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Protein Crystal Processing Using a Deep-UV Laser

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We propose a new protein crystal processing technique, pulsed UV laser soft ablation (PULSA). This is the first report on the successful processing of protein crystals using an optical light source without causing significant damage to the crystal. Photoablation without thermal degradation of the protein crystal structure was achieved using deep-UV laser pulses at a wavelength of 193 nm. The PULSA technique did not affect the quality of the crystals used for X-ray data collection. Moreover, complicated and precise processing of protein crystals, which is difficult using conventional mechanical tools, has been achieved using PULSA. This technique will undoubtedly be a powerful tool for processing protein crystals.

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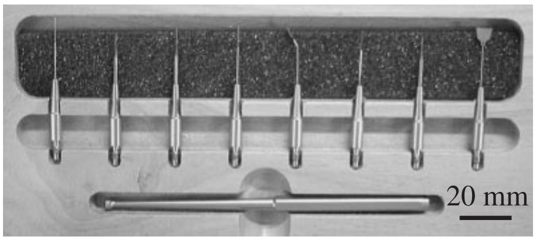
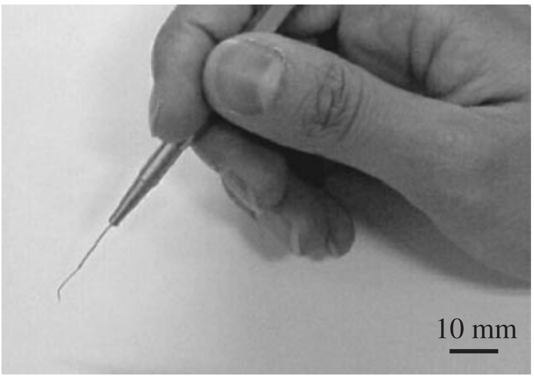
The structural and functional analysis of protein molecules has direct applications to human-disease therapy and drug design. X-ray crystallography is a fundamental tool used to determine the three-dimensional structures of protein molecules with atomic resolution. Precise measurement of X-ray diffraction (XRD) data necessitates high-quality protein single crystals with adequate size and appropriate shape. However, the optimization of crystallization and growth conditions for many proteins of interest is still a challenging task for crystallographers.[1] Problems often occur during the process of crystal growth (*e.g.*, poor crystallinity, polycrystal formation, and adhesion to the growth vessel). Moreover, the shape of protein crystals strongly reflects their molecular structure and growth conditions, and many as-grown crystals are not appropriate for XRD measurements due to their unsuitable shapes (*e.g.*, needle-like, narrow pillars, and thin plates). There exists a clear need for reliable methods of manipulating crystals, for example, the separation of a single crystal from a polycrystal, removal from the growth vessel, detachment of denatured or damaged parts, and reshaping it into an ideal form.

Protein crystals are, however, much softer and more fragile than inorganic or organic crystals and can be easily damaged by mechanical shock.[2,3] Moreover, protein crystals are frequently hypersensitive to temperature variation. Heating of protein crystals often causes denaturation. Therefore, the equipment that is widely used for mechanical processing of hard materials cannot be applied to protein crystals. The processing methods currently employed for protein crystals are at a low level of advancement and generally rely on the use of a microknife and needle. These mechanical tools are commercially available; Fig. 1 shows an example (Micro-Tools[4] manufactured by Hampton Research, USA). As a common practice, crystallographers manipulate the crystals using tiny tools under microscopic observation. The success of this manual operation ultimately depends on the experience and fortuity of the protein crystallographer. Even a skilled manipulator frequently faces problems in protein crystal processing, resulting in poor accuracy and low reproducibility. Consequently, many protein crystals, although produced with considerable effort, are not appropriate for XRD measurements due to the lack of useful processing tools. These problems delay the progress in protein research.



(a)



(b)

Fig. 1. Protein crystal processing Micro-Tools, provided by Hampton Research, USA.

We have developed a novel protein crystal processing technique based on a nonmechanical photoablation procedure, in which a pulsed deep-UV laser is used. This method is more convenient, reliable, flexible, and accurate than using conventional mechanical tools. Repeated irradiation of deep-UV pulses onto the crystal surface was found to be useful for processing protein crystals without significant damage to the structure. We call this technique pulsed UV laser soft ablation (PULSA). In this letter, we report three processing patterns using the PULSA procedure. The crystallinity of the laser-processed crystals was then evaluated by XRD measurement to confirm the success of the PULSA technique.

