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In Situ Tracking of Enzymatic Breakdown of Starch Granules by Synchrotron UV Fluorescence Microscopy

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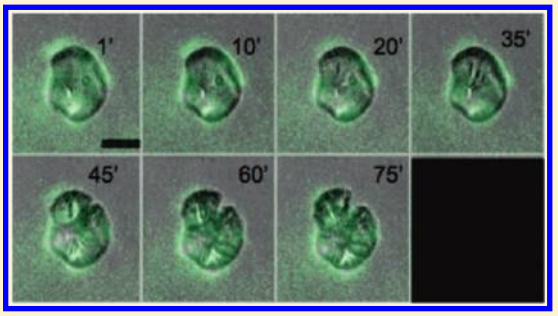
ABSTRACT: Synchrotron UV fluorescence microscopy was used for the first time to visualize the adsorption and diffusion of an enzyme while degrading a solid substrate. The degradation pathway of single starch granules by two amylases, optimized for biofuel production and industrial starch hydrolysis, was followed by tryptophan fluorescence (excitation at 280 nm, emission filter at 300−400 nm) and visible light imaging. Thus, both the adsorption of enzyme onto starch granules at 283 nm resolution and the resulting morphological changes were recorded at different stages of hydrolysis. It is the first time that amylases were localized on starch without staining or adding a fluorescent probe at such high resolution. This technique presents a very high potential for imaging proteins in complex systems. Its sensitivity was demonstrated by the detection of GBSS (the granular bound starch synthase) at high recording times, GBSS being present at very low levels in maize starch granules.



Starch is the major energy reserve of a large variety of higher plants and is biosynthesized as granules that have dimensions ranging from 1 to 100 μm.[1] Their properties are strongly dependent on their crystalline ultrastructure. The crystallinity varies from 15 to 45%, and the crystalline domains are present in semicrystalline shells which consist of alternating crystalline and amorphous lamellae repeating at 9−10 nm.[1,2]

Starch and its two main constituents, amylose and amylopectin, are degraded by α-amylases which are the main enzymes involved in the hydrolysis of α(1→4) glycosidic bonds.[3−5] Amylases are very important in biological functions such as fermentation or digestion but also widely used at the industrial level for the production of glucose syrup and of bread products for antistaling or in detergents to remove starch-based stains. Many applications of amylases are carried out at high temperatures where starch is gelatinized, and there is extensive literature on the mechanism of action of these enzymes on soluble starch.[3−5] Contrary to homogeneous phase hydrolysis where both substrate and enzyme are in solution, the hydrolysis of solid starchy substrates strongly depends on the structure of the substrate[6−8] which limits the diffusion of enzyme and restricts its accessibility to glycosidic linkages. The mechanisms involved in the hydrolysis of the native starch granule are not well-known despite the growing interest in biofuels, low temperature glucose syrup, and slowly digestible food, i.e., resistant starch.

Recently, two new α-amylases, referred to as AFA and RA, were cloned from *Anoxybacillus flavothermus* and *Rhizomucor* sp., respectively. They were studied for their use in bioethanol and low temperature glucose syrup production and were proved to be very efficient in hydrolyzing raw starch granules. This result raises the question of the accessibility of the different domains in the starch granule to amylases, their adsorption onto the starch granule, and the way amylases diffuse within the granule.

Very few techniques allow the assessment of the mechanisms involved in enzymatic hydrolysis of solid starch substrates. Besides the classical determination of hydrolysis kinetics and structural study of residues at different stages of hydrolysis,[6−8] microscopy has rarely been used for monitoring the hydrolysis of single starch granules.[9,10] In the same way, few studies have been reported on the diffusion of amylases within the starch granule, and those reported in the literature use either probes with a size potentially leading to modification of the enzyme behavior[9] or indirect staining of the substrate.[11] Moreover, the resolution reached is strongly dependent on the probe used and is rarely less than 1 μm with conventional confocal laser scanning microscopes.

