Experiment title: Protein induced lattice distortion in chitin crystals in spider cuticles: possible conformation changes and reduction in symmetry

Experiment number: SC-4038

Beamline: ID-22

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Report:

Here we report on our high-resolution powder x-ray diffraction experiments at ID-22 aimed at determining the role of protein in lattice distortion in biomaterials: chitin crystals and biogenic calcites.
We collected data from various biological materials: (1) chitin from lobsters and crab tendon (instead of the spider tendon). (2) Sea urchin spines, which are single crystals of calcite containing protein (0.1wt.%), both native and annealed at several temperatures to remove protein. Calcite from a mollusk shell was used a positive control for the annealing treatments.
We performed Rietveld refinement to determine lattice distortions, crystallite sizes and microstrains of the different biogenic systems and show how these parameters can drastically change among systems and upon removal of the incorporated protein by heat treatment.
Finally, an unknown synthetic $CaCO_3$ phase was also measured at ambient temperature and at 100 K for *ab initio* structure determination.

Experimental Procedure

Sample preparation:
Tendons from the claw of lobster (*Humarus Americanus*) and crab (*Cancer irroratus*) were extracted from the animals. Part of each of the samples was demineralized in 0.5 M EDTA in 0.1 M phosphate buffer for five days, isolated by centrifugation and rinsed with deionized water.
Sea urchin spines were severed from the test of sea urchins and washed with sodium hypochlorite under agitation during one day. Several spines were then annealed at different temperatures (180°C, 250°C and 350°C) for two hours. Temperatures were chosen according to thermal gravimetric and heat flow analysis of sea urchin spines (Figure 2A): between 180°C and 250°C the transformation of ACC into calcite occurs and after 350°C no more organic molecules are present.
The unknown $CaCO_3$ phase was prepared by dosing 2 mL of a Mg-Ca solution at a rate of 10 mL/min into a 100 mL reaction vessel filled with 48 mL of carbonate solution under stirring. The reaction solution was then filtrated and washed with ethanol after ~70 min of reaction. The resultant dry powder was stored in a vacuum desiccator.
All samples were ground into fine powder and mounted in glass capillaries.

Data acquisition:

Measurements were performed at ID-22. Thanks to the high resolution of the beamline a short wavelength of 0.3997 Å was used to cover a high Q range without compromising peak overlap due to instrumental resolution. The beam-size was 1 x 1 mm^2.
The chitin samples were run for 4 hour each to get satisfying statistics, as the organic nm-size chitin crystals only have a weak scattering signal. To ensure no beam damage over time, a cryo-stream was used and several fast scans were run and compared. In addition, in situ heat treatments were performed so as to follow strain relaxation if present via the heat degradation of the proteins (as opposed to chemical treatment). Crystalline biogenic carbonate samples (sea urchins spines and mollusk shell), were measured for one hour. Finally, the unknown $CaCO_3$ phase was measured at 100 K using a cryo stream and at room temperature.
The samples were measured from 3 to 21° 2theta for chitin, from 5 to 25° 2theta for carbonates and from 3 to 50 ° 2theta for the unknown $CaCO_3$ phase. All measurements were done with 2degrees/min giving a step size of 0.002 degrees (2theta).

Data evaluation:
Rietveld refinement was performed using the FullProf_suite program 3.00 or GSAS and EXPGUI [1]. We could determine the lattice distortions, crystallite sizes and microstrains of the different crystals. For the unknown $CaCO_3$ phase, unit cell indexing was attempted using the Dicvol and Topas softwares.

Results and discussion:
Chitin samples
Data from the native and demineralized lobster tendon is shown in Figure 1. The data from the native sample (blue curve) contains signals from both calcite, amorphous calcium carbonate, (ACC), and α-chitin (a reference spectrum is shown in black). The data from the demineralized sample (red curve) shows that the demineralization process was successful as no calcite or ACC signals are observed. When comparing selected chitin diffraction peaks from the native and demineralized samples, significant and anisotropic peak shifts towards higher q values are observed. This indicates that the chitin crystals unit cells decrease in size upon demineralization. Besides the peak shifts, the peaks from the demineralized samples are a bit narrower than from the native sample. This indicates that the chitin in the native sample is more disordered than in the demineralized samples. Both of these observations suggest a significant degree of interaction between the organic chitin and inorganic calcium carbonate within this biological sample [2]. To quantify these changes in the diffraction signals in order to evaluate the nature of the interactions between the chitin and calcium carbonate phases, Rietveld refinements will be performed. Because of the complexity of these data, full Rietveld refinements may not be possible and single peak analysis of selected isolated diffraction peaks might be favored.

