resolution range (Å)	25.86-3.00 (3.78-3.00)	38.17-1.79 (2.33-1.79)
No. of reflections, used	2989 (1423)	7461 (3933)
R_{work}/R_{free}	0.215 /0.270	0.171/0.226
No. of atoms		
Protein	1001	1001
Water	0	83
R.m.s. deviations		
Bonds (Å)	0.004	0.002
Angles (°)	0.743	0.547

Owing to the increased flux density provided by the synchrotron source, the resolution and number of observed reflections are greatly improved. **Figure 3.4** shows the resulting structure and an electron density map around the active site. The active site is clearly visualized at side chain level.

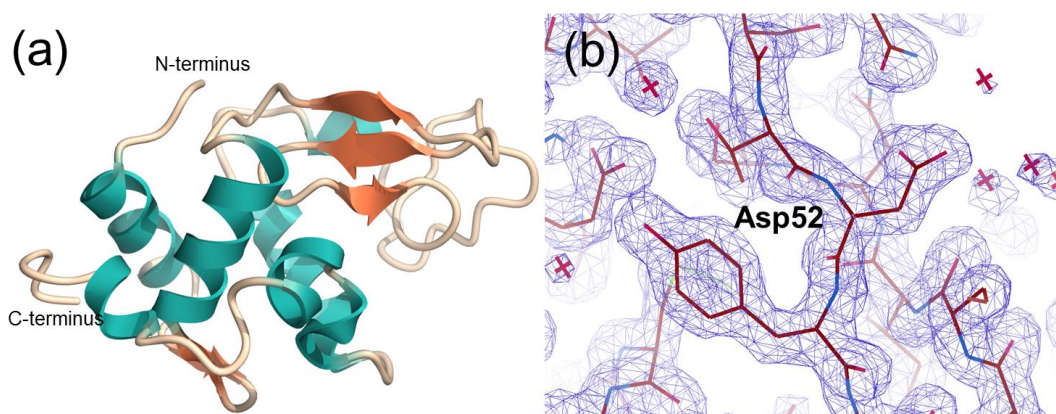


Figure 3.4. (a) Molecular structure determined and (b) the electron density map around the active site.

The total completeness is approximately 85%, which is lower than that usually obtained for measurements from single crystals. This is because a diffuse ring around 3–5.5 Å arising from the matrix resin caused inaccuracy in the integration of the intensities. If other matrices such as gels are used instead of resin, the background scattering may be reduced and thus the completeness could be improved.

The MOMA used in this chapter was of a cylindrical shape of size 3.5 mm in diameter and 5 mm in height, containing approximately 20 % (wt/wt) protein crystals. The FWHM of the X-ray beam was by 180 μm (vertical) 87 μm (horizontal) and the

MOMA was irradiated with the X-ray beam almost perpendicular to the cylindrical axis of MOMA (**Figure 3.5**). Thus, the diffracting volume was approximately 0.055 mm^3 , and this contained approximately 1,400 microcrystals. This means that the diffraction intensity was approximately 1,400 times higher than for a single microcrystal if the incident X-ray intensity was the same. Conversely, $1/1,400$ of the incident X-ray intensity would be sufficient to obtain the same diffraction intensity. In addition, the

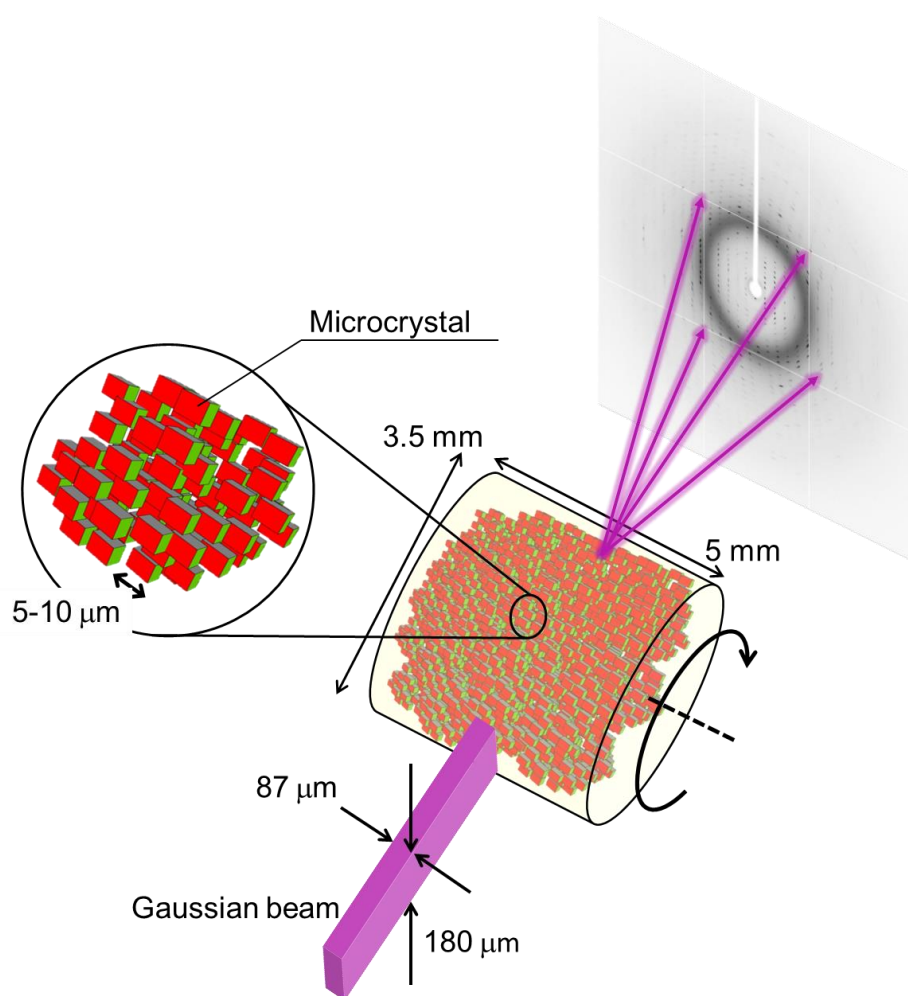


Figure 3.5. The size of a MOMA is compared with that of the X-ray beam in order to estimate the number of microcrystals existing in the diffracting volume. The beam size is much smaller than that graphically displayed.

