A Novel Method of Crystal Structure Analysis Using *In-Situ* Diffraction Measurement of Magnetically Oriented Microcrystal Suspension

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Introduction

Biomass has been considered a viable solution to resource and energy problems in recent years. There are physical, chemical, and biochemical methods involved in the material conversion of biomass, but the biochemical method, particularly, conversion of biomass using enzyme reactions, is the one with good characteristics and compatibility.[1] However, because there are various problems with the practical use of enzyme activity and heat tolerance, improving the activation and heat tolerance of enzymes[2,3] becomes an important issue. Hence, information about the higher-order structure of enzymes is very important, as it has been used for the first time to improve the enzyme characteristics and ensure reaction control.[4] In addition, not only the structural information of the enzyme, but also that of the substrate on which it acts is important. Because the enzyme reaction is known, the steric structure of both the enzyme and the substrate can be understood for the first time. Furthermore, information on the structural connection between the enzyme and the substrate is important.[5,6]

The most common method to evaluate the structure of the enzyme and the substrate is **crystal structure analysis**, and the most commonly used means of crystal structure analysis is single-crystal X-ray diffraction (XRD) measurement.[7-9] However, a single crystal of at least 50 μm is required for the measurement, and many enzymes (proteins) and substrates do not grow to the sufficient size for single-crystal structure analysis.[10] As a result, the analysis relies on XRD

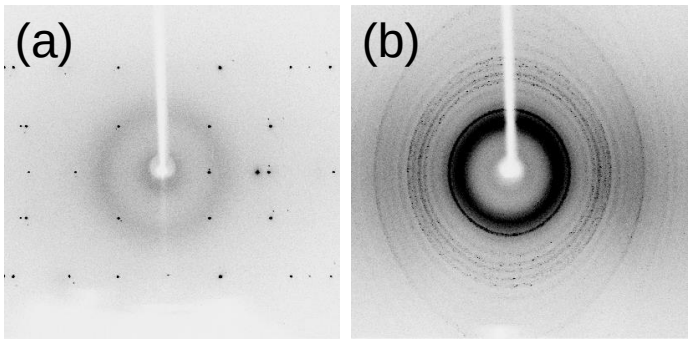

Figure 1. X-ray diffraction images of (a) a single crystal and (b) a microcrystalline powder of L-alanine. The diffraction peaks appear as spots for a single crystal and as rings for a microcrystalline powder.

measurement of a powder sample (microcrystalline powder).[11,12] However, information related to diffraction is very limited for powder samples. Figure 1 shows a diagram of L-alanine single crystal and powder XRD to explain the difference. The promptness and accuracy of crystal structure analysis are inferior for powder samples as compared to those for single-crystal samples. In order to solve this, we propose a three-dimensional (3D) orientation of microcrystals using a magnetic field.

The main problem associated with powder X-ray structure analysis is that the microcrystals randomly orient in powder samples. If the microcrystal orientation in the powder can be restricted three-dimensionally, it is possible to obtain diffraction images similar to those from a single crystal (Figure 2).[13]

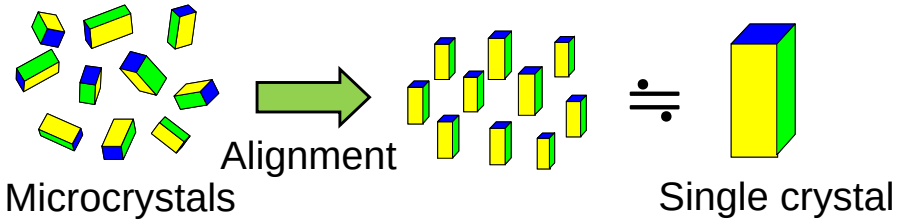

Figure 2. Three-dimensional alignment of a microcrystalline powder.