This paper highlights the very high potential of using a new full-field fluorescence microscope using synchrotron radiation for monitoring the enzymatic hydrolysis of solid substrates. More generally, this method could be used to follow in real time the behavior of unprobed molecules that present natural UV fluorescence. This equipment was used for the first time to assess the hydrolysis of maize starch granules both in visible light through the morphological changes induced by enzyme action and in fluorescence emission after 280 nm excitation through the location on and within the starch granule. This excitation wavelength allowed the imaging of the tryptophans present in the amylases,

Received: October 19, 2010
Accepted: November 30, 2010
Published: December 23, 2010

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and their adsorption and diffusion on and within the starch granules were followed at a not previously attained spatial resolution.

The only other tryptophan-containing component in maize starch is the biosynthetic granular bound starch synthase (GBSS) which is still present after granule biosynthesis[1] at a level between 0.05 and 0.1%. Its contribution to the global fluorescence was checked at different exposure times and with the waxy mutant of maize starch which is known to be deficient in GBSS.[1,12]

EXPERIMENTAL SECTION

Material. Normal maize starch was obtained from Cerestar (Cargill Vilvoorde, Belgium) and waxy maize starch from Roquette Frères (Lestrem France). Purified preparations of RA and AFA were provided as respectively 3 $mg \cdot mL^{-1}$ and 4 $mg \cdot mL^{-1}$ solutions in sodium acetate buffer from Novozymes A/S, Denmark.

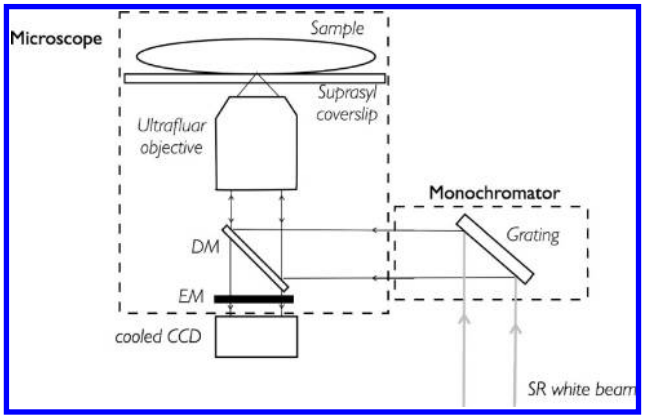

Figure 1. General setup used for fluorescence imaging, showing the monochromatic beam focused onto the sample after reflection on the dichroic mirror (DM). The collected light is filtered by an emission filter (EM).

Methods . *Enzymatic Hydrolysis.* Three milligrams of maize starch was mixed with 10 μL of acetate buffer pH 4.5 on a glass plate, and 2 μL of enzyme solution was then added. The changes in the granule morphology were observed at different time scales as previously determined from hydrolysis curves.[13]

GBSS detection. Three milligrams of maize starch granules or waxy maize starch was mixed with 10 μL of acetate buffer pH 4.5 on a glass plate, and fluorescence emission was studied at different recording times ranging from 10 to 60 s.

Microscope setup. The white beam (180−600 nm) of DISCO beamline at Synchrotron SOLEIL[14] was monochromatized before coupling (Figure 1) with the entrance of a modified Zeiss Axio Observer Z1 (Carl Zeiss, Germany). The synchrotron beam monochromatized by an iHR320 (Jobin-Yvon Horiba, Longjumeau, France) was equipped with a 100 grooves per millimeter grating with a 250 nm peak efficiency (Spectrum Scientific, Inc., Irvine, CA) with entrance and exit slits fixed at 0.1 mm, corresponding to a bandpass of 0.137 nm. The monochromatized light at 280 nm was then reflected toward the microscope beamsplitter. A sharp 300 nm beamsplitter (Omega Optical, Brattleboro, VT) reflected the 280 nm incident monochromatic light upward through a Zeiss Ultrafluar 40× objective (N.A. 0.6). The emitted light was filtered through a XF3000 bandpass emission filter (Omega Optical) and recorded with a Rollera XR cooled CCD camera (QImaging, Surrey Canada). We used UV grade suprasyl 0.17 mm coverslips (Heraeus Glasses, Grenoble, France) for best objective correction, and glycerin (Zeiss, Oberkochen, Germany) was added onto the objective before sample positioning. The recording time was fixed at 10 s per emission fluorescence image.