diffracting volume is almost all refreshed for every ω scan, since new material rotates into the beam so that most of the individual microcrystals in a MOMA apart from those located near the centre are irradiated just once. This is in marked contrast to the case in which a single microcrystal of the same size is used for diffraction measurements. In this scenario, the single microcrystal is irradiated continuously throughout the whole data acquisition, suffering much more radiation damage.

For the MOMA used here, 9.6 mg protein is contained in the 3.5 mm by 5 mm cylinder volume, but this large size is not required in order to obtain the data for the current analysis. Since a diffracting volume of 3.5 mm diameter by 87 μm (X-ray beam horizontal FWHM) is large enough (Figure. 3.5), the 0.17 mg of the protein crystals contained within this volume is sufficient.

3.4 Conclusions

A magnetically oriented microcrystal array (MOMA) was prepared on a microcrystalline suspension (5-10 μm) of lysozyme and X-ray diffraction measurements were performed on BL38B1 at SPring-8. A 1.8 Å resolution structure was determined from microcrystals of 5-10 μm by using a MOMA. These results showed that MOMAs may have great potential for use in circumstances where samples of protein crystals are available only in small sizes and amounts.

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Chapter 4

Neutron and X-ray single crystal diffraction from protein microcrystals via magnetically oriented microcrystal arrays in gels

4.1 Introduction

The function of proteins is closely related to their tertiary structures, and thus the determination of protein structures at atomic levels is of primary importance in fundamental as well as in applied research areas. For the purpose of structure solution, single-crystal X-rays (Kendrew et al., 1958) and neutron (Blakeley et al., 2008) diffraction are the most powerful. A bottleneck for these single-crystal techniques is that many protein crystals do not grow to sizes large enough for the necessary measurements. In particular, for neutron diffraction, crystals in the millimetre size range are required, but such large sized crystals can only be grown from a very few proteins. This greatly hinders the use of the neutron diffraction technique.

We proposed a technique to collect single crystal diffraction data from organic (Kimura et al., 2006; Kimura et al., 2010) and inorganic (Kimura et al., 2009) microcrystals which had been magnetically aligned and then ‘fixed’ in their aligned orientation. To prepare these microcrystal samples, a suspension of microcrystals using an appropriate liquid medium is first prepared. The microcrystals are then magnetically aligned, followed by consolidation of the suspending medium to fix the crystal alignment. We call this composite a MOMA (magnetically oriented microcrystal array). The diffraction from MOMAs are equivalent to those obtained from a corresponding piece of single crystal (Kimura et al., 2014).

In the chapter 3, we applied the MOMA technique to protein microcrystals (Kimura et al., 2011). In these works, we used ultra-violet (UV) light curable monomers (liquid) for suspending media. However, the use of these monomers has drawbacks, and among these is that the microcrystal had to be partially dried in air. In this chapter, aqueous gels of biological origin are used instead of synthetic monomers. The advantages of these gels are their high affinity to proteins and the possible recoverability of precious protein samples. In particular, the background in neutron measurements are drastically reduced by using aqueous gels prepared with D₂O. In this chapter, single crystal neutron diffraction from lysozyme microcrystals (ca. 10 μ m x 10 μ m x 50 μ m) are reported for the first time to a resolution of 3.4 Å using D₂O gel MOMA that contains a vast number of magnetically oriented microcrystals. Also, the structure determinations by X-ray diffraction measurements of H₂O gel MOMAs at in-house (2.42 Å resolution) and synchrotron facility (1.76 Å resolution) are reported.

4.2 Materials and method

Preparation of gel MOMA for neutron diffraction measurements

High-purity freeze-dried hen egg white lysozyme (HEWL) (Eisai Food Chemical, Inc.) was crystallized without any further purification. HEWL powders were dissolved in an acetate buffer (50 mM, pH 4.5) to obtain HEWL solution (400 mg/ml). The same acetate buffer containing 4 % (wt/wt) NaCl and 16 % (wt/wt) polyethylene glycol (MW=4,000) was prepared for the precipitating solution. These two solutions were filtered through a 0.20 μ m membrane filter. The HEWL solution and the precipitating solution were mixed droplet-wise, followed by further stirring for 2 h at 313 K. The mixture was then maintained at 313 K for 2 days to obtain

microcrystals. The precipitated HEWL microcrystals were separated using a centrifuge (1,000 *g*, 30 min). These microcrystals were soaked with a D₂O acetate buffer (50 mM, pH 4.5) containing 4 % (wt/wt) NaCl and 8 % (wt/wt) polyethylene glycol (MW=4,000) and centrifuged to recover the microcrystals. This soaking/centrifuge process was repeated three times to increase the content of D₂O in the suspension. Then, the microcrystals in the D₂O acetate buffer were allowed to stand for 2 weeks at 313 K, followed by being centrifuged to remove the supernatant. The D₂O treated microcrystals were mixed with the D₂O acetate buffer containing 3 % NaCl and 12 % gelatin (Gelatin 21, Nitta Gelatin Inc.), followed by stirring to prepare the microcrystal suspension. A quartz capillary (inside diameter: 4.20 mm, outside diameter: 4.97 mm, height: 20 mm) was filled with the suspension and sealed with dental wax.

