ESRF	**Experiment title:** Phosphorus and sulfur imaging on starch.	**Experiment number**: SC-2367
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1. Scientific context of the experiments

Starch accumulates as a complex granular structure containing two distinct polysaccharides: quasi-linear amylose and moderately branched amylopectin [1]. Amylopectin, the major fraction of starch, is thought to be chiefly responsible for the semicrystalline organization of the granule while amylose is generally considered as an amorphous polymer. Among the numerous enzymes involved in starch biosynthesis, GBSS1 (Granular Bound Starch Synthase 1) is one of the various elongation enzymes only responsible for amylose biosynthesis. GBSS1-deficient starches lack more or less completely the amylose fraction (waxy maize for example). GBSS1 naturally represents up to 95% of the proteins bound to the granule where it is active. Depending on growth conditions, tissue origin, or genetic background, GBSS1 content may correspond to up to 0.5% of the mass of the granule. *Chlamydomonas reinhardtii*, a unicellular eukaryotic green alga, has been widely used as a model to investigate the genetics of plant starch biosynthesis [2]. The *Chlamydomonas* system is also the best available so far to study amylose biosynthesis since the algal GBSS1 is 10 to 50 times more active *in vitro* than the corresponding enzymes in vascular plants [3]. synthesis by incubation of *C. reinhardtii* native starch granules (that contain GBSS1) in a buffer containing ADP-glucose, the unique precursor for starch synthesis. Under these *in vitro* conditions of incubation, it has been shown that GBSS1 can synthesize a significant amount of amylose However, *in vitro* synthesis of amylose has also been successfully achieved with maize and *Arabidopsis thaliana* starches [4,5].

The second aspect of this project deals with the imaging of phosphorus within the starch granule. Starch is naturally phosphorylated on some of its glucose residues at C3 and C6 positions. Specialized enzymes are responsible for starch phosphorylation: Glucan Water Dikinase and Phosphoglucan Water Dikinase. Like GBSS1, both enzymes are bound to the starch granule but represent only tiny amount of the whole proteins present in the granule. Mutations for these enzymes lead to reduced starch-phosphorylation and to higher starch content [6]. Phosphorylation is therefore a key process that triggers starch degradation. However, it is not precisely known yet how phosphorylation occurs during the diurnal cycle

2. Experimental method

Samples : Starch granules from maize and *Curcuma zeodaria* (10-20 µm, Fig. 1a), Dianella potato (20-30µm) *Chlamydomonas reinhardtii* (1-3µm, Figs . 1b et 1c) and *Arabidopsis thaliana* (1-3 µm) starch were used. For bigger granules (potato and maize), thin sections (6-7 µm) were prepared using classical resin embedding and microtomy.

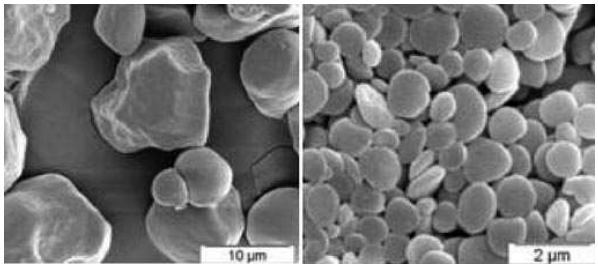

Figure 1. Scanning electron micrographs of native starch granules : a) maize, b) *C. reinhardtii* (strain 137C).

X-ray microfluorescence : By tuning the exciting energy at 2.5 keV, X-ray fluorescence sulfur and phosphorus K-lines were used to map the distribution of those two elements in the samples. A beam size of approximately $0.3 \times 0.6\ \mu m^2$ was obtained thanks to a Fresnel zone plate. Other experiments in a more defocused mode were performed to have the global amount of phosphorus and sulfur on a total single granule or a set of granules. Fluorescence spectra were recorded from 0.3 to 3 keV with a HPGe solid-state detector. XANES measurements were acquired at the S edge (2.45-2.55keV). Data were treated with PyMca.

3. Results

3.1. Global S and P contents in defocused mode

The measurements were first carried out on the bigger starch granules, i.e., from maize and potato, to evaluate the level of response from such samples. Mutant maize and potato starches lacking GBSS1, the major S source in starch, and phosphorylating enzyme (GWD), respectively, were used in order to have very discriminating responses in S and P, respectively. Typical results are shown in Table 1.

Table 1.

sample	WT maize	Wx maize	WT Potato	asGWD potato
MCA (scan n°)	7	4	107	129
P	2.71e+04	1.97e+04	9.66e+04	1.07e+04
S	8.57e+04	5.52e+03	6.87e+03	9.47e+03
XANES (scan n°)	20	19	128	150
$\mathbf{S^{2-}}$	0.0065	n.s.	0.011	0.013
$\mathbf{S^{6+}}$	0.0035	200	n.s.	0.012*

n.s. : not significant

The amount of sulphur is 7 times higher in wild-type (WT) maize when compared to the waxy mutant which does not contain GBSS1. Moreover, when considering the XANES results, no significant amount of S^{2-}, the sulfur species present in proteins is detected. The phosphorus amount in the antisense GWD is 10 times lower than that of WT potato starch, which is in agreement with the strong decrease in GWD activity, a starch phosphorylating enzyme, induced by the antisense expression of the corresponding mRNA.

