ESRF	**Experiment title:** Time-resolved monitoring of structural modifications during acid hydrolysis of "amylose-only" barley starch granules	**Experiment number:** 02-01-889
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Project outline

Contrary to standard starch granules that contain a mixture of linear and branched homopolymers of glucose (amylose and amylopectin) [1], a new type of granule that only contains amylose has recently been biosynthesized in a specifically modified barley cultivar (amylose-only barley starch, or AOBS in the following) [2]. The morphological and structural features of the AOBS granules can be studied by mild and selective acid degradation. Our preliminary WAXS and solid-state NMR analyses (**Figure 1**) showed that the initial granules have a low crystallinity and contain a mixture of two allomorphs: "B-type", made of amylose double helices, and "V-type", made of single helices complexed with the lipids present in the granules. During hydrolysis in dilute HCl, over the course of one hour to a few days, the V-type rapidly disappears while the B-type significantly increases. In addition, while the granules are initially optically non-birefringent, the birefringence rapidly builds up. This project aimed at investigating the molecular mechanisms underlying these ultrastructural modifications.

In particular, we wanted to address the following questions:

1) Are the structural phenomena occurring during the first hours of hydrolysis related or independent ?

2) Are the molecular reorganizations detected by WAXS linked to ultrastructural modifications at a larger length scale and detectable by SAXS ?

3) Do the washing / drying / rehydration steps used to prepare our specimens for the WAXS analysis on our laboratory set-up influence the collected data ? In other words, would the results be different if the hydrolysis was followed *in situ* by X-ray scattering without washing and drying the specimens ?

To address these questions, we have set-up time-resolved experiments on the D2AM beamline to collect scattering data *in situ* from the AOBS granules soaked in dilute acid medium, simultaneously at the molecular (WAXS) and supramolecular (SAXS) lengthscales.

Experimental method

Sample preparation. A purified batch of AOBS granules has been obtained from the Department of Plant and Environmental Sciences (Copenhagen, Denmark). AOBS granule slurries in dilute HCl (2.2 N) have been poured in 3 mm glass tubes. Control specimens have also been prepared from standard barley and waxy potato starch granules. In advance, we have also prepared samples pre-hydrolyzed at specific times (2 to 21 days) to "simulate" longer experiments covering up to 3 week of hydrolysis [3].

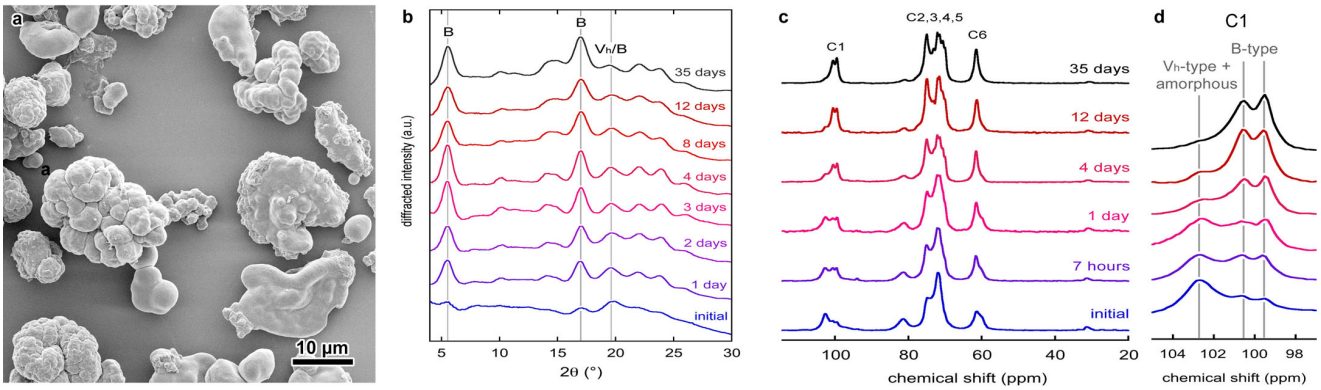

Figure 1. *a) Scanning electron microscopy image of native AOBS granules; b) WAXS profiles of hydrated AOBS granules washed with water after HCl hydrolysis at 36°C (recorded on laboratory equipment); c) corresponding ^{13}C CP/MAS solid-state NMR spectra; d) detail of the evolution of the signal at C1 carbon position in the glucose units.*