The glass capillary was mounted on the sample rotating unit placed at the centre of a cryogen-free superconducting magnet (Sumitomo Heavy Industry) generating a horizontal magnetic field of 8 T. The rotation axis was horizontal and the rotation speed was switched between $\omega_{\text{slow}} = 30$ rpm and $\omega_{\text{fast}} = 65$ rpm every 90°. The HEWL microcrystal suspension was rotated for about 90 min at 308 K, during which the suspension remained a sol. Then, the temperature of the suspension was reduced to 293 K to transition the suspension to a gel to fix the alignment of microcrystals.

Preparation of gel MOMA for in-house X-ray crystal structure analyses

The HEWL powders were dissolved in the acetate buffer (50 mM, pH 4.5) to obtain the HEWL solution (400 mg/ml). The solution was filtered through a 0.20 μm membrane filter. The acetate buffer containing 5 % (wt/wt) NaCl and 16 % (wt/wt)

polyethylene glycol ($M_w=4,000$) was filtered through a $0.20\ \mu\text{m}$ membrane filter and used for the precipitating solution. An equal amount of HEWL solution and the precipitating solution was mixed with vigorous stirring at 313 K for 12 h, followed by standing for two days at 313 K. The resulting HEWL microcrystals were separated using a centrifuge (1,000 g , 30 min). The microcrystals and the acetate buffer (50 mM, pH 4.5) containing NaCl (3 %) and gelatin (12 %, Gelatin 21, Nitta Gelatin Inc.) were mixed and stirred to prepare a microcrystal suspension that contains approximately 20 % microcrystals. The HEWL microcrystal suspension thus obtained was poured into 3 mm ϕ Lindemann glass capillary and sealed with dental wax. The magnetic alignment was performed under the same conditions as those for preparation of the MOMA for neutron samples.

Preparation of gel MOMA for synchrotron X-ray crystal structure analysis

HEWL microcrystals were obtained in the same manner as already described with slight modifications: the precipitating solution was the acetate buffer (50 mM, pH 4.5) containing 7 % (wt/wt) NaCl and 16 % (wt/wt) polyethylene glycol ($MW=4,000$). All other procedures were the same as detailed above.

X-ray and neutron diffraction measurements

X-ray diffraction of HEWL MOMAs was measured with an in-house X-ray diffractometer and at BL38B1 of SPring-8. The in-house measurements were performed with a Rigaku RAXIS-VII detector and an RA-Micro7 rotating-anode generator. The $\text{CuK}\alpha$ radiation from the RA-Micro7 generator was monochromatized and focused at the sample position using a Si/W multi-layered mirror. The diameter

of the collimator was 0.5 mm. 240 images to a resolution of 2.42 Å were collected under the condition of $\Delta\phi=0.5^\circ$, 3 min/frame, and a camera distance of 200 mm. For the synchrotron experiment, 720 images to a resolution of 1.76 Å were collected under the condition of $\Delta\phi=0.5^\circ$, 2 sec/frame, and a camera distance of 170 mm using a Rayonix MX225HE detector. The full width at half maximum of the X-ray beam was 100 μm (vertical) by 50 μm (horizontal). The neutron diffraction experiments were performed at iBIX J-PARC (Tanaka et al., 2009; Kusaka et al., 2013). The parameters for the measurements and the alignment of the detectors are shown in **Table 4.1** and **Figure 4.1** respectively.

Table 4.1. The conditions for neutron diffraction measurement

Accelerator power	300 kW
Measurement time	15 hours and 21 minutes (1070075 pulses)
Temperature	Room temperature
Range of wavelength	3.15-7.15 Å
Goniometer angle	$\phi, \chi, \omega = 0^\circ$

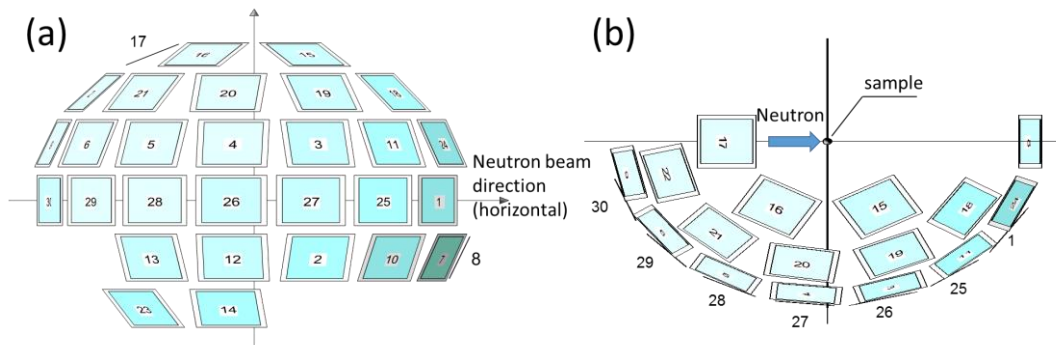


Figure 4.1. Detector arrangement of iBIX. (a) side view and (b) top view. The detector numbers are indicated.