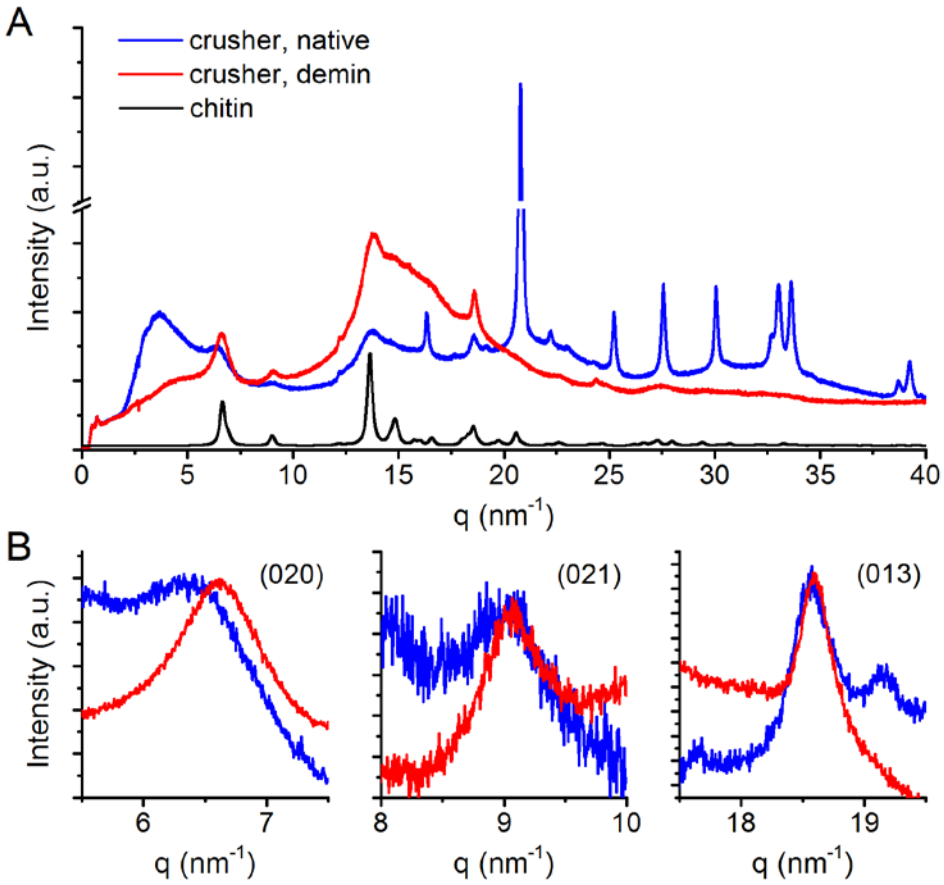

Figure 1: Chitin diffraction results. (A) selected part of the diffractograms from the native (blue) and demineralized (red) tendon from the crusher claw of the lobster. The black curve is a reference curve of α-chitin taken from the crystallography open database #1516344 (Sikorski et al., 2009).
(B) 'Zoom-in' of selected chitin peaks where the intensity of the data from the demineralized sample has been adjusted to highlight the peak shifts. The (hkl) for the most intense peak is indicated in each of the regions

Sea urchin spine samples

Sea urchin spines annealed at 180°C show shifted peaks compared to native samples whereas heated spines at 350°C show peak broadening. This trend is easily identified for the (006) peak diffraction (Figure 2 B). Shifted peak gives information on the lattice distortions happening in the crystal and peak broadening informs on the crystallites sizes and microstrains. Thanks to Rietveld refinement we determined the lattice distortions along the c-axis and the (ab) plane (Figure 2 C). Anisotropic lattice distortions were observed with the maximum distortion in the c-axis direction for 180°C when the transformation of ACC into calcite starts. After 180°C, lattice distortions relaxed (Figure 2 C). Finally, crystallite sizes and microstrains have been determined: crystallite sizes decrease rapidly before 180°C and microstrains increase quickly after 180°C. Similar trends have been observed for *Pinna nobilis* samples which were due to the presence of organic molecules inside the crystals [3]. In Pinna, a correlation was found between the decrease of crystallite sizes, the increase of microstrain and the decrease of lattice distortions. In sea urchins spines, this correlation is different and interestingly the lattice distortion along the c-axis is the same at 25°C and 350°C. These particularities might be due to the presence of ACC in sea urchin

spines (8 at.%) absent in the other biogenic systems studied until now. Thus, the sea urchin spines reflect a new mechanism of strains and distortions due to the simultaneous roles of ACC and organic molecules on lattice distortions of calcite in sea urchin spines. These are not yet understood and will be further investigated in our next beamtime, if granted.

