	Experiment title:	Experiment number:
	Microdiffraction experiments on a biomimetic starch system.	SC-2237
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1. Scientific context of the experiments

Starch granules exhibit a complex ultrastructure of two polymers of glucose (almost-linear amylose and branched amylopectin). Amylopectin, the major fraction, is thought to be responsible for the semicrystalline organization of the granule, while amylose is generally considered to be amorphous. The A- and B-type crystal lattices describe the two structures reported for cereal and tuber starches, respectively. If the understanding of most enzymatic systems involved in starch biosynthesis has considerably increased during the last decade, the granule formation and, in particular, its crystallization process, remain largely unknown. *Chlamydomonas reinhardtii*, a unicellular eukaryotic green alga, has been widely used as a model to investigate the genetics of plant starch biosynthesis. This system is the best available so far to study amylose biosynthesis since GBSS1 (Granule Bound Starch Synthase 1), an elongation enzyme responsible for amylose biosynthesis within the granule, is 10 to 50 times more active than its orthologues in vascular plants. We have developed an *in vitro* biomimetic system in which *C. reinhardtii* native granules (that contain GBSS1) are incubated in a buffer containing ADP-glucose, the unique precursor for starch synthesis. Under these conditions, it has been shown that the amylose synthesized by GBSS1 induced the formation of B-type crystallites.

During these experiments, we first investigated the molecular orientation in native or recrystallized starch samples with various shapes, sizes and crystal types. This allowed us collecting reference spectra from moderately to highly crystalline specimens. Then, we collected microdiffraction data from ultrasmall *C. reinhardtii* granules before and after *in vitro* biosynthesis of amylose. The goal was to monitor and possibly localize the changes of allomorphic type resulting from the growth of amylose in the granules.

2. Experimental method

• **Samples :** Medium-sized (10-20 µm) granules from avocado, lotus *(Fig. 1a)* and maize *(Fig. 1b)* starch were used for molecular orientation studies. These samples exhibit the three main diffraction 'signatures' for native starch, namely A (maize), B (potato) and C (lotus), a mixture of A and B. In addition, spherocrystals were prepared by recrystallizing a 2 wt% aqueous solution of synthetic amylose (degree of polymerization 40-80) and used as a highly crystalline standard *(Fig. 1c)*. For *in vitro* biosynthesis experiments, we used maize starch granules as well as very small (1-2 µm) lenticular granules grown from two strains of *C. reinhardtii* (wild type '137C' and mutant 'I97' - *Figs. 1d,e*, respectively) cultivated in media with or without nitrogen supplementation. To promote amylose synthesis, maize and *Chlamydomonas* starch samples were incubated for various times in a suitable buffer, in the presence of ADP-glucose, with or without additional maltotriose.

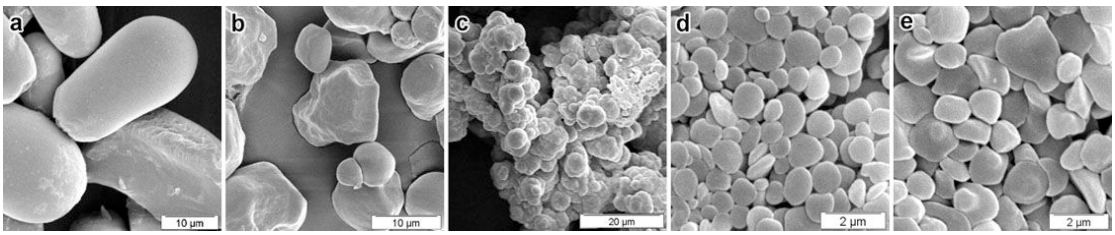

Figure 1. SEM images of samples used in this study: a,b) starch granules from lotus and maize, resp.; c) synthetic amylose spherocrystals; d,e) starch granules from *C. reinhardtii* (strains 137C and I97, resp.).

