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Universal Processing Technique for Protein Crystals Using Pulsed UV Laser

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We demonstrated the laser processing of three types of soft protein crystals using a 193 nm deep-UV pulsed laser. In particular, high-solvent-content crystals, phosphoenolpyruvate carboxylase (PEPC) crystals, were successfully processed without causing significant damage. An unchanged resolution limit between pre- and post-processings in X-ray diffraction measurements showed that laser irradiation has little influence on crystallinity. These results reveal that the processing using a deep-UV laser is effective for various protein crystals and will be very useful in accelerating protein structural analysis. [DOI: 10.1143/JJAP.43.L873]

KEYWORDS: laser processing, protein crystal, UV laser, photoablation, X-ray diffraction

Structural biology research, in the three-dimensional (3D) structural analysis of proteins, has received considerable attention because it contributes to the development of new pharmaceutical products and the investigation of various phenomena of life. Crystallization remains as a major bottleneck in the process of protein structure determination by X-ray crystallography. Moreover, the strength of bondings, such as hydrogen bond and van der Waals force, between each molecule in protein crystals are much weaker than that in inorganic or organic crystals, so that protein crystals are very soft and fragile.[1,2] A slight mechanical shock easily cuts the bonding between molecules, leading to the destruction of the crystal structure. It is very difficult for crystallographers to process protein crystals using a tiny mechanical tool, which is the only available means. There has been minimal effort exerted to establish reliable methods of processing protein crystals, even though crystallographers frequently obtain crystals that require some processing. Thus, an effective processing method is desired.

Compared with a mechanical approach, a photochemical approach using a light source has a great potential to serve as a universal method of processing delicate protein crystals. We have proposed a novel processing technique for protein crystals using pulsed UV laser soft ablation (PULSA).[3] UV light has a sufficiently high photon energy to induce the photochemical decomposition of protein molecules. The risk of denaturation caused by heating can be suppressed using deep-UV laser pulses with a short pulse duration. Previous experiments revealed that the irradiation of laser pulses at a wavelength of 193 nm leads to the successful processing of a model protein crystal, hen egg-white lysozyme (HEWL),[4] without significant damage to the crystal structure of the nonirradiated part.[3,5] A HEWL crystal is, however, not only chemically and thermally stable but also relatively hard. Most protein crystals, which cannot be identified by a 3D structural analysis, are considered to be more difficult to handle than HEWL crystals. It cannot be concluded that the PULSA technique is universally effective for a variety of protein crystals, according only to the success of HEWL crystal processing. To confirm the universal usefulness of PULSA, the ablation properties of other protein crystals should be investigated in detail. In this study, we demonstrate the laser processing of three types of protein crystals, in which an extremely soft protein crystal is also examined. The validity of PULSA technique is then discussed based on the results of X-ray diffraction (XRD) pattern recordings.

Table I. Molecular weights and solvent contents of the examined protein crystals.

	Molecular weight (kDa)	Solvent content (%)
HEWL	14	39
HL	15	38
GI	173	56
PEPC	400	60

Human lysozyme (HL),[6,7] glucose isomerase (GI),[8] and phosphoenolpyruvate carboxylase (PEPC)[9–11] were chosen as sample proteins, and their single crystals were grown by a hanging-drop vapor-diffusion technique. Table I summarizes the molecular weights and calculated solvent contents of the above protein crystals and a HEWL crystal. Since the most spatial part of the protein crystals is occupied with various solvents taken up during growth, solvent content is one of the indices that represent the softness and friability of protein crystals. In general, protein crystals with a higher solvent content are relatively difficult to crystallize and handle. By calculation, the solvent content in protein crystals usually ranges from 23% to 65%, and rarely reaches 90%.[12]

The resistance against mechanical shock in protein crystals is so small that it is quite difficult to quantitatively measure their hardness. The only reported Vickers hardness of protein crystals is 0.2 kgf/mm^2 for a HEWL crystal.[13] This is two or three orders of magnitude smaller than that of typical inorganic and organic crystals.[14,15] One may accomplish the processing of hard protein crystals using mechanical tools. However, the success rate in this mechanical operation drastically drops as softer protein crystals are treated. The PEPC crystal applied in this work is classified