X-ray data processing and structure refinement

The in-house and synchrotron X-ray diffraction patterns were processed using the MOSFLM software (Leslie & Powell, 2007) and scaled with AIMLESS (Evans & Murshudov, 2013) from the CCP4 suite (Winn et al., 2011). Phases were determined by the molecular replacement method using the program Phaser with PDB code 1VDQ as the starting model (McCoy et al., 2007). The model was refined using REFMAC5 and Phenix.refine from the Phenix software suite (Vagin et al., 2004; Adams et al., 2010). Further cycles of manual iterative modelling in COOT and refinement were then performed (Emsley et al., 2010).

4.3 Results and discussion

Principle of 3D alignment

Biaxial crystal alignments are achieved by the following mechanism (Kimura et al., 2012). The magnetic anisotropy of biaxial crystals including the orthorhombic, monoclinic, and triclinic systems is described by their magnetic susceptibility tensor χ that is characterized by its principal values χ_1 , χ_2 , and χ_3 (we define $\chi_1 > \chi_2 > \chi_3$) and corresponding principal axes. The relationship between crystallographic axes and magnetic axes depends on the particular crystal system. These two axes systems coincide in the case of the orthorhombic crystal system (Nye, 1985). For example, for the lysozyme crystals used in this chapter, the axes have the following relationships: $\chi_1 \parallel a$, $\chi_2 \parallel b$, and $\chi_3 \parallel c$. For static magnetic fields, χ_1 axis aligns parallel to the applied magnetic field, while for rotating magnetic fields, χ_3 axis aligns perpendicular to the rotating plane of the magnetic field. In actual experiments, the sample is rotated instead of rotating the magnetic field. We have shown (Kimura et al., 2009) that a combination of static and

rotating fields shown in **Figure 4.2** can bring about biaxial alignments. In this rotation scheme, the sample is rotated in a static magnetic field, but the rotation speed is switched between ω_{slow} and ω_{fast} ($>\omega_{\text{slow}}$) every 90° . There are several types of possible rotation schemes used to achieve biaxial alignments (Yamaguchi et al., 2013).

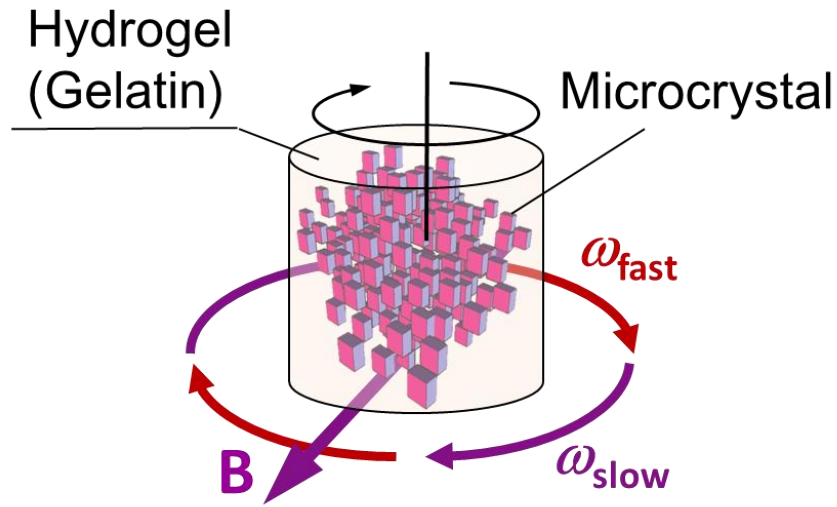


Figure 4.2. Schematic view of non-uniform rotating magnetic field applied to HEWL microcrystal suspensions. A HEWL microcrystal suspension was rotated at speeds ω_{slow} and ω_{fast} ($>\omega_{\text{slow}}$) in a static magnetic field **B**.

Neutron diffraction from gel MOMA

Microphotographs of lysozyme microcrystals used for neutron measurements are shown in **Figure 4.3**. Crystal sizes were ca. $50\ \mu\text{m} \times 10\ \mu\text{m} \times 10\ \mu\text{m}$. A single piece from these microcrystals is not enough to produce measurable diffraction intensities. In a gel MOMA, several millions of these microcrystals were aligned three-dimensionally in the gel matrix as shown schematically in **Figure 4.4**. The

measured diffraction then becomes the sum of the diffraction from the individual microcrystals, which means that the diffraction intensity is enhanced by several million orders of magnitude. The MOMA used for the neutron measurements was cylindrical in shape (4.2 mm ϕ , 20 mm in height) and the size of the iBIX neutron beam was 5 mm ϕ at full width. Since the incident neutron beam was perpendicular to the cylinder axis of the MOMA, the diffracting volume was estimated as ca. 4.2 mm ϕ by 5 mm in height. By using the values of 1.1 g/cm³ and 1.3 g/cm³ for the densities of the MOMA and the HEWL crystal, respectively, and 20 % for the mass fraction of HEWL crystals in the MOMA, the total volume of the microcrystals included in this diffraction volume was estimated as 11.7 mm³ (equivalent to a cube shaped crystal of sides 2.3 mm), which means that there were ca. 2 million microcrystals in the diffraction volume. Since all these microcrystals are aligned three dimensionally, the diffraction intensity is 2 million times higher than that from a single microcrystal existing in the MOMA.