• **Wide-angle microdiffraction :** The hydrated starch granules and spherocrystals were sandwiched between two collodium films supported on 200 mesh TEM copper grids *(Fig. 2a)*. The grids were then glued on the tip of a glass rod *(Fig. 2b)* and placed on a motorized specimen holder *(Fig. 2c)*. The samples were X-rayed using a beam with a diameter of about 0.9 µm, at a wavelength of 0.976 Å. Mesh-scans were carried out with 2 µm steps. The experiments were performed at room temperature, the hydration of the starch granules being preserved by the residual water trapped between the collodium films *(Fig. 2d)*. The diffraction patterns were recorded on a MAR 165 CCD camera (1024×1024 pix^2) during 2 or 4s exposures. The data were processed and displayed using the Fit2D software.

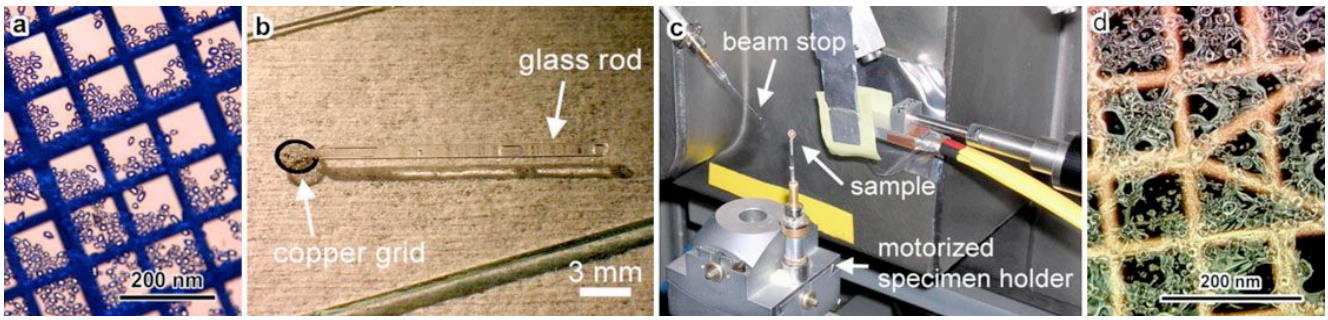

Figure 2. Experimental set-up: a) transmission optical micrograph of starch granules sandwiched between two collodium films supported by a TEM copper grid; b) the specimen grid was glued on the tip of a glass rod; c) the sample was fixed on a motorized specimen holder and place on the beamline ; d) reflection optical micrograph of the specimen using the camera available on the beamline.

3. Results

3.1. Reference patterns of allomorphs A and B

Reference microdiffraction patterns were collected from pure A- and B-type oriented semicrystalline domains in maize and avocado starch granules, respectively *(Fig. 3)*. In particular, allomorph B could be easily recognized by the presence of a typical 100 reflection (*d*-spacing: 1.6 nm).

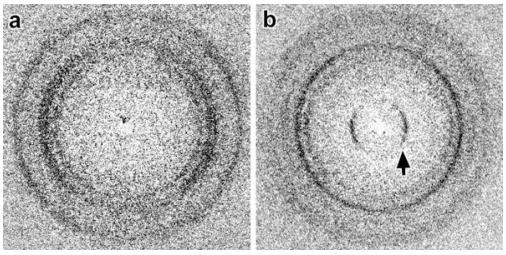

Figure 3. Typical oriented microdiffraction patterns corresponding to A- and B-starch (a and b, respectively), recorded from maize and avocado granules, respectively, and displayed up to a 0.28 Å^{-1} resolution. The arrow indicates the characteristic 100 reflection of allomorph B.