Experimental set-up. WAXS and SAXS data have been simultaneously collected over two *q*-ranges corresponding to the crystal structure (0.1 to 3 $Å^{-1}$) and molecular ultrastructure (0.1 to 1 nm^{-1}), using the WOS and D5 detectors, respectively. A temperature-controlled automatic sample changer has been used. For the time-resolved *in situ* experiments, we have collected data during 24 h since preliminary results had shown that the first few hours of hydrolysis were crucial to monitor the structural modifications. For comparison, data have been collected on a series of specimens prepared in conditions similar to those used for the XRD analyses on our laboratory set-up, *i.e.* neutralized by extensive washing in water and analyzed soaked in pure water. All experiments have been carried out at 36 °C using a heating sample changer. Scattering patterns have been collected from the tubes containing the starch slurries during 30 s exposures, with a beam energy of 18 keV. The tubes have been sometimes shaken in order to homogenize the acid / granules mixtures.

Results

The WAXS profiles reveal the allomorphic composition of the crystalline fraction of the starch granules. We have considered 3 allomorphs: A-type (typical of cereals), B-type (typical of tubers and amylose-rich starches) and V-type (complexes with endogenous lipids). As shown in **Figure 2**, native waxy maize starch contained a majority of A-type with a very small amount of B-type (characteristic peak at $q \sim 4$ nm^{-1}). Over 24 h of hydrolysis, the profiles did not change much, which means that the crystal structure was rather stable.

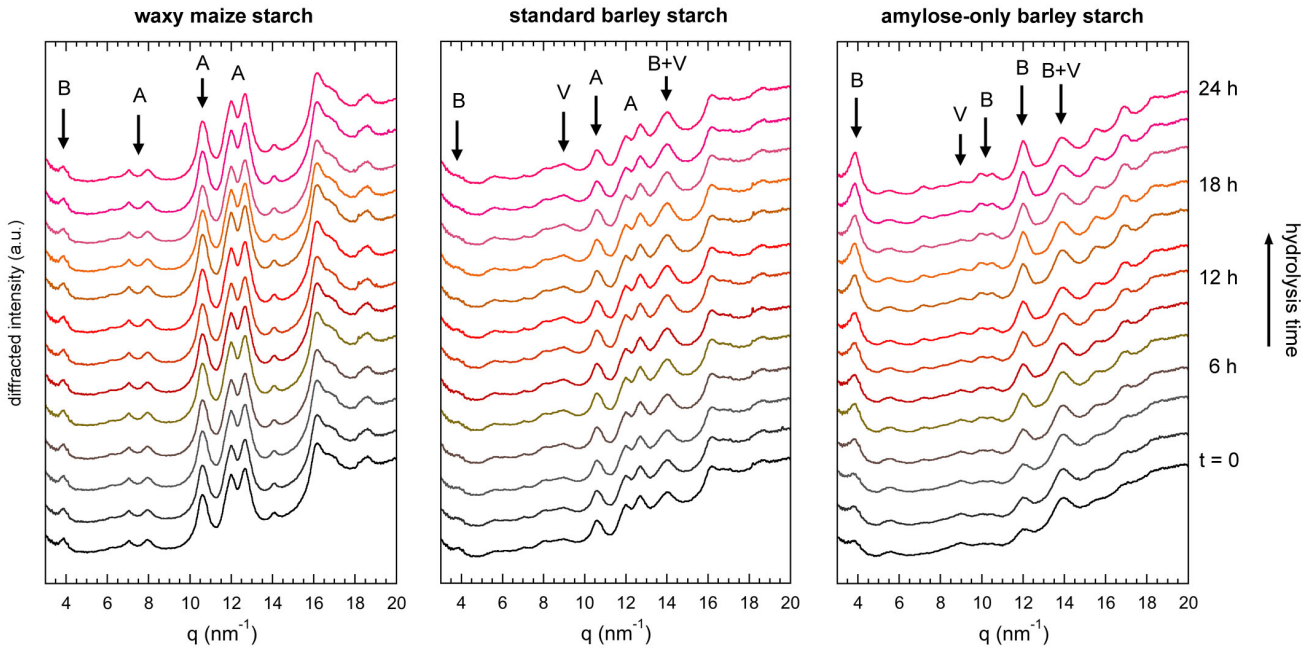

Figure 2. *Time-resolved evolution of the WAXS profiles of waxy maize, standard barley and amylose-only barley starch granule slurries with acid hydrolysis time.*