These results were confirmed by a study of 3 mutant starches from Dianella potato (Table 2) with a lower amount of sulfur in the GBSS antisense line and a lower amount of phosphorus in the asGWD line in comparison to the wild type. Here the high amount of phosphorus in the antisense line deficient in branching enzyme activity (asSBE) is quite remarkable and was never observed before.

Table 2.

Dianella sample	asGBSS	asSBE	WT	asGWD
MCA (scan number)	63	85	107	129
P	1.11e+05	3.63e+05	9.66e+04	1.07e+04
S	4.66e+03	8.35e+03	6.87e+03	9.47e+03

Similar results were obtained on model plant (*Arabidopsis*) and algal (*Chlamydomonas*) starches as examples of a lower amount of phosphorus for sex1-3 Ara, the *Arabidopsis* mutant deficient in GWD, and a lower amount of sulphur for BAFR1, the *Chlamydomonas* mutant deficient in GBSS, respectively. Moreover, a systematic difference was observed between *Chlamydomonas* starches (wild type (137C) and related mutants (I97 and BAFR1) grown in nitrogen supplied or nitrogen starvation conditions. The phosphorus response is much higher when algae were grown in –N conditions (Table 3) which induces massive starch accumulation in the cells. The biological significance of this increase in phosphorus content with starch accumulation is not known.

Table 3.

Chlamydomonas sample	137C+N	137C-N	I97+N	I97-N	BAFR1+N

P	3.19 e+04	2.15 e+05	3.20 e+04	1.39 e+05	7.98 e+04

3.2. Phosphorus and sulfur mapping in single starch granules

With a beam size of approximately 0.3×0.6 µm, S and P were mapped at 2.5 keV. For potato starch which has the biggest size, 5 to 10 µm thick sections were used in order to prevent from averaging on the whole thickness. Maize starch granules are both smaller and flatter which allowed to get specific information about the edges of the granule. Starch from the model plant *Arabidopsis* and model alga *Chlamydomonas* are much smaller, between 1 and 5 µm, and it was quite challenging to map such granules and to get a pertinent information from 5-10 points.

3.2.1 Phosphorus and sulfur location in sectioned potato starch (wild type and amylose-.free mutant)

Figure 2 shows the location of phosphorus in sectioned starch granules from potato, for the wild type and the amylose-free (AMF) mutant which is deficient in GBSS1, the enzyme responsible for amylose biosynthesis.

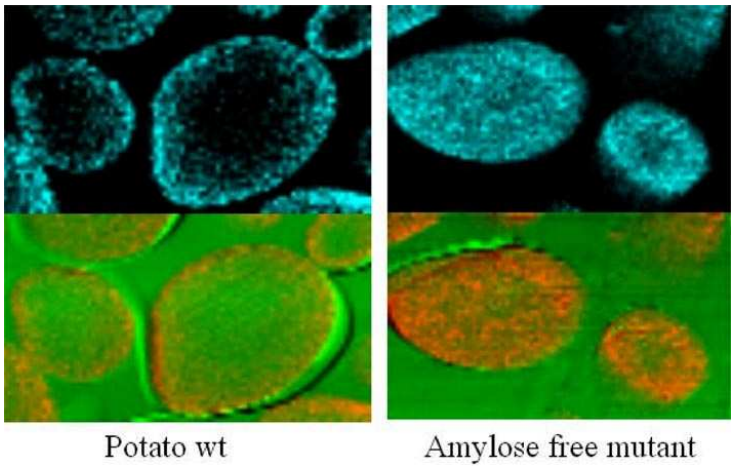

Figure 2: Location of phosphorus in thin sections of WT and AMF mutant potato starch. The two starches were sectioned after embedding in polyhydroxy-ethyl metacrylate. In the lower part P maps were superimposed to phase contrast images

In WT potato, phosphorus stemming from phosphate groups is obviously on the more external layers of the starch granules while it is homogeneously distributed all over the granule for the AMF mutant. This specific location which has never been shown before is in good agreement with the knowledge that the peripheral domains of the potato starch granules are more crystalline and better organized than in the center [7] and that the phosphate groups have to be present in the starch crystalline areas to make them degradable by phosphorylases in the starch metabolic cycle. Moreover, Blennow et al. [8] have shown that only amylopectin, which is also thought to be responsible for the semi-crystalline architecture of starch, is phosphorylated. Therefore, our results could show that amylopectin is more present on the edges of the granule than in the center. On the contrary, the AMF mutant which only contains amylopectin has phosphate groups spread over the entire granule.