3.2. Lotus starch granules

The microdiffraction patterns recorded from lotus starch confirmed the C-type nature of the sample and that A- and B-type crystallites coexisted within the granules *(Fig. 4)*. Like for smooth pea and green banana starch, previously studied using the same technique, we deduced that allomorph B was located at the center of the granule whereas A-type crystallites were present at the periphery

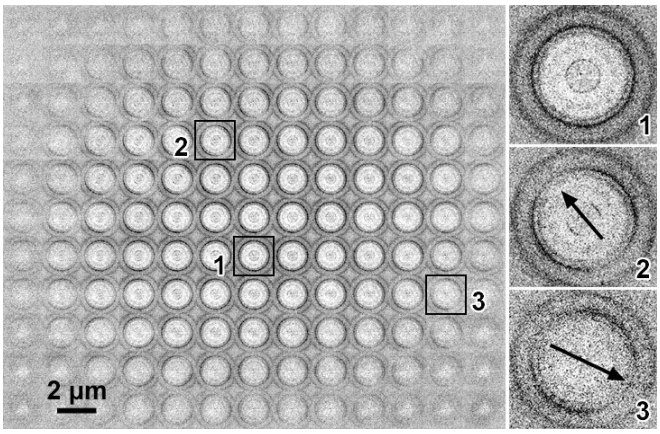

Figure 4. Microdiffraction mesh-scan recorded from a lotus starch granule. Individual patterns framed in 1, 2 and 3 have been enlarged on the right. 1 is a C-type powder pattern collected close to the center of the granule. 2 is a C-type fiber pattern collected closer to the edge and revealing the molecular orientation, indicated by the arrow. 3 is an A-type pattern recorded on the edge. All patterns are displayed up to a 0.28 Å^{-1} resolution.

3.3. Amylose spherocrystals

This sample of amylose spherocrystals had previously been used for an experiment at ESRF on the D2AM beamline (see report #02-01-723 in 2007). WAXS powder patterns had been recorded and suggested that the sample was a mixture of allomorphs A and B, in a 30/70 ratio. An example of mesh-scan recorded from this sample is shown in *Fig. 5a*. As expected, the average diffraction pattern corresponds to C-type *(Fig. 5b)*. Individual patterns are of powder type, not showing any preferential orientation. This is not due to a lack of molecular orientation since polarized light optical micrographs of the particles had clearly shown strongly birefringent domains. As the spherocrystals were rather small (about 5 µm) and highly aggregated *(Fig. 1d)*, it is likely that for each pattern, our microbeam crossed through several particles. Depending on the scanned area, the powder diffraction diagrams clearly varied from nearly pure B-type to mixed C-type patterns containing a high fraction of A-crystallites *(Fig. 5c)*. No pure A-type pattern was observed. Therefore, we could not discriminate between two possibilities: either the particles were a mixture of both allomorphs in varying proportions, or a minor population of A-type particles was mixed with B-type.

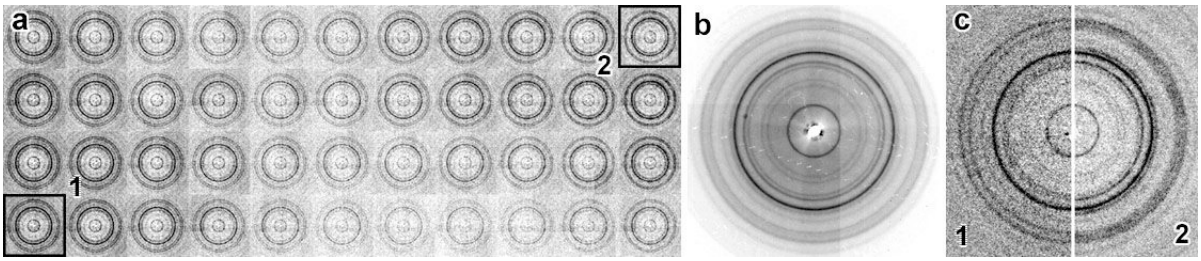

Figure 5. a) 20×6 µm^2 strip of microdiffraction patterns extracted from a mesh-scan recorded from amylose spherocrystals (displayed up to a 0.28 Å^{-1} resolution); b) C-type average powder pattern calculated using all the original patterns, without background subtraction); c) comparison of individual patterns corresponding to those framed in a. 1 and 2 correspond to B- and A-type-rich regions, respectively.