A suitable photoablation light source should be chosen carefully since protein crystals are very soft, fragile, and sensitive to temperature. The widespread laser-processing techniques of using an infrared source (or a visible source) are based on the thermal process and are therefore not suitable for processing protein crystals. In contrast, UV light has high photon energy, which causes direct photochemical decomposition of C-C bonds and C-N bonds in the peptide linkage. In general, the ablation threshold (minimum laser fluence for processing) depends on the absorbance of the target. Proteins have a broad absorption spectrum below a wavelength of 300 nm. In particular, the absorption coefficient below 200 nm is known to be more than twenty times greater than at the 280 nm absorption peak.[5,6] The use of UV pulses with high absorbance at the crystal surface enables low-power photoablation, which would be desirable for avoiding heating effects to the crystal. In addition, photoablation using highly absorbed light is confined to the irradiated crystal surface, and this interaction makes it possible to precisely control the laser-affected part. Therefore, we considered using a 193-nm pulsed deep-UV laser as a light source for processing protein crystals. By repeated irradiation of deep-UV pulses, the crystal can be softly processed from its surface layer by layer.

We applied the PULSA processing technique to a well-known protein crystal, hen egg-white lysozyme (HEWL). Commercially available HEWL (Seikagaku Kogyo, Japan) was used without further purification. We prepared a solution containing 25-mg/m*l* HEWL in 3.0% sodium chloride - 0.1 M sodium acetate at pH 4.5. HEWL was crystallized by sitting-drop and floating-drop[7] vapor diffusion methods at 293 K. A protein drop of 10 μ*l* volume was equilibrated against a 400 μ*l* reservoir solution containing 6.0% sodium chloride and 0.1 M sodium acetate at pH 4.5. Single crystals of HEWL were obtained over a period of two weeks. The protein solution was not transparent at the wavelength of 193 nm. After the removal of the protein solution, precipitates and buffers around the target crystal, the growth vessel was sealed with a UV-grade fused-silica cover glass to avoid dehydration of the protein crystal.

The experimental setup for protein crystal processing is shown in Fig. 2. The 193 nm light source consisted of a distributed-feedback laser diode (1547 nm wavelength), an erbium-doped fiber amplifier system, and a five-stage frequency-conversion system constructed using nonlinear optical crystals. Details of the lasing scheme have been described in our previous work.[8] A 193 nm output beam has a pulse energy of 1 μJ, a spectral width of <10 pm, and a pulse duration of 1 ns at a repetition rate of 1 kHz. After it passed through a mechanical shutter, the 193 nm laser beam was directed at the HEWL crystal along the *Z*-axis using a fused-silica focusing lens ($f = 100$ mm). Energy density at the crystal surface was adjusted to 50 mJ/cm^2/pulse, with a spot diameter of 25 μm. The growth vessel containing the target HEWL crystal was placed on a computer-controlled *XY*-stage, and the irradiation position was precisely aligned by observation through a stereoscopic microscope. During the irradiation, the laser spot at the crystal surface was continuously scanned in the *XY*-plane at a constant velocity of 0.5 mm/s.

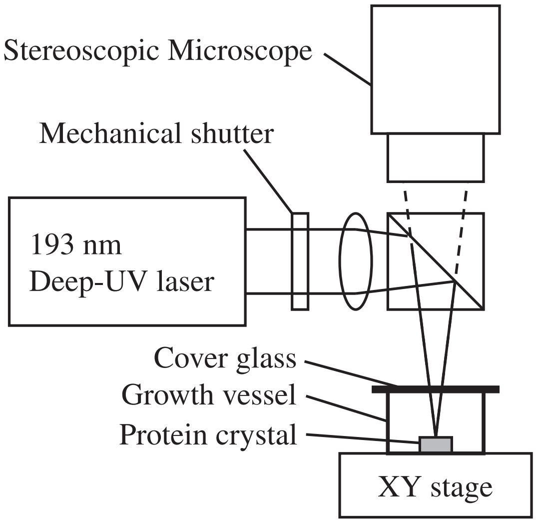


Fig. 2. Experimental setup for processing protein crystals.

Using the PULSA technique, we were able to cut the crystal into two halves. Figure 3 shows photographs of the HEWL crystal before and after the laser processing procedure. The separation of the halves was achieved with 5×10^4 laser shots. The repeated back-and-forth motion of

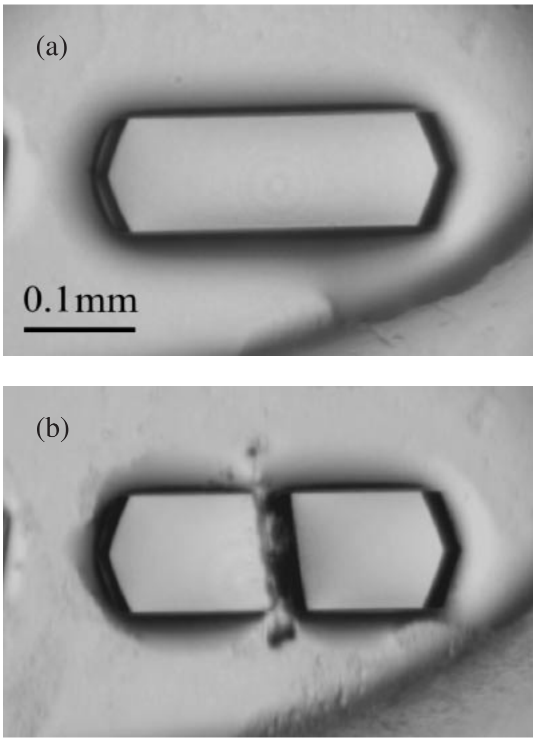


Fig. 3. Photographs of a HEWL crystal before and after PULSA processing. The crystal was cut into two halves. (a) HEWL crystal before irradiation. (b) HEWL crystal after irradiation.