A powerful technique to align microcrystals is magnetic orientation.[14-16] Magnetic orientation is applicable to all materials with magnetic anisotropy.[17] Because most organic crystals show magnetic anisotropy, it is possible to align them using a magnetic field.[18-20] Particularly, many biaxial organic crystals (triclinic, monoclinic, and orthorhombic crystals) have three different values of magnetic susceptibility ($\chi_1 > \chi_2 > \chi_3$) as shown in Figure 3, and the magnetic response is different for each of the three orthogonal directions. This suggests that three-dimensional orientation of biaxial crystals is possible by application of a magnetic field having different strengths three dimensionally.[13]

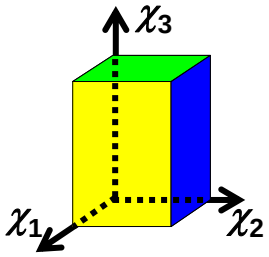

Figure 3. Magnetic susceptibility of biaxial crystal.

In order to conduct X-ray crystal structure analysis of 3D-oriented microcrystals, a fixation method using UV-curable resin is often used for orientation in actual practice.[21,22] This three-dimensionally oriented microcrystal/resin

composite is called magnetically oriented microcrystal array (MOMA). It shows the same diffraction image as a single crystal in actual measurements (Figure 4).[21,22] There are reports of successful X-ray crystal structure analysis using MOMA on inorganic crystal (cobalt lithium phosphate[22]), organic crystal (sucrose[23]), and protein crystal (lysozyme[24]). In addition, a combination of techniques using magnetic field orientation and UV-curable resin is called the MOMA method. The MOMA method allows for single-crystal structure analysis from powder, but it has various problems that will be discussed below.

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The MOMA method has the following four drawbacks.

(1) It cannot collect microcrystal samples.[25]

The suspended microcrystals cannot be collected once the UV-curable resin has hardened. If sample collection is difficult, a rare sample cannot be analyzed using the MOMA method. Methods using thermoplastic resin dispersant and casting with polymer have also been considered theoretically, but because thermal denaturation of protein often occurs with temperature changes, the effect described in (2) is often encountered, thus none of these methods are realistic.

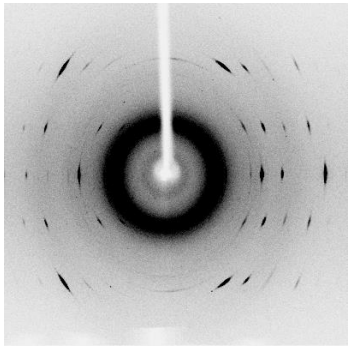

Figure 4. X-ray diffraction images of a magnetically oriented microcrystal array of L-alanine. This pattern is nearly the same as that of a single crystal.

(2) Distortion of microcrystal orientation due to hardening of the resin.[25]

UV-curable resin shrinks with hardening. This shrinkage becomes a large factor in disturbing the orientation of microcrystals. Compared to the case of other orientation fixation methods such as the casting method, the degree of shrinkage of the UV-curable resin is small. If there is no resin shrinkage, improvement of the orientation is feasible.

(3) Large limitations on the suspension medium.[25]

There are few options for suspending mediums because it is impossible to use resins other than UV-curable resins. The microcrystal sample should not be dissolved or aggregated in the UV-curable resins, but the resins satisfying these conditions are sometimes difficult to find.

(4) The appropriate applied magnetic field conditions for microcrystal orientation are not known.

In the MOMA method, the process of magnetic orientation is important, but in actual practice, a quantitative correlation between the magnetic condition and microcrystal orientation is not understood. Thus, it is important to conduct a carpet-bombing experiment in order to create a good MOMA of a microcrystal sample.

One of the objectives of this study is to develop a MOMA method without hardening the resin. This method should be like that the magnetically orientated microcrystal is not fixated in the suspension medium and goes directly for *in-situ* XRD measurement. For this purpose we perform *in-situ* XRD measurement of a magnetically oriented microcrystal suspension (MOMS). Because there is no hardening process in the MOMS method, sample collection is simple. Moreover, there is no effect of orientation disturbance due to hardening of the resin. Furthermore, there are more options for the suspending medium. Thus, problems (1), (2), and (3) of the MOMA method are solved, and crystal structure analysis using magnetic field orientation becomes possible for many other substrates.

In order to develop the MOMS method, first, a device was designed and manufactured for simultaneous measurement of the applied rotating magnetic field and XRD in this study. This was followed by actual *in-situ* XRD measurement of magnetically oriented microcrystals in suspension to analyze their crystal structure.

