Functional analysis of water molecule in the catalytic process of inverting glycoside hydrolases for efficient utilization of biomass

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1. Introduction

Cellulose and hemicellulose, key components of plant cell walls, represent some of the most abundant biomasses on Earth. These polysaccharides are crucial in developing a sustainable, low-carbon society, serving as potential alternatives to fossil fuels. Understanding the enzymatic reactions involved in their breakdown is vital for efficient biomass utilization. However, identifying the water molecules involved in the reactions of stereospecific inverting enzymes, where active amino acid residues directly activate water molecules, poses a significant challenge. A major obstacle in cellulose-based biomass utilization is the degradation of crystalline cellulose, which is inherently resistant to decomposition. Notably, the cellulase from the basidiomycete *Phanerochaete chrysosporium*, *Pc*Cel6A, is known to efficiently degrade crystalline cellulose, but its mechanism, presumably diverging from the typical hydrolysis reaction of stereospecific inverting enzymes, remains elusive. Neutron crystallography analysis has facilitated new insights into the reaction mechanisms, as demonstrated with *Pc*Cel45A by visualizing protons (Nakamura A. et al. *Science adv.* 2015). Employing a similar methodology could be instrumental in deciphering the reaction mechanism of *Pc*Cel6A, particularly in understanding how it activates nucleophilic water. Therefore, this study aims to unravel the reaction mechanisms of inverting cellulases using neutron crystallography, focusing on determining the position, orientation, and state (such as protonation) of water molecules involved in enzymatic activity.

2. Experiment

The cellulase *Pc*Cel6A was recombinantly produced by the yeast *Pichia pastoris*, purified, and crystallized in the similar crystallization condition described previously (Tachioka M. et al. *Acta Cryst. Sect. F*, 2017). Obtained crystals were soaked in deuterated crystallization buffer and enclosed in quartz capillaries. The time-of-flight (TOF) neutron diffraction measurement was performed at room temperature at 205 kW for two hours for each crystal, and the best-diffracted crystal was moved onto the overnight experiment (Exposure time: 16 hours). The neutron diffraction data was analyzed by STARGAZer.

3. Results

The millimeter-sized crystals of *Pc*Cel6A (Fig. 1A) were mounted on the quartz capillaries (Fig. 1B). During the two-hour neutron diffraction measurement, diffraction spots of the highest resolution 3.3 Å and 2.8 Å were detected on detectors #5 and #26, respectively. After expanding the exposure time to 16 hours, the spot of the highest resolution reached 1.9–2.0Å, which is feasible to proceed to the full data collection.

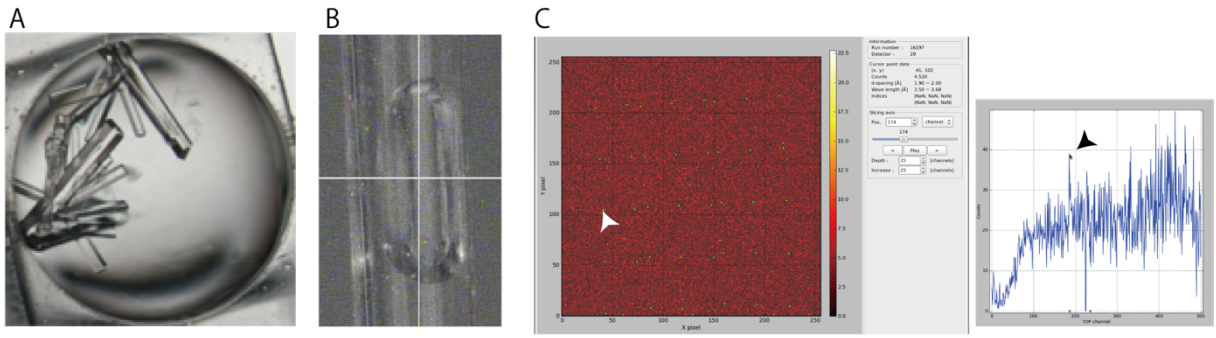

Fig.1 Results of test neutron diffraction measurement of *Pc*Cel6A-apo crystal. (A) Crystals of *Pc*Cel6A. Drop size: 5 mm in diameter. (B) A Crystal for the overnight experiment, mounted on the quartz capillary. (C) After a 16-hour experiment, the spot was detected in the highest resolution of 1.9–2.0Å in detector #29. The spot and diffraction signal are indicated by arrows.

4. Conclusion

As good neutron diffractions could be obtained under these experimental conditions, we could proceed to further neutron diffraction measurements. After the experimental conditions for deuterium exchange and co-crystallization with ligands were further verified, neutron structures of *Pc*Cel6A from a total of four crystals were successfully determined. By leveraging the strengths of neutron structural analysis, we were able to determine unexpected protonation states of amino acids, and identify the orientation of water molecules. We will submit a paper proposing a new mechanism for glycoside hydrolase family 6 cellulases based on these findings.