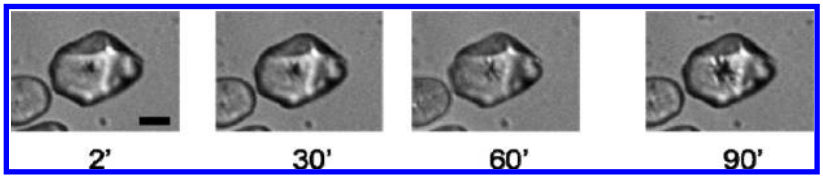

Figure 2. Visible light imaging of maize starch granules during hydrolysis by AFA at 2, 30, 60, and 90 min. The scale bar is 10 μm.

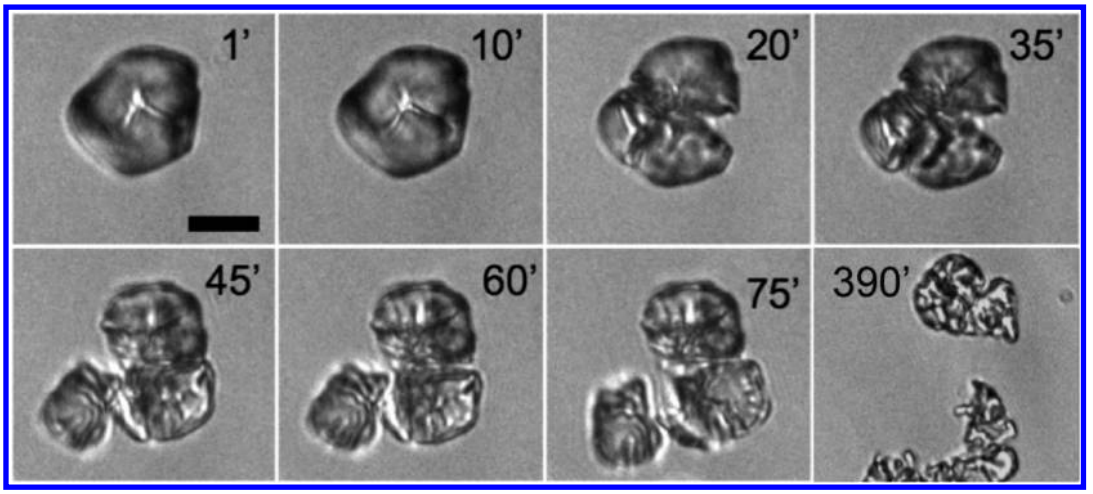

Figure 3. Visible light imaging of maize starch granules during hydrolysis by RA at different stages of hydrolysis (from 1 to 6.5 h). The scale bar is 10 μm.

dx.doi.org/10.1021/ac1027512 |*Anal. Chem.* 2011, 83, 989–993

RESULTS AND DISCUSSION

About the New Setup. To record full field UV fluorescence images of living specimens, a real deep DUV (180−600 nm range) microscope with only one transmission optic has been installed on the DISCO beamline imaging branch.[15] All the optics are of UV grade quality, the excitation is provided by the synchrotron beam, and the emission is filtered by bandpass filters. This setup allows real UV excitation/emission of samples, benefiting all the molecules that do fluoresce when excited with UV below 350 nm. Because of the diffraction limit, the lateral resolution is always increased when looking in the UV range. Indeed, using the Rayleigh criterion, the lateral resolution obtained in this case for tryptophan observation is 340 nm/(2 × 0.6) = 283 nm. The detection limit was obtained by measuring the GBSS within the starch granules (see section 3).

Visible Light Observation of Starch Granule Breakdown. Figure 2 shows typical changes of a single maize starch granule upon the action of AFA. The degradation rate is low, this enzyme being optimized to work at higher temperatures. Nevertheless, it is obvious that the most visible enzymatic degradation starts from the center of the granule and expands to the surrounding area. The center of the granule is known as the hilum and is the point from which granule development starts.[16] This domain is known to be poorly organized and is a weak point for enzyme attack. Some cracks appear, starting from the hilum and spreading radially while the degradation is progressing. In all cases, the granular integrity is retained during the hydrolysis.