One more objective of the present study is to better understand the orientation of microcrystals in a magnetic field. This can solve problem (4) of the MOMA method by determining the optimal applied magnetic field conditions for microcrystal orientation. *In-situ* XRD measurement is also very effective for this investigation because magnetic field application and the resulting microcrystal orientation condition can be immediately confirmed. In this study, we mainly investigated microcrystal orientation in static and rotating magnetic fields. In addition, with these magnetic fields applied to the microcrystals *in-situ*, the optimal conditions for microcrystal 3D orientation and XRD measurement are established.

Furthermore, while a major part of this study deals with the use of XRD measurement, we also attempted the application of the MOMA and MOMS methods for neutron diffraction measurement. Because the position of hydrogen atoms in a crystal can be easily identified with neutrons,[26] which is difficult when using X-rays, this would be very useful for analyzing the positions of hydrogen atoms, which is often

important in the structure analysis of enzymes.[27] However, compared to X-ray analysis, neutron crystal structure analysis requires a large (millimeter order) single crystal. However, because this drawback rarely occurs with the MOMA and MOMS methods, these methods would have good compatibility with neutron structure analysis. In the present study, neutron diffraction measurements of a MOMA were conducted, and the possibility of and problems related to crystal structure analysis were summarized. In addition, MOMS neutron diffraction measurements were conducted, and its possibility was suggested.

The summary of this study is presented in Figure 5.