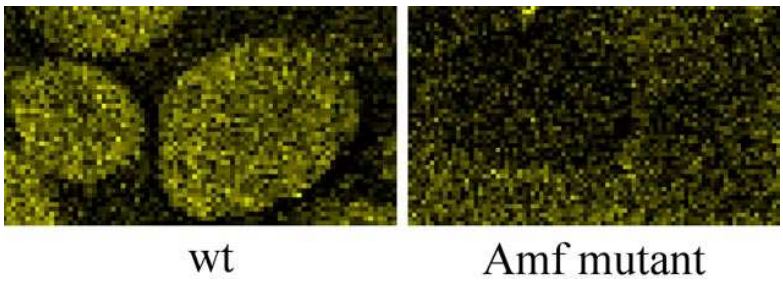

Figure 3: Sulfur mapping of potato starch (wild type and AMF mutant) sections

Figure 3 shows the phosphorus location in potato starch. No significant amount was found in the AMF mutant (starch granules appear as black holes) in which GBSS1 is not present while the mapping obtained on the wild type shows that this enzyme is present all over the starch granule. A similar result was found by S mapping of maize starch granules, the waxy mutant which has no GBSS1 showing no significant amount of sulfur on contrary to the wild type.

3.2.2. S and P mapping in ultrasmall starch granules

Scanning the smallest granules (<1-2 µm) for which only a few pixels were recorded per granule was tricky (Fig. 4a). Using the microscope, it was necessary to define some larger mapping domains

enclosing several starch granules. Very nice S and P maps were obtained on 3-5 µm granules from both *Arabidopsis* and *Chlamydomonas* starches, as shown on Fig. 4b for the *Arabidopsis*. SS4 mutant.

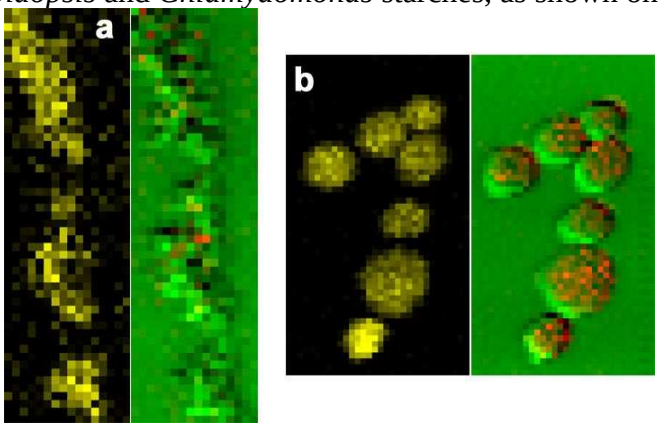

Figure 4: Sulfur and phosphorus (+ phase contrast) location in : a) WT Chlamydomonas (1-2 µm), b) Arabidopsis SS4 mutant (3--4 µm) starch granules.

3.2.3. S and P amounts in single starch granules

The S and P maps were integrated for each granule to check the potential granular variability within a given starch source. To prevent from experimental discrepancies between the different maps and scans, as incident flux or recording time, only the S/P ratios were used for the sake of comparison between the different granules. The results are very heterogeneous and, due to the small number of studied granules and also their small size, especially for Arabidopsis and Chlamydomonas, the statistics is probably not good enough to conclude on inter-granule variability or heterogeneity. Nevertheless, some reproducible S/P ratios were found for potato WT and AMF (5 granules) with $0.3<S/P<0.5$ and $0.12<S/P<0.17$, respectively. The ratio is lower for the AMF mutant due to lower content in sulphur. Good results were also obtained for smaller granules as for example $5.2<S/P<7.4$ on 6 granules for the SS4 mutant of Arabidopsis shown in Fig. 4.

4. Conclusion

X-ray microfluorescence experiments were performed on starch granules for the first time. The results are very promising and open the route to challenging studies on starch biosynthesis. Sulfur and phosphorus amount and location within the starch granule are directly connected to important biological issues like starch biosynthesis and/or metabolism in seeds and leaves. Global measurements of P and S content in non-focused mode on series of starch granules of different sources (including mutants either for enzymes involved in phosphorylation or for the major enzyme involved in amylose synthesis) allowed to clearly observe the direct impact of specific biosynthesis schemes. Some new results were also obtained for the first time on the location of P and S in major starch sources like potato and maize and their amylose-free mutants. These results, especially in terms of mapping, shoud have to be confirmed by future thorough experiments on a more limited number of samples.

This first study has enlightened some experimental constraints especially in terms of mapping. It is quite tricky even impossible, even with the very small beam presently available on the ID21 beamline, to map with a sufficient statistics granules smaller than 2 µm. For the bigger starch granules, it is necessary to use some 5-10 µm-thick sections to prevent from averaging over all the sample thickness and therefore determine the location of the elements of interest within the starch granule. An extreme purity of the sample is also necessary, given the sensitivity of the technique. In some samples (*Arabidopsis* and *Chalmydomonas*) still containing some traces of Percoll or MOPS used for extraction and purification, important response in silicium and/or sulfur was found. XANES spectra allowed to show that this sulfur was stemming from sulfonate present in MOPS and different from that linked to the presence of the GBSS enzyme (S^{2-} and SH).

5. References

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