Figure 3 illustrates the characteristic breakdown of maize starch granules during hydrolysis by RA. The first observed action of RA is a preferential attack at the center of the granules which rapidly expands through wide breaches along the whole width of the granule, leading to breakage of the granule in three to four pieces after 15−45 min. In the same time, the surface of the granule and subsequently each granule fragment is rapidly attacked, in contrast to that observed for AFA, and becomes rougher and thinner over time. At the end of hydrolysis, some smaller thin fragments are observed with a very different texture (Figure 3). The fragments look like a gel or a recrystallized polymer. It could correspond to the B-type structure observed at very high extents of hydrolysis.[13] This structure was shown to originate from recrystallization of linear fragments of macromolecules released by amylase and was present in the hydrolysis end products. It is the first time that enzymatic breakdown on single maize starch granules has been monitored. Literature reports usually refer to images of a population of starch granules at different stages of hydrolysis,[7,9,17,18] noting the most critical changes and linking them to the different degradation stages.

Detection Limits with GBSS on Wild and Mutant Maize Starch Granules. To ensure that the observed fluorescence does not originate from a starch component, fluorescence of a suspension of maize starch granules was studied without amylase. No fluorescence was observed at recording times used for enzyme location (10 s) which validates the experiments. At longer recording times (>30 s), a uniform fluorescence background was observed on all granules (Figure 4). This fluorescence was not observed on waxy maize starch (Figure 4), a mutant deficient in GBSS. It shows that observed fluorescence originates from GBSS. It is the first time that GBSS has been imaged in a starch granule, and the results strongly indicate that this biosynthetic enzyme is spread throughout the starch granule. This experiment also shows the sensitivity of the used method to detect a tryptophan-containing component. Seven tryptophans are contained in the whole sequence of GBSS (609 amino acids, M_w = 66,859 KDa),[19] i.e., tryptophans represent around 2% of the total amount of GBSS. Assuming that the GBSS content is usually less than 0.1% of starch, the sensitivity of the technique is less than 2×10^{-4}.

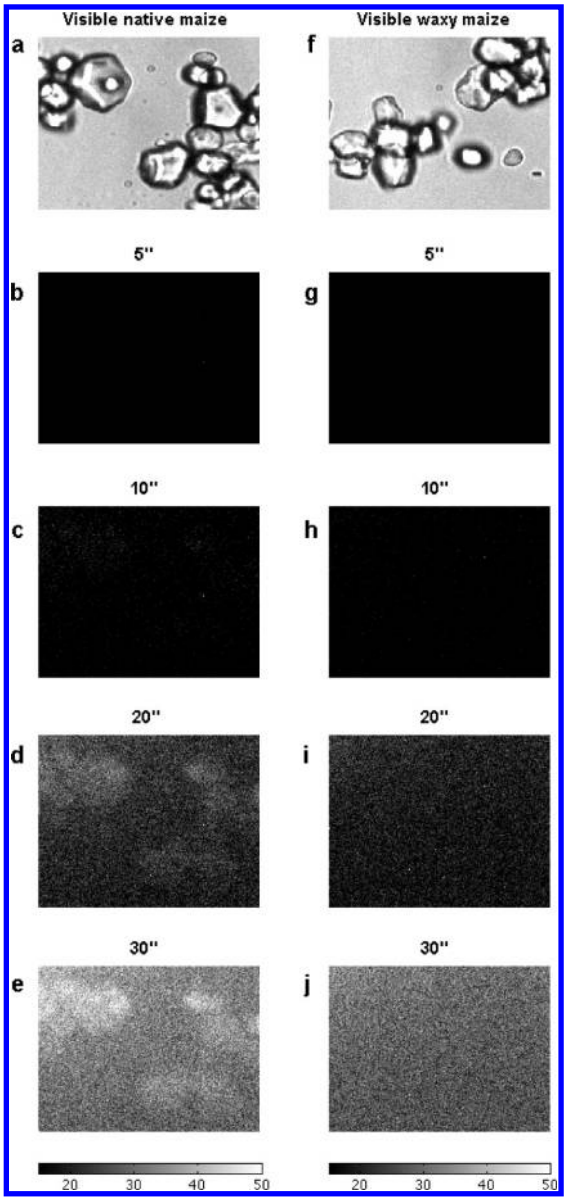

Figure 4. Typical fluorescence and transmitted light imaging of maize (left) and waxy maize (right) starch granules at increasing recording time. GBSS fluorescence is clearly detected in maize starch (bottom left) but is absent for waxy maize starch (bottom right).