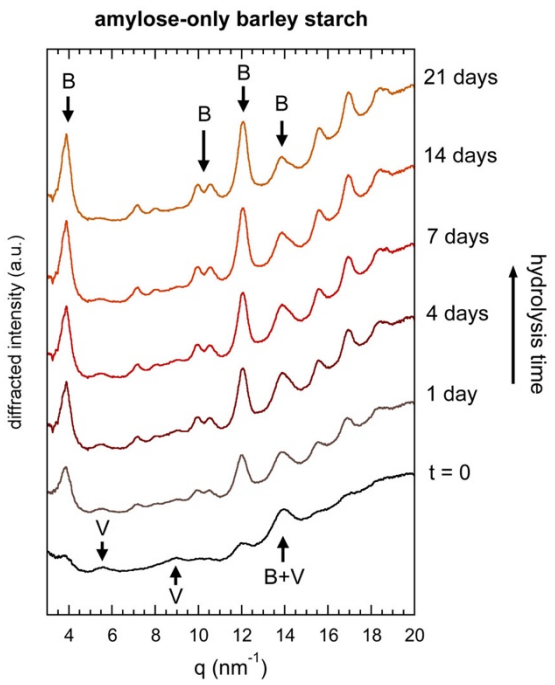

Figure 3. *Evolution of the WAXS profiles of an amylose-only barley starch granule slurry during 3 weeks of acid hydrolysis.*

The standard barley starch granules were less crystalline and corresponded to a mixture of A-type, V-type and a very small amount of B-type. Again, over 24 h, the hydrolysis did not modify the structure much. AOBS granules initially contained a mixture of V-type and a small amount of B-type. The WAXS pattern clearly evolved during hydrolysis. The amount of B-type decreased. It also seems that the V-type concomitantly decreased but a more precise quantitative analysis should be performed. This overall tendency is in agreement with the data collected in our laboratory.

Over a longer hydrolysis time, the tendency observed for AOBS during 24 h was clearly marked (**Figure 3**). The V-type decreased during the first few days while B-type became dominant. It has to be noted that while over a 24-h hydrolysis, the granular morphology is intact, the granules are disrupted after a few weeks of acid treatment. The amorphous domains are etched away and the WAXS signal comes from the more resistant crystalline regions. Therefore, a quantitative analysis should be carried out to evaluate this apparent recrystallization phenomenon.

The collected SAXS data revealed a structural evolution at larger lengthscales. The overall scattered intensity increased with hydrolysis time and the position of the peak maximum in the Kratky plot shifted to lower *q*-values (**Figure 4**), corresponding to characteristic dimensions / distances of 8 nm at t = 0 to 12 nm after 24 h and 18 nm after 3 weeks. The tendency observed during the 24-h *in situ* experiment was also confirmed for the 3-week hydrolysis experiment (**Figure 5**). However, it is premature to propose a description of the initial ultrastructure and its modification upon acid hydrolysis. Several effects can be involved and superimposed:

- initial variation of granule density due to the swelling by the acid;
- molecular reorganization due to a decrease of the chain length, promoting an increase of local orientation (hence the increase in optical birefringence) and formation of recrystallized domains;
- over longer times, as the amorphous regions are dissolved, disruption of the granule architecture and fragmentation into smaller crystal units (supported by TEM images) resulting in a general increase in crystallinity.