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into very soft protein crystals, as associated with the fact that the crystal has a high solvent content. Although we attempted to manipulate PEPC crystals using a mechanical tool, the handling of PEPC crystals was much more difficult than that of the other crystals listed in Table I. It was almost impossible to yield a designed processing pattern using a micro knife and needle. If PEPC crystals can be easily processed by laser irradiation, the PULSA technique will be very helpful for various soft protein crystals.

Single crystals of HL, GI, and PEPC, $250 \times 600 \times 200\,\mu m^3$, $200 \times 1000 \times 150\,\mu m^3$ and $250 \times 500 \times 200\,\mu m^3$, respectively, were processed by laser irradiation. The experimental setup was basically the same as that for HEWL crystals, as described in our previous work.[16] We adopted a deep-UV solid-state laser operating at 193 nm as a light source for the processing. A growth vessel containing the crystal set on a XY-stage was exposed to laser pulses. An output laser beam, with a pulse duration of 1 ns at a repetition rate of 1 kHz, was focused onto the crystal surface with a spot diameter of 25 μm. The laser fluence was adjusted to $50\,mJ/cm^2$/pulse. Until the completion of the designed ablation, the laser spot on the target was continuously scanned to ablate all aimed areas that was much larger than the beam diameter. Using these experimental procedures, we cut the HL and GI crystals, and reshaped the PEPC crystal.

Under the same laser-irradiation conditions, the HL and GI crystals were easily divided without trouble into two halves with $\sim 1 \times 10^5$ laser shots. The ablated volume of each crystal was less than $0.01\,mm^3$. Similarly, 1×10^5 laser shots modified the shape of as-grown PEPC crystal into a pentagon. The schematic illustration of the processing and photographs of the PEPC crystal before and after laser irradiation are shown in Fig. 1. The hatched region in Fig. 1(a) indicates the position of the laser-irradiated area. After the laser irradiation, no considerable damage or denaturation was observed in all the processed crystals under microscopic observation. The cross section was sharp, and the non-irradiated parts of the crystals retained their original shape. These results reveal that PULSA successfully achieve a desired processing pattern for various protein crystals.

We then measured the XRD patterns of the softest crystal among the three sample proteins, PEPC, before and after the processing to evaluate whether laser irradiation influenced the crystallinity. An as-grown PEPC crystal with a dimension of $800 \times 1800 \times 800\,\mu m^3$ was transferred into a glass capillary tube, which was then sealed after the removal of the protein crystallization solution around the crystal. First, we recorded the XRD pattern of the target PEPC crystal before processing. Data were collected at room temperature on a Rigaku R-AXIS IV^{++} imaging plate. Cu Kα radiation was produced using a Rigaku ultraX18 rotating anode generator operated at 50 kV and 100 mA. The X-ray beam with a diameter of 0.3 mm was irradiated on the middle part of the crystal. The detector was positioned 250 mm from the crystal, and the time per image and crystal oscillation angle were set at 5 min and 1°. Figure 2(a) shows the XRD pattern derived from the nonirradiated PEPC crystal at a resolution limit of 3.0 Å.

After the first XRD-pattern recording, the capillary tube containing the target PEPC crystal was set on the laser-processing equipment. We trimmed away one edge of the as-grown PEPC crystal in the capillary tube by laser irradiation because PULSA is noncontact processing. The ablated volume was about $0.01\,mm^3$, after applying 1×10^6 laser shots. The laser-processed crystal in the capillary was again set on the XRD equipment to measure the resolution limit of diffraction. The time per image and crystal oscillation angle were set at 30 min and 2°. The detector was positioned 150 mm from the crystal. Figure 2(b) shows the XRD pattern derived from the processed crystal. The post-irradiation crystal diffracted beyond 3.0 Å resolution, which was the same as that obtained for the pre-irradiated crystal. The 193 nm deep-UV irradiation seemed not to affect crystallinity in XRD data collection. We can say that the processing technique using a UV laser is undoubtedly effective not only for hard protein crystals but also for soft protein crystals that