Fluorescence Observation of Enzyme during Enzymatic Digestion. Fluorescence images of maize starch granules during hydrolysis by AFA are shown in Figure 5 for three starch granules at different times of hydrolysis. This demonstrates that it is possible to monitor enzymes without adding an external probe and only using the endogenous tryptophans present in the enzyme. This has never been demonstrated previously. Fluorescence images clearly show that AFA accumulates on the sides and more specifically on the edges of the polygonal granules. It appears that the specific area has a major impact on enzyme adsorption during the first stages of hydrolysis. These images are

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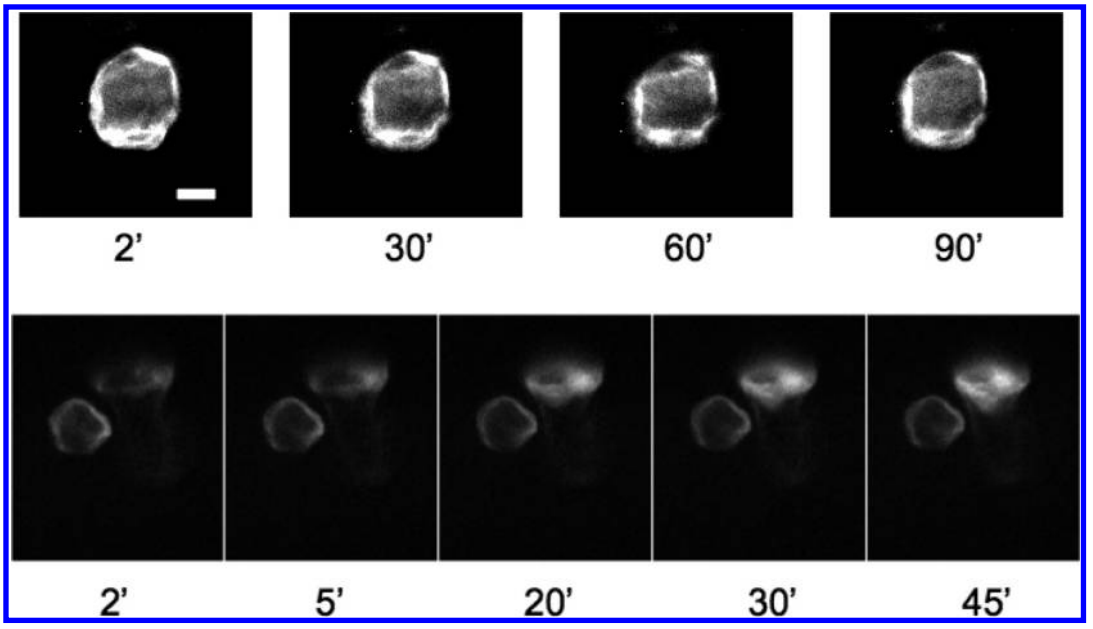

Figure 5. Fluorescence images of amylase adsorption on maize starch granules during hydrolysis by AFA (λ excitation 280 nm, recording time 10 s). Bar is 10 μm. Mean intensity measured in the presence of AFA (per surface and time unit): 1385 counts pixel^{-2} s^{-1}.