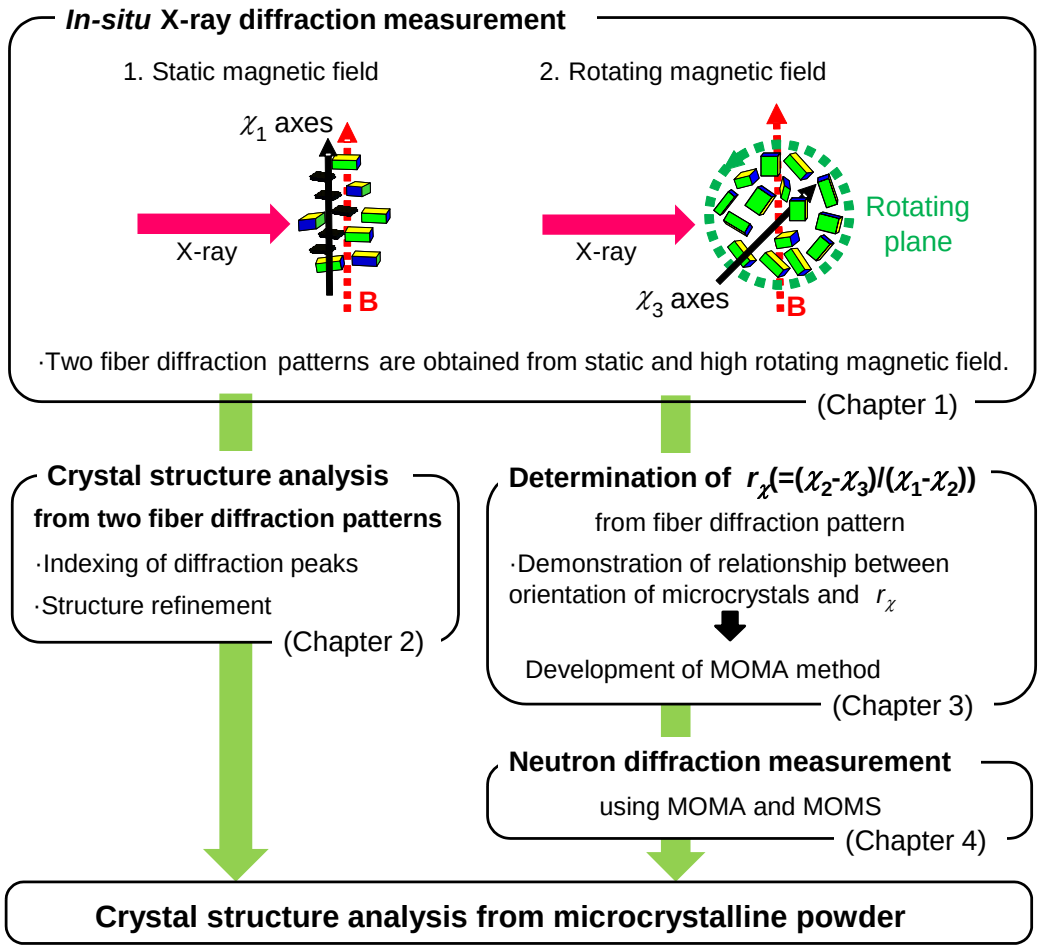

Figure 5. Schematic representation of the outline of this thesis.

Chapter 1

We successfully obtained two fiber X-ray diffraction patterns from *in-situ* X-ray diffraction measurement with and without rotation of the microcrystal suspension (crystal size of ca. 2-20 μm) in a magnetic field (1 T). Diffraction spots were sharp (ca. 2-3^{o} in half width) and well separated. The present results suggest a potential use of MOMS for crystal structure analysis of solid materials that do not grow into large crystals but are obtained in the form of microcrystal suspensions.

Furthermore, we discovered the following facts about orientation of biaxial crystals in a rotating magnetic field. With a small rotation speed (SRR), the χ_1 axis of the microcrystal follows the rotation and remains near the magnetic field. In this case, the χ_2 and χ_3 axes are not oriented. As the rotation speed increases (ARR), the χ_1 axis of the microcrystal cannot follow the rotation of the magnetic field. On the other hand, the χ_3 axis is oriented in the direction of the rotation axis. The degree of orientation of the χ_3 axis depends on the rotation speed.

Chapter 2

We have demonstrated the potential of MOMS method as a means to determine structure by a single crystal X-ray analyses. In this study, a microcrystalline powder of L-alanine was used for the purpose of demonstration. *In-situ* X-ray diffraction measurement was performed on an L-alanine microcrystalline suspension that was subjected to a static or a rotating magnetic field. From the two diffraction patterns obtained, the space group and the lattice constants of L-alanine were determined with no knowledge. The observed 44 independent diffraction intensities were in good agreement with the values calculated from the single crystal data.

Chapter 3

We determined the ratio r_χ of the diamagnetic anisotropy of L-alanine and D-mannnitol by *in-situ* X-ray diffraction measurements of their microcrystalline suspensions. The results obtained were in good agreement with those estimated by corresponding single crystal data reported in literature. The technique proposed here provides a facile method for determination of the ratio of the magnetic susceptibility from a microcrystalline sample. If the size of microcrystals under investigation is well characterized, the determination of the absolute values of three magnetic susceptibilities is possible.

The ratio r_χ is an important parameter when fabricating MOMAs. We prepared an L-alanine MOMA under various experimental conditions of the time-dependent

magnetic field, and found that the ratio r_χ is in fact useful to determine the experimental conditions that make possible the equalization of the half widths of diffraction spots.

Chapter 4

We have demonstrated the potential of the MOMA and MOMS methods as a powerful means to determine structure by neutron single-crystal analyses. Characteristic features of these methods are summarized as follows: (i) A MOMA and a MOMS are prepared from microcrystalline powder with a particle size as small as tens of micrometers if one uses magnetic fields of 8.0 and 1.0 T, respectively. Microcrystals of even smaller sizes can be used if a stronger magnetic field is applied. (ii) The sample size of a fabricated MOMA can be as large as several millimeters to several centimeters. (iii) The half width of the diffraction spot can be on the order of 4º (MOMA) and 2° (MOMS) under the fabrication conditions employed in the present study. It will be improved if higher magnetic fields are used. (iv) The signal-to-noise ratio on a MOMA method is sufficiently high even though a commercially available resin precursor is used. This is because coherent diffractions from the microcrystals are much higher than the incoherent scattering due to the resin owing to the three-dimensional orientation of microcrystals in the MOMA. Finally, it should be noted that the use of a low temperature resin (STYCAST, GE varnish) as a matrix resin might allow the low temperature measurement with MOMA.

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