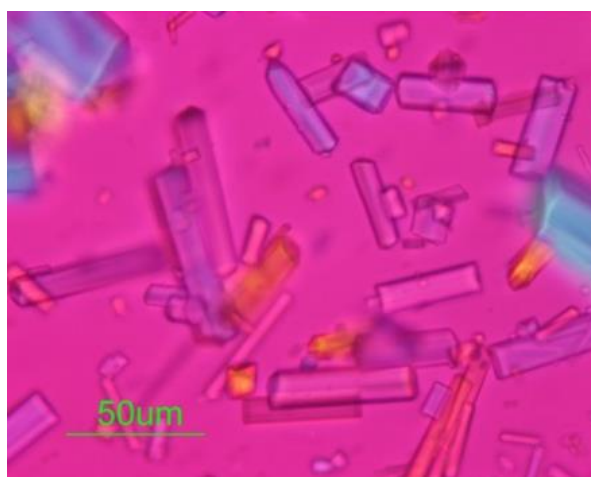


Figure 4.3. Polarized optical micrograph of lysozyme microcrystals used for neutron measurements. The size of microcrystals is less than 50 $\mu\text{m} \times 10 \mu\text{m} \times 10 \mu\text{m}$.

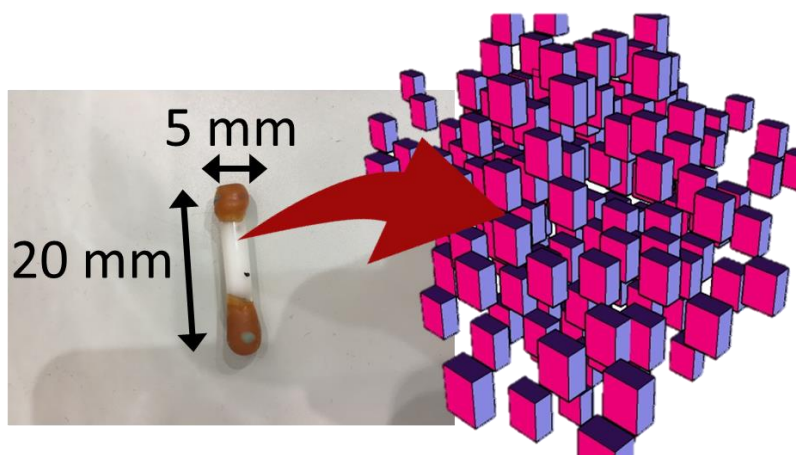


Figure 4.4. A D₂O gel MOMA in a quartz capillary for neutron diffraction measurements, in which microcrystals are aligned three dimensionally as shown schematically.

Reflections to a resolution of $3.4 \text{ \AA}$ were observed. In the present measurement, we were unable to collect a full data set which is required for the structure determination because of the limitation of machine time (one day). Several diffraction spots having high intensities are shown in **Figure 4.5**. The background was rather high and signal to noise ratio (S/N) was not sufficiently high. This may be attributed to the residual protons in the gelatin that were not exchanged to deuterium in the soaking procedure. The S/N could be improved by reducing the gelatin concentration or using other gelling agents like agarose. Also, increasing the protein concentration in MOMAs might improve the S/N. The collection of a full data set is planned.

Single crystal X-ray diffraction analyses of gel MOMA

In **Figure 4.6**, the X-ray diffraction images obtained at the in-house and SPring-8 sources are shown. The data collection, structure solution, and refinement

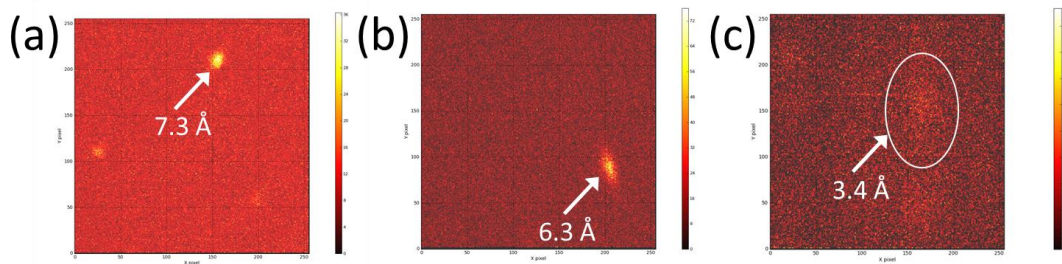


Figure 4.5. Typical neutron diffraction diagrams of HEWL MOMA at the respective detector numbers designated in Figure 4.1. The images (a), (b), and (c) are recorded by detectors Nos. 7, 11, and 26, respectively. The resolutions of the diffraction spots indicated by arrows are shown in the figure. The diffraction spot of the highest resolution was observed with detector No. 26.

statistics are summarized in **Table 4.2**. From these diffraction data, the structure was successfully determined at resolutions of 2.42 Å and 1.76 Å, respectively. In our previous studies at BL38B1 at SPring-8, we reported the structure analyses from microcrystal lysozyme for which partial drying was necessary in order to prepare homogeneous suspensions with UV-curable acrylate monomers. Thereby, the mosaicity was 2.29°, which is compared with the present value of 1.37°. In addition, the lattice parameters in the previous study were $a=30.11$, $b=49.40$, and $c=61.41$ Å. This should be compared with the present result, $a=30.46$, $b=56.51$, and $c=73.64$ Å, shown in **Table 4.2**. We find that the lattice parameters in this chapter are larger. These lattice parameters are almost the same as those determined from a single lysozyme crystal: $a=30.38$, $b=56.57$, $c=73.55$ Å.

We performed XRD measurements of a single piece from the microcrystals used for gel MOMA at in-house diffractometer. We obtained diffraction to a resolution of 8 Å. On the other hand, we obtained much higher resolution of 2.42 Å as seen from **Table 4.2** when the measurement was performed through a gel MOMA. This clearly demonstrates the advantage of using MOMAs.