the *XY*-stage gradually made the groove deeper during the irradiation, and this process was continued until cutting was completed. As can be seen in Fig. 3(b), the cut crystal did not exhibit any visible sign of cracks or denaturation. The cross section was very sharp, and nonirradiated parts of the crystal retained their original shape.

To confirm the quality of the laser-irradiated crystal, we recorded the XRD pattern of the halved HEWL crystal. The crystal was sealed in a glass capillary tube. 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 detector was positioned 150 mm from the crystal, and the time per image and crystal oscillation angle were set at 30 minutes and 2°. The beam diameter of the X-ray was adjusted to 0.3 mm, which covered the laser-irradiated section. The crystal diffracted beyond a 1.9 Å resolution, which was the same as that of the as-grown HEWL crystal obtained under identical growth conditions. This result clearly showed that PULSA processing did not alter the crystallinity.

We then trimmed away the edges of the as-grown hexagonal HEWL crystal, and produced a rectangular shape, using the PULSA technique. The pre-irradiation crystal dimensions were $450 \times 1000 \times 300\,\mu\text{m}^3$. Four sides of the HEWL crystal were completely removed after 3×10^6 laser shots. The crystal was then easily reshaped into $350 \times 750 \times 300\,\mu\text{m}^3$. We collected XRD data also for the reshaped rectangular HEWL crystal. The experimental procedure for XRD measurements was the same as that mentioned above. The resolution limit for the analysis (1.9 Å) indicated that there was no PULSA-induced deterioration of the crystal. This result demonstrates that PULSA is a powerful tool that can be used for various processing procedures.

In addition, we also carried out another shaping experiment on the protein crystal using the PULSA technique. The schematic illustration and photographs of the HEWL crystal before and after processing are shown in Fig. 4. The hatched region in Fig. 4(a) indicates the position of the laser-irradiated area. The 193 nm laser beam was directed at the central part of the crystal. The formation of a rectangular hole with 1×10^6 laser shots modified the crystal into a doughnut-like shape. We did not observe any significant damage or denaturation after the processing. The manipulations detailed here are extremely difficult to accomplish using conventional mechanical tools. By the PULSA technique, however, the desired trimming was easily achieved.

In conclusion, we have developed a new protein crystal processing technique, which we call PULSA. Various processing patterns were achieved using a 193 nm deep-UV laser. This light source produces low-power photoablation and localized interaction with low thermal effects. X-ray data were successfully collected from the processed crystals. The results indicated that no significant damage was induced by the laser irradiation. This new approach will enable us to reliably process biological macromolecular crystals, and to expand the limits of crystal species that can be used for XRD measurement.

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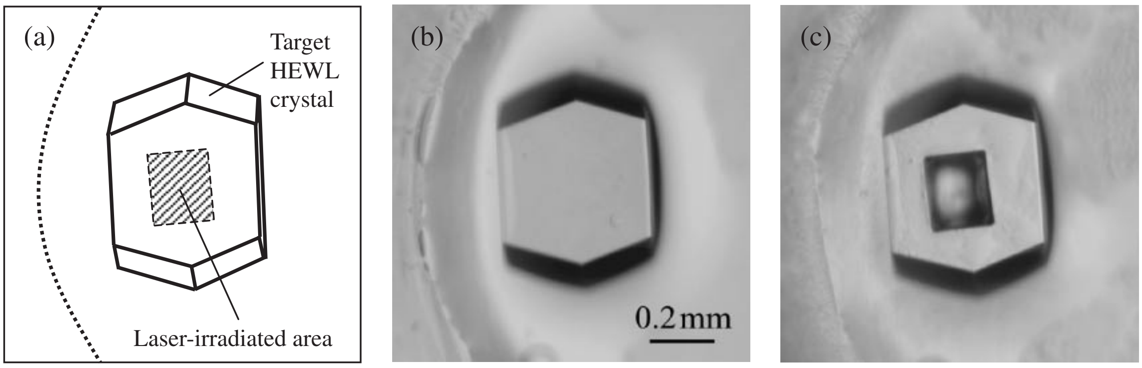

Fig. 4. Schematic illustration and photographs of the processed HEWL crystal. The crystal was trimmed into a doughnut-like shape. (a) Illustration of the processing scheme. Hatched region indicates the position of the laser-irradiated area. (b) HEWL crystal before irradiation. (c) HEWL crystal after irradiation.