3.4. Maize starch granules - *in vitro* biosynthesis

Microdiffraction mesh-scans were recorded from polygonal wild-type maize starch granules. The patterns corresponded to allomorph A. Those collected at the periphery of the granules showed a significant orientation *(Fig. 6a)*, indicating that the molecules were radially oriented. A similar mapping was carried out on maize-GBSS granules after incubation with ADP-glucose during 24 h in the presence of maltotriose. We did not observe any clear modification of the microdiffraction patterns that might have resulted from the biosynthesis of amylose. In particular, we did not observe the 100 reflection ring indicating the formation of B-type crystallites *(Fig. 6b)*.

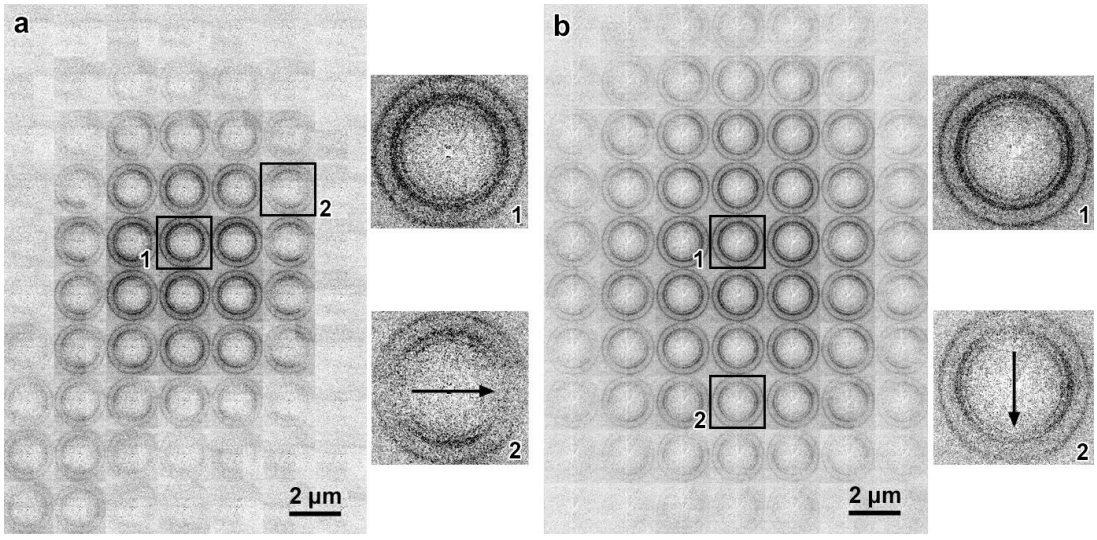

Figure 6. Microdiffraction mesh-scans recorded from a granule of wild-type maize starch (a) and a granule of maize GBSS starch after *in vitro* biosynthesis of amylose in the presence of maltotriose. In a and b, the individual patterns framed in 1 and 2 have been enlarged. 1 is a powder pattern collected close to the center of the granule while 2 is a fiber pattern collected on the edge and revealing the molecular orientation, indicated by the arrow. The patterns are displayed up to a 0.28 Å^{-1} resolution.

3.5. Chlamydomonas starch granules - *in vitro* biosynthesis

The starch granules from *C. reinhardtii* are generally lenticular with a width of 1-2 µm and a thickness not exceeding 300 nm *(Figs. 1d,e)*. Consequently, the intensity in the microdiffraction diagrams was significantly lower than that recorded from larger native granules from maize starch. The use of averaging procedures over patterns collected from several granules was mandatory in order to identify the corresponding allomorphic type. Initial granules from the wild-type strain (137C) exhibited an A-type average diagram *(Fig. 7a)*. After a 24 h incubation with 3.2 mM ADP-glucose and 50 mM maltotriose, the presence of a weak 100 diffraction ring suggested that B-type crystallites had been formed during the synthesis of amylose *(Fig. 7b)*. The average patterns calculated from mesh-scans of granules from the I97 strain before and after *in vitro* biosynthesis were both of the A-type *(Fig. 8)*.