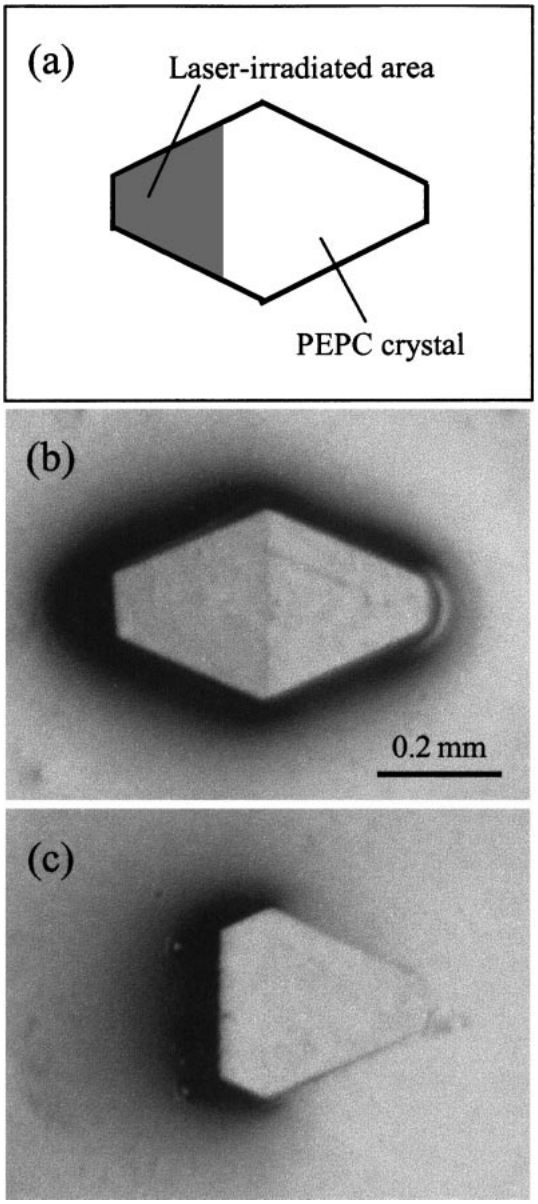

Fig. 1. Schematic illustration and photographs of processed PEPC crystal. The crystal was reshaped by laser irradiation. (a) Illustration of processing scheme. The hatched region indicates the position of the laser-irradiated area. (b) As-grown PEPC crystal. (c) PEPC crystal processed by laser irradiation.