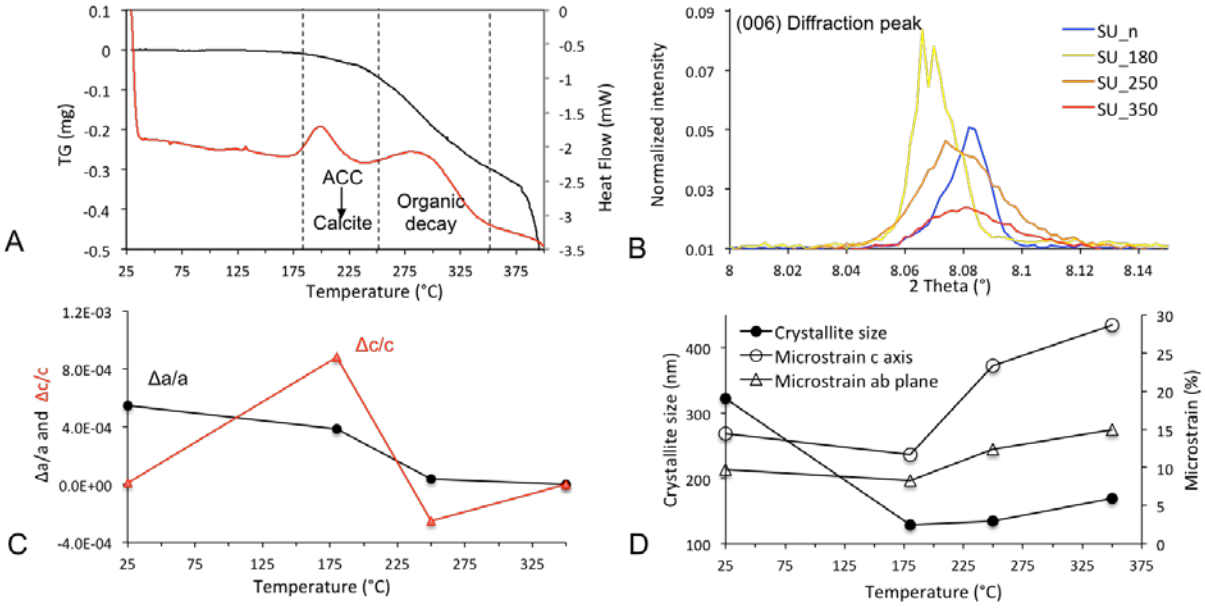

Figure 2: A) Thermal gravimetric (black) and heat flow (red) analysis of SU spines, B) (006) diffraction peak for native spines (25°C: SU_n), annealed spines at 180°C (SU_180), 250°C (SU_250) and 350°C (SU_350), C) temperature-dependent lattice distortions, Δc/c and Δa/a respectively along the c-axis and the (ab) plane obtained using Rietveld refinement D) crystallite sizes and microstrain along the c and ab directions for the different temperatures.

Unknown $CaCO_3$ sample

The unit cell indexing with Dicvol and Topas softwares failed because of high peak overlap. Therefore, we plan to first determine the unit cell of the unkown phase by HRTEM and second to perform Rietveld refinement of the HRXRD data obtained here in order to gain high accuracy of unit cell parameters, atom positions etc.

We would like to thank Carlotta Giacobbe for invaluable assistance in using beamline ID-22 and help in critical online data evaluation and Andy Fitch for additional discussion.

References:

[1] J. Rodriguez-Carvajal, Recent developments of the program FULLPROF, Commission on powder diffraction (IUCr). Newsletter 26, 12-19.
[2] Sikorski, P., Hori, R., Wada, M., Revisit of alpha-chitin crystal structure using high resolution X-ray diffraction data, Biomacromolecules 2009, 10, 1100-1105
[3] Pokroy, B., Fitch A. N., Zolotoyabko, E., The microstructure of biogenic calcite: a view by high-resolution synchrotron powder diffraction, Advanced materials 2006, 18, 2363-2368.