Crystalline Ultrastructure of Starch Granules Revealed by Synchrotron Radiation Microdiffraction Mapping

A. Buléon,[†] B. Pontoire,[†] C. Riekel,[‡] H. Chanzy,*[,§] W. Helbert,[§] and R. Vuong[§]

Institut National de la Recherche Agronomique, B.P. 71627, 44316 Nantes Cedex, France, European Synchrotron Radiation Facility, B.P. 220, 38043, Grenoble Cedex, France, and Centre de Recherches sur les Macromolécules Végétales, CNRS, B.P. 53, 38041 Grenoble Cedex 9, France, affiliated with the Joseph Fourier University, Grenoble, France

Received January 31, 1997

Starch, the main source of stored energy in higher plants, is also the major carbohydrate nutrient for humans and animals. In addition, starch has extensive industrial uses relating to paper, inks, and adhesives. Starch normally occurs in the form of semicrystalline birefringent granules with dimensions ranging from 1 to 100 μm. Depending on their botanical origin, these granules present a diversity in shape and chemical composition[1−3]. Diversity seems to be the rule with starch as even in a given sample from the same plant cell, there is also substantial variation in the shape and morphology of sister granules. Thus, one may say that no two starch granules are identical.[4]

Advances in the science and technology of starch depend on a thorough understanding of its chemical and biochemical structure. The knowledge of its ultrastructure and morphology is another determinant aspect. Indeed, most processes involving starch are initiated as heterogeneous reactions. In these, the reactivity toward given chemicals or biological reagents is governed by the accessibility of the starch building blocks.[5] Therefore, the ultrastructure of starch plays a major role in its reactivity. In recent years, substantial advances have been made to unravel the chemistry and biochemistry of starch. However, its physical ultrastructure and in particular the description of its crystalline microstructure are still far from being complete. The semicrystallinity of bulk starch sample is well established and documented.[1,6,7] Indeed X-ray powder patterns are easily obtained with starch. These patterns indicate that there are two main allomorphs of starch: A starch occurring essentially in cereals and B starch in tubers. C starch, a third allomorph, which is believed to be the sum of A + B, is also sometimes observed in legume starch.[8]

Most common starch granules are much too small to be studied individually by solid state polymer techniques such as spectroscopy or diffraction analysis. Up to now, there is only a single case where an oriented X-ray diagram has been obtained from an isolated starch grain. This diagram, published by Kreger in 1951,[9] was obtained with an X-ray microcamera operating on only one sector of a gigantic grain extracted from the pseudobulb of the orchid *Phajus grandifolius*. This unique experiment proved without ambiguity that the molecular orientation of the polymer segments was perpendicular to the growth rings of this unusually large granule.

At present, if one wants to get some ideas on the structural changes of a given starch granule during a chemical, physical, or biochemical process, one has to rely on optical microscopy observations in polarized light. The resulting images present useful birefringence data, but their resolution is far too small to get some insight into the molecular details of the starch crystalline blocklets either in the initial samples or during any subsequent transformation. Transmission electron microscopy (TEM) carried out on ultrathin sections has also been used successfully to approach the ultrastructural modification of starch upon enzymatic[