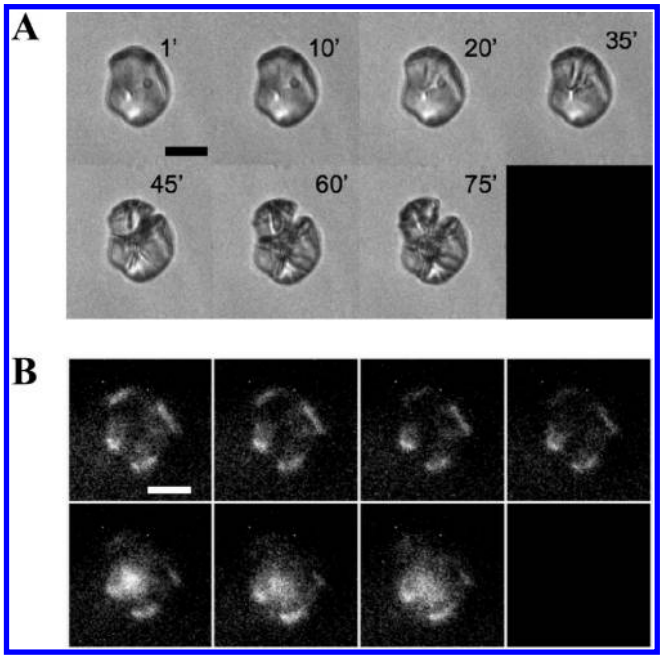

Figure 6. Hydrolysis of maize starch by RA: (a) starch granule morphological changes (visible light) and (b) adsorption/diffusion of amylase (fluorescence imaging). The time series are the same for visible light and fluorescence imaging. Bar is 10 μm. Mean intensity measured in presence of RA (per surface and time unit): 444 counts pixel^{-2} s^{-1}.

difficult to interpret given the low degradation rate of the granules. This intense adsorption on the granule edges does not lead to a visible degradation at the surface of the granule at the available resolution in visible light. On the contrary, the fluorescence intensity is lower in the central part of the granule from which the degradation of the starch granule starts. This means that enzyme molecules are diffusing within the starch granules through the cavity resulting from the first stages of hydrolysis. In the second series of images (Figure 5, bottom), diffusion and adsorption of enzyme over all of the granule surface are observed except in the most degraded regions of the granule when the enzyme has diffused within the granule.

Figure 6 shows a typical series of visible and fluorescence images recorded on the same starch granule during hydrolysis by RA. In this case, fluorescence accumulation on the edges of the granule is less obvious compared to that observed for AFA since enzyme adsorbs and diffuses very rapidly over the whole surface of the granule as shown by the morphological changes observed in the first stages of hydrolysis (Figures 3 and 6A). This is probably due to the added starch binding domain which makes adsorption easier. Very few enzyme molecules are detected in the major cracks and crevices resulting from the RA action, probably due to its diffusion within the granule. Then, when the granule starts to break, the enzyme diffuses into all the resulting cavities and progressively over all the surface of the broken granule, which becomes increasingly rough. Enzyme is still adsorbed in the last stages of hydrolysis when the texture of the granule remnants becomes gel-like (data not shown).

CONCLUSION

We present a very innovative technique that allows us to follow in real time the fate of an unstained enzyme on its substrate. This method could also be used to follow the movements and localization of any protein that presents a fluorescent amino acid residue when excited in the UV down to 200 nm. The resolution is directly proportional to the observed wavelength, i.e., experiments with probes that fluoresce in the ultraviolet are naturally better resolved. The sensitivity of the system was estimated by recording the residual fluorescence of the GBSS protein. Our next step will be to develop a multi z acquisition in order to follow the diffusion of the enzyme in three dimensions.

The study of protein dynamics, interactions, and localization at high resolution has always been a major challenge. First, antibody staining was introduced after fixation and permeabilization

dx.doi.org/10.1021/ac1027512 |*Anal. Chem.* 2011, 83, 989–993

of cells. Other trials were done with modified fluorescent amino acid analogues to visualize protein activity in real time.[20] The ability to label proteins of interest with fluorescent mutant protein probes changed cell biology paradigms in the late 1990s and refreshed the field of protein study inside cells. However, grafting a 27 kDa molecule to an active protein may impair its movements and function. Therefore, autofluorescence detection of natural occurring proteins has always been a challenge[21] even if cells are full of proteins and do not permit the monitoring of one particular protein. Enzymology is one of the fields that will benefit greatly when one single protein can be followed when acting on a non-UV-fluorescent substrate. Moreover, the fact that we worked in the UV range allowed us to record images with a better lateral resolution than ever seen previously. The potential for imaging proteins in a complex medium has been demonstrated. Our method should facilitate the understanding of enzymatic hydrolysis of solid substrates and also of bioindustrial applications such as biorefining and biofuels production on the molecular level.

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