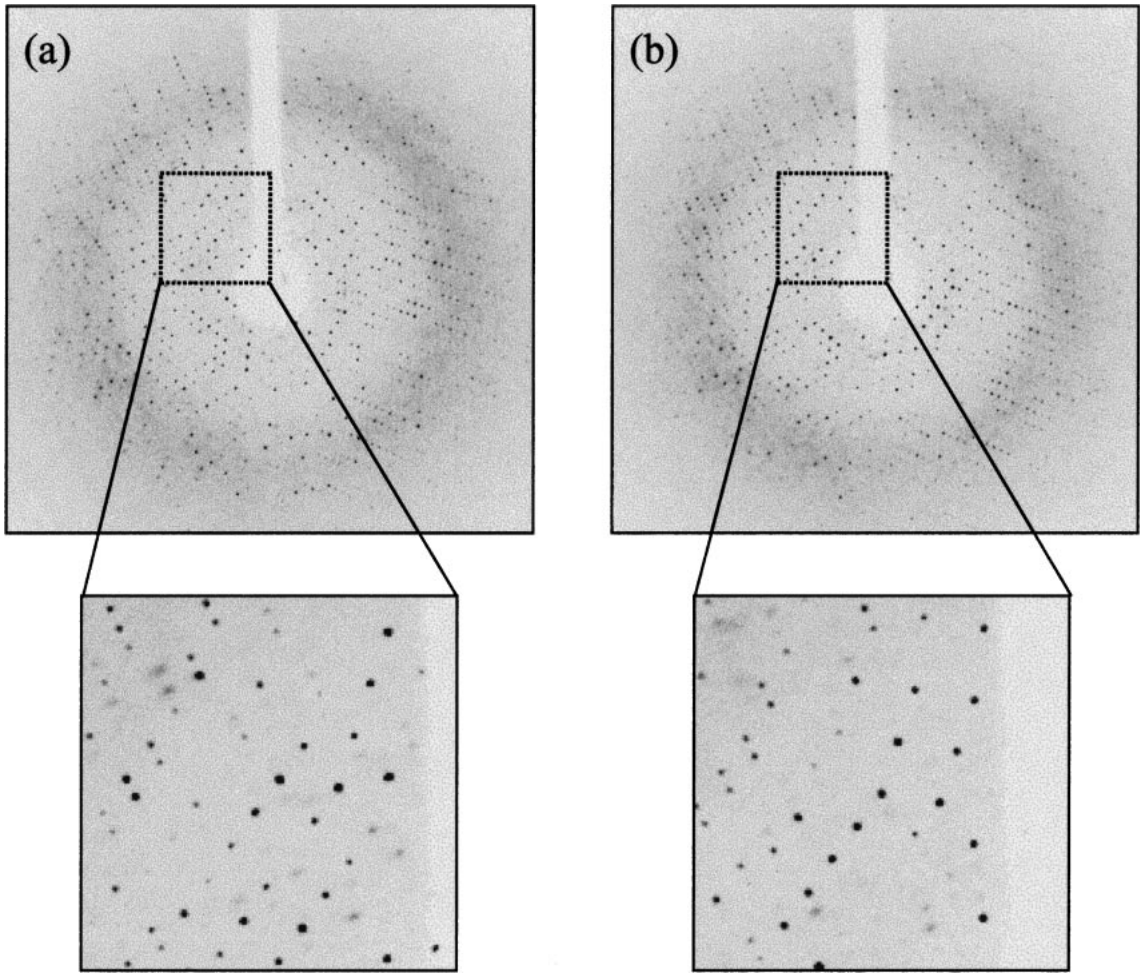

Fig. 2. XRD patterns of PEPC crystal. (a) Before laser irradiation. (b) After laser irradiation.

are too fragile to handle using mechanical tools.

All the laser-processing experiments mentioned above were performed under identical irradiation conditions (in terms of laser fluence and pulse repetition rate among other) except for the number of total laser shots. There was no need to carefully adjust laser power for each crystal. Although the protein crystals used in this work had different molecular weights and moisture contents, they showed similar ablation properties. The ablation rates of the three types of examined crystals did not differ much from that of a HEWL crystal.[5] This result implies that the photoablation mechanisms for all protein crystals are essentially identical. Namely, the PULSA technique is applicable to various protein crystals without changing laser-irradiation parameters, which must be very advantageous from a practical viewpoint.

At present, the final 3D structure of protein crystals is determined by measurements using synchrotron radiation. As a preliminary examination, however, XRD equipment based on electron beam (EB) pumping is widely used to measure such diffraction. An EB-pumped X-ray beam cannot be tightly focused on to a spot of less than 100 μm diameter, while synchrotron radiation produces a small beam with a diameter of down to approximately 10 μm. One sometimes encounters the problem that some damage in protein crystals disturbs to yield a clear XRD pattern because the irradiated X-ray beam covers the damaged areas. In the past, for lack of an effective processing method for protein crystals, crystallographers had to repeat screenings until they found the best growth conditions and had to obtain an ideal single crystal that necessitates minimal processing treatment. The PULSA technique, which enables a selective processing in an easy operation, will moderate the demand for optimized crystallization conditions and shorten the period of protein structure analysis.

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