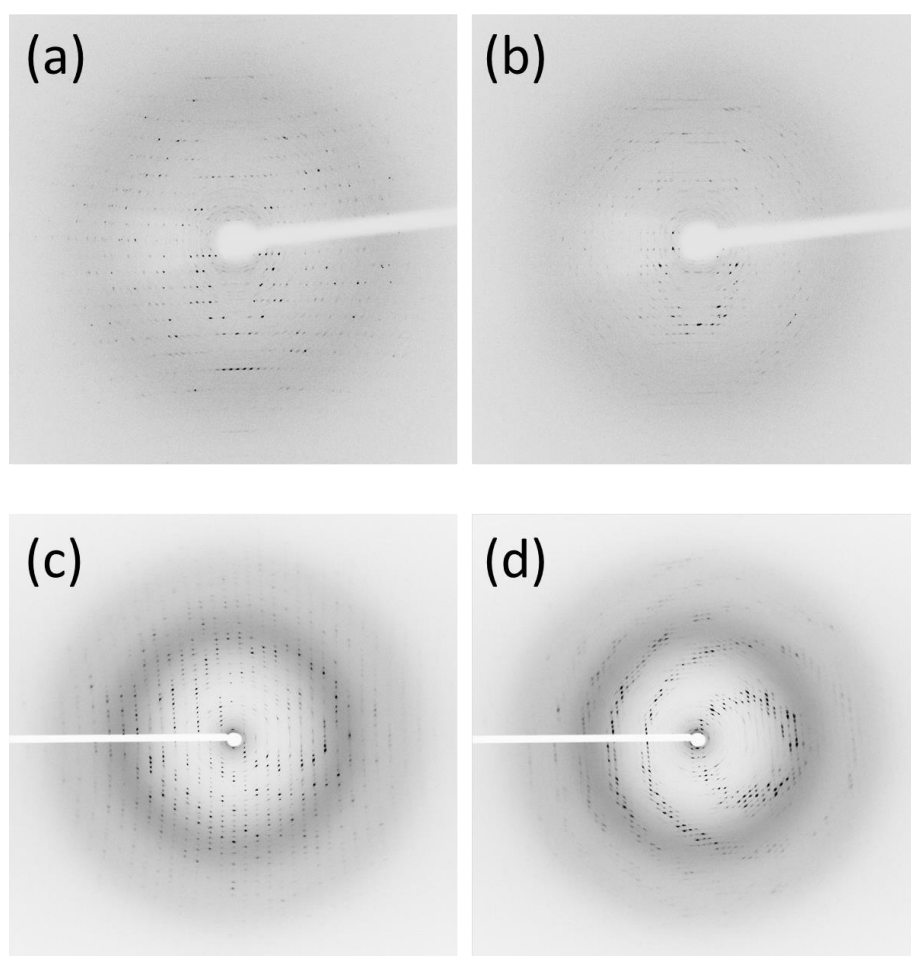


Figure 4.6. Typical X-ray diffraction images taken at two angles differing by 90°. (a) and (b) were taken on an in-house diffractometer with a 0.5° oscillation for 180 s. (c) and (d) were taken at SPring-8 with a 0.5° oscillation for 2 s.

Table 4.2. Data collection, structure solution and refinement statistics

Data collection		
Diffraction source	inhouse	synchrotron
Wavelength (Å)	1.5418	1.0000
Temperature	Room Temperature	Room Temperature
Space group	$P2_12_12_1$	$P2_12_12_1$
a, b, c (Å)	30.53, 56.42, 73.34	30.46, 56.51, 73.64
Resolution range (Å)	56.42-2.42 (2.52-2.42)	56.51-1.76 (1.79-1.76)
Total No. of reflections	22095 (2164)	157228 (8590)
No. of unique reflections	4958 (488)	12616 (704)
Completeness (%)	96.8 (93.3)	96.2 (95.1)
Multiplicity	4.5 (4.4)	12.5 (12.2)
$mean\ I/\sigma(I)$	6.9 (3.6)	13.6 (3.5)
R_{merge}	0.144 (0.296)	0.113 (0.473)
$CC_{1/2}$	0.967 (0.896)	0.997 (0.844)
Mosaicity (°)	1.03	1.37
Structure solution and refinement		
Resolution range (Å)	30.75-2.42 (2.76-2.42)	26.38-1.76 (1.90-1.76)
No. of reflections, used	4932 (1442)	12587 (2344)
R_{work}/R_{free}	0.171/0.188	0.161/0.190
No. of atoms		
Protein	1001	1001
Water	23	104
R.m.s. deviations		
Bonds (Å)	0.004	0.006
Angles (°)	0.622	0.835
Average B factors (Å ²)	31.00	19.29

4.4 Conclusion

Single crystal neutron reflections to a resolution of 3.4 Å from microcrystalline powders of lysozyme are reported here for the first time. This was achieved using magnetically oriented microcrystalline powders consolidated in D₂O/gelatin gels. Single crystal X-ray diffraction from H₂O gel MOMAs was collected with an in-house diffractometer and at SPring-8, from which data the structures were determined successfully with the resolutions of 2.42 Å and 1.76 Å, respectively. The advantage of using gels is that the protein microcrystals can be measured in a state that is as close to as native conditions.

We believe that the gel MOMA technique will have large applications when we need the single crystal analyses of proteins that do not grow to enough sizes. In particular, this technique is quite powerful when applied to neutron diffraction analyses because D_2O is available as suspending media.