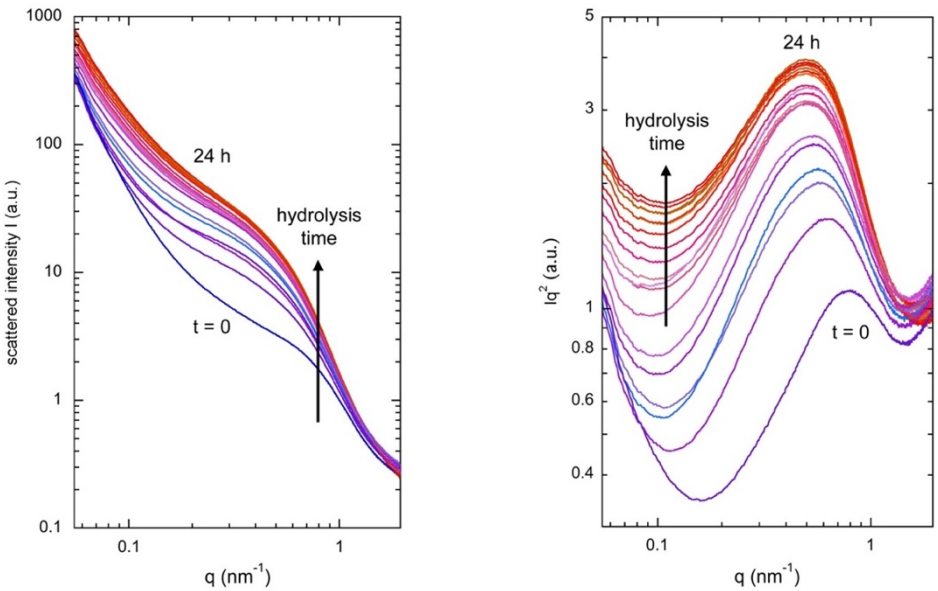

Figure 4. *Time-resolved evolution of the SAXS profiles (left) of AOBS granules during a 24-h acid hydrolysis and corresponding Kratky plot (right). The displayed profiles were collected approximately every hour.*

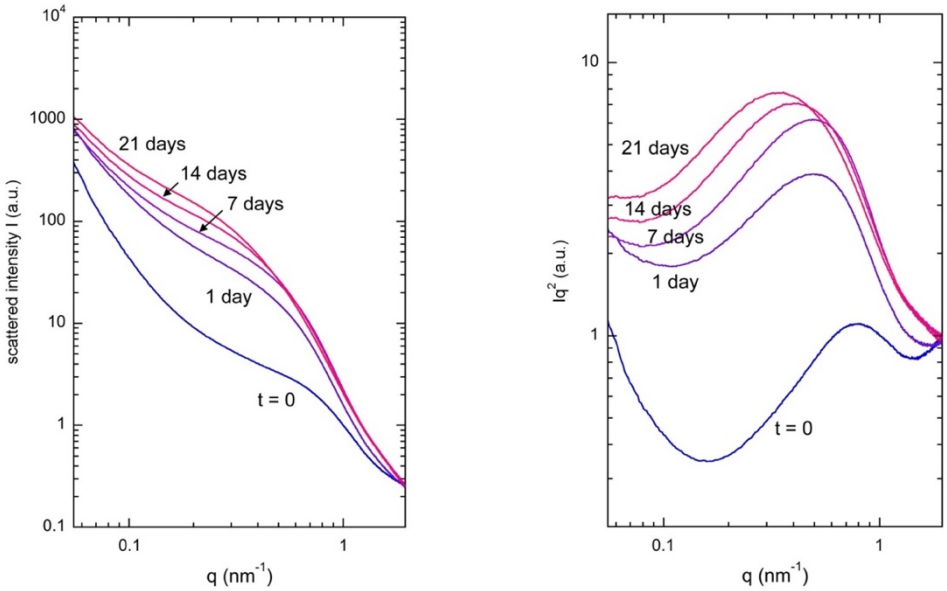

Figure 5. *Time-resolved evolution of the SAXS profiles (left) of AOBS granules over 3 weeks of acid hydrolysis and corresponding Kratky plot (right).*

Conclusion

Our experiments confirmed that the effect of acid hydrolysis on the ultrastructure and crystal structure of the starch granules could be monitored *in situ* during the first 24 h of treatment. The evolution was particularly marked in the case of AOBS. The WAXS data were analyzed in terms of allomorphic composition, showing that the B-type rapidly became dominant, which suggested a recrystallization process. This result has previously been reported for acid-hydrolyzed standard starch granules and has been interpreted as a molecular reorganization due to the higher mobility imparted by a chain length decrease [4]. To check whether this hypothesis is valid for our system, the chain length distribution of our hydrolyzed fractions is being analyzed by chromatography in Denmark. The SAXS profiles are more difficult to understand since, to date, we have no definite hypothesis on the molecular organization of amylose in the AOBS granules. The data are being processed to retrieve more information on the nature, size and distribution of the relevant structural "domains" detected within this *q*-range and propose an ultrastructural model. Finally, the results should help us assess if and how the concomitant phenomena observed at different lengthscales are indeed related.

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References

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[4] Wang S., Copeland L. *Crit. Rev. Food Sci. Nutr.* 55 (2015) 1081.