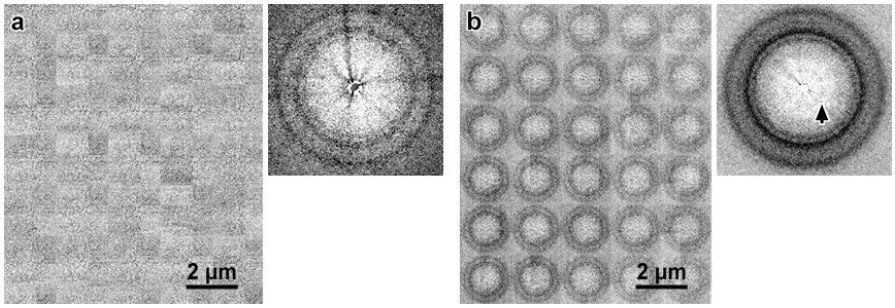

Figure 7. Microdiffraction mesh-scans of wild-type *C. reinhardtii* granules (137C): a) initial sample purified from cells grown in a nitrogen-supplied medium. The average pattern corresponds to A-type. b) Granules after 24 h of incubation with 3.2 mM ADP-glucose and 50 mM maltotriose. The average patterns corresponds to C-type since a weak 100 signal from B crystallites can be seen. The patterns are displayed up to a 0.28 $Å^{-1}$ resolution.

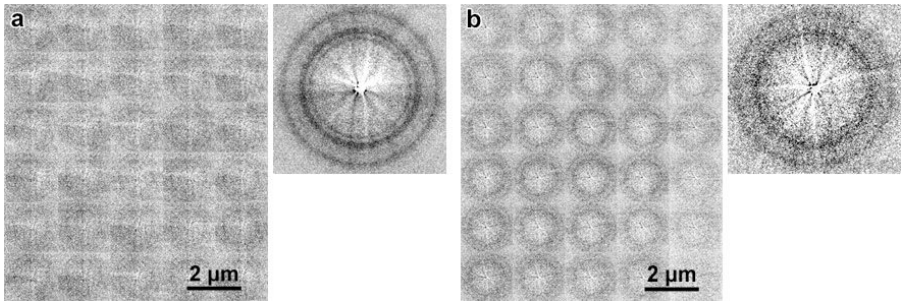

Figure 8. Microdiffraction mesh-scans of granules from the *C. reinhardtii* mutant I97: a) initial sample purified from cells grown in a nitrogen-supplied medium; b) granules after 24 h of incubation with 3.2 mM ADP-glucose and 50 mM maltotriose. In both cases, the average pattern corresponds to A-type. The patterns are displayed up to a 0.28 $Å^{-1}$ resolution.

4. Conclusion and perspectives

Although part of the data is still being processed, several interesting preliminary results could be obtained during this run of experiments:

- The radial orientation of the molecules in A-type maize starch granules was validated for the first time, despite a polygonal shape that made the recording of oriented patterns at the periphery more difficult than in larger spheroidal B-type potato granules.
- The peripheral localization of allomorph A in C-type lotus starch granules confirmed the results previously obtained using microdiffraction mapping of C-type granules from other cultivars.
- Well-resolved microdiffraction patterns were recorded for the first time from highly crystalline amylose spherocrystals. The data collection would be improved if larger and more individual spherulites with a single allomorphic structure could be prepared.
- *Chlamydomonas* starch granules were the smallest (1-2 µm) ever probed by ID13's microfocus beam. The diffraction signal was very weak, mostly due to the low thickness of the lenticular granules. Nevertheless, averaging procedures were successfully used to identify the allomorphs. In particular, we detected the presence of B-type crystallites formed after *in vitro* biosynthesis of amylose within the granules. However, it was not possible to localize the site of amylose synthesis within the granule which was the primary goal of this experiment. The intensity being also limited by beam damage, the data collection would certainly be improved by working at low temperature using the cryo-flow system that maintains the sample at 100 K. The further reduction of the beam diameter would also greatly help getting information about the localization of amylose synthesis within the granules.