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Chapter 5

General conclusions

In Chapter 2, the kinetics of magnetic orientation of rod-like samples was studied in an attempt to explore a novel orientation technique. Macroscopic rod samples that have well-defined directions of magnetic axes were prepared for the kinetic study of the magnetic orientation under conditions of SRR. The orientation kinetics strongly depended on the direction of the magnetic axes with respect to the rod axes. This observation was attributed to the fact that the orientation behavior is dominated by the balance of the magnetic and hydrodynamic torques under conditions of SRR. In some cases, the 3D orientation was achieved, but in general, the desired orientation was not achieved. These results are in good agreement with the theoretical prediction. Since the microcrystals to be aligned are individually different in shape, it was concluded that the orientation under conditions of SRR was not to be used to achieve 3D orientation.

In Chapter 3, the MOMA of lysozyme microcrystals (ca. 10 μm) prepared with UV-curable monomers was subjected to the XRD measurement at beamline BL38B in SPring-8 and the structure was successfully determined at a resolution of 1.8 $\text{\AA}$. This resolution was in marked contrast to that of 3.0 $\text{\AA}$ achieved previously at an in-house diffractometer. Usually, at this beamline, a piece of single crystal of larger size is needed to determine the crystal structure. The lattice parameters determined here are shorter than those determined from a real single crystal because the microcrystals had to be partially dried in order to prepare a uniform suspension with UV-curable monomers.

This was a drawback of using UV-curable monomers for fixing 3D alignment. The solution to this problem is shown in the next chapter.

In Chapter 4, the use of aqueous gels was proposed in order to be free from drying protein crystals during MOMA preparations. Gel MOMAs exhibited sharp XRD spots, resulting in high resolution. When consolidated, the gels exhibit less shrinkage than UV-curable monomers and have a good affinity for protein crystals; thus the deterioration of crystal structure is minimized.

In **Figure 5.1**, the HEWL structures determined by the MOMAs prepared using UV-curable monomers (Chapter 3) and using gels (Chapter 4) are compared by superposition. Though the both are similar in structures, the unit cell of that prepared with UV-curable monomers shown in **Figure 5.2** is largely shrunk. This shrinkage is attributed to the loss of water molecules existing between protein molecules; water molecules bound to the protein molecules were not removed by drying. However, this type of dehydration is exceptional: usually bound water molecules are removed by drying, resulting in large change in structures occurs. Therefore, gel MOMAs are superior to polymer MOMAs in making natural structures of proteins as intact as possible.

Gel MOMAs are most suited for ND measurements. Firstly, large MOMAs of several cubic millimeters in size needed for ND can be prepared. Secondly, D₂O can be used for preparing aqueous gels, which is of primary importance for reducing the background in ND measurement. For these reasons, gel MOMAs provide opportunities to conduct ND measurements for microcrystals that do not grow to millimeter sizes.



Figure 5.1. HEWL structures determined by the MOMAs prepared using UV-curable monomers (Red) (Chapter 3) and using gels (Yellow) (Chapter 4)

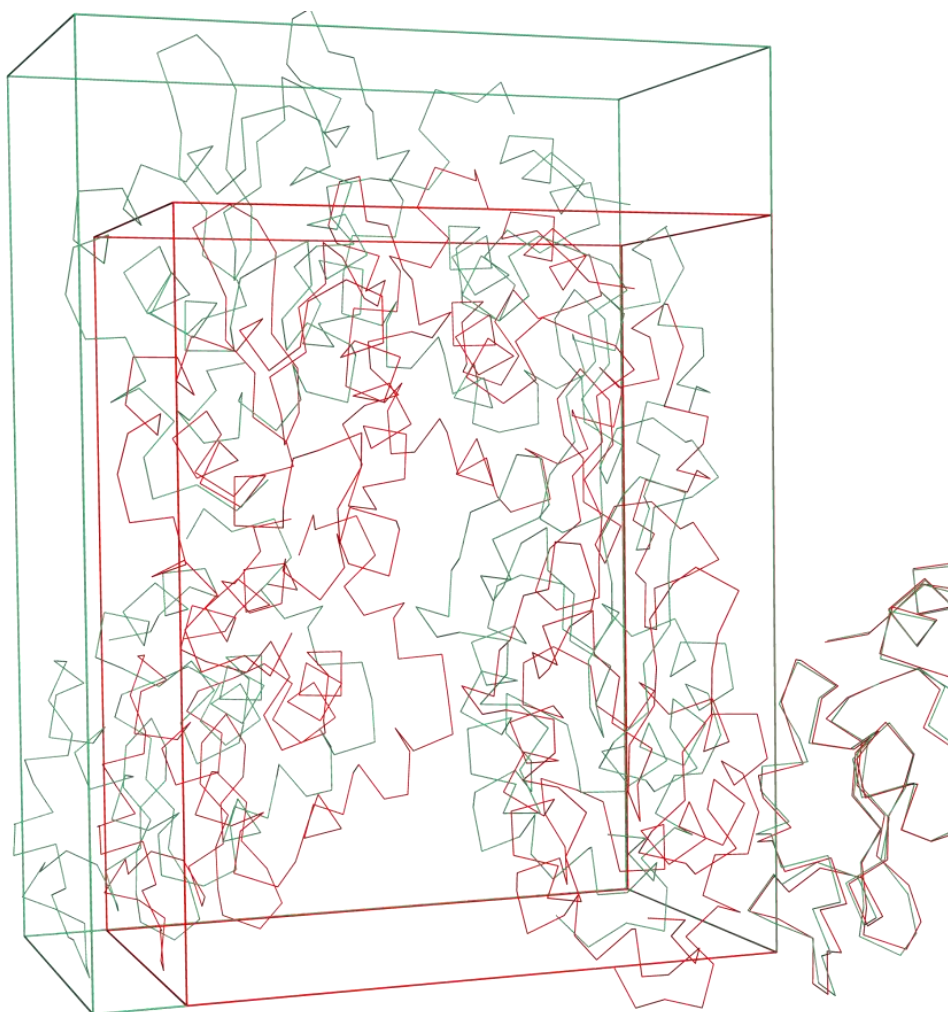


Figure 5.2. The unit cells determined by the MOMAs prepared with UV-curable monomers (Red) and with gel (Green)

A magnetic technique for 3D alignment of microcrystalline powders has been applied to lysozyme microcrystals to determine its structure. It was demonstrated for the first time that the crystal structure was successfully determined at resolution of 1.8 Å from microcrystals (< 10 μm in size) using MOMAs. Protein crystals of this size are usually difficult to analyze at conventional high-throughput beamlines in synchrotron facilities, but the magnetic technique employed here has enabled XRD

measurements for this crystal size even at in-house X-ray diffractometers. It was also demonstrated that the use of gels with D₂O for preparing MOMAs effectively reduced the background in neutron diffraction, thus for the first time, enabling ND spots to be obtained from microcrystals of 10 µm sizes.

List of publications

The contents of this thesis have been described in the following publications

Original papers

1. Magnetic Alignment of Magnetically Biaxial Diamagnetic Rods Under Rotating Magnetic Fields, Shu Tsukui and Tsunehisa Kimura, *Japanese Journal of Applied Physics*, **2012**, 51, 057301. **(Chapter 2)**
2. X-ray Crystal Structure Analyses of Magnetically Oriented Microcrystals of Lysozyme at 1.8 Å Resolution, Shu Tsukui, Fumiko Kimura, Elspeth F. Garman, Seiki Baba, Nobuhiro Mizuno, Bunzo Mikami and Tsunehisa Kimura, *J. Appl. Crystallogr.*, **2016**, accepted. **(Chapter 3)**
3. Neutron and X-ray single crystal diffraction from protein microcrystals via magnetically oriented microcrystal arrays in gels, Shu Tsukui, Fumiko Kimura, Katsuhiro Kusaka, Seiki Baba, Nobuhiro Mizuno and Tsunehisa Kimura, **2016**, submitted. **(Chapter 4)**

International conference

1. Kinetics of magnetically biaxial rods under rotating magnetic field, Shu Tsukui and Tsunehisa Kimura, The International Chemical Congress of Pacific Basin Societies (PACIFICHEM), Hawaii USA, December, 2010.

2. Kinetics of magnetically biaxial rods under rotating magnetic field, Shu Tsukui and Tsunehisa Kimura, International Symposium on Renewable Energy & Materials Tailoring, Kyoto Japan, September, 2011.
3. X-ray Diffraction Analysis of Magnetically Oriented Microcrystal Array of Lysozyme, Shu Tsukui, Fumiko Kimura, Kimihiko Mizutani, Bunzo Mikami and Tsunehisa Kimura, International Conference on Magneto-Science 2013, Bordeaux France, October, 2013.
4. X-ray Diffraction of Magnetically Oriented Microcrystals of Protein, Shu Tsukui, Fumiko Kimura, Kimihiko Mizutani, Bunzo Mikami and Tsunehisa Kimura, Twenty-Third Congress and General Assembly of the International Union of Crystallography, Biological Macromolecules, Montreal Quebec Canada, August, 2014.
5. X-ray Diffraction Analysis of Magnetically Oriented Microcrystals of Protein, Shu Tsukui, Fumiko Kimura, Kimihiko Mizutani, Bunzo Mikami and Tsunehisa Kimura, 6th International Workshop on Materials Analysis and Processing in Magnetic Fields, Okinawa Japan, July, 2014.
6. Application of magnetically oriented microcrystal array to X-ray and neutron crystallography, Shu Tsukui, Fumiko Kimura and Tsunehisa Kimura, The 29th European Crystallographic Meeting, Rovinj Croatia, August, 2015.
CrystEngComm poster prize

Other publications of the author based on the work at Laboratory of Fibrous Biomaterials, Division of Forest and Biomaterials Science, Graduate School of Agriculture, Kyoto University are specified below.

1. X-ray Diffraction of a Magnetically Oriented Microcrystal Suspension of L-alanine, Kenji Matsumoto, Fumiko Kimura, Shu Tsukui and Tsunehisa Kimura, *Cryst. Growth Des.*, **2011**, 11(4), 945-948.

Other international presentation of the author based on the work at Laboratory of Fibrous Biomaterials, Division of Forest and Biomaterials Science, Graduate School of Agriculture, Kyoto University is specified below.

1. In-situ X-ray Diffraction Measurements Using Magnetically Oriented Microcrystal Suspension of L-alanine, Fumiko Kimura, Kenji Matsumoto, Chiaki Tsuboi, Shu Tsukui and Tsunehisa Kimura, International Conference on Magneto-Science 2013, Bordeaux, France, October, 2013.
2. Application of Magnetically Oriented Powder Crystal to X-ray and Neutron Diffraction Measurements, Fumiko Kimura, Tsuboi Chiaki, Shu Tsukui, Hiroaki Kikuchi, and Tsunehisa Kimura, International Conference on Magneto-Science 2015, Matsumoto, Japan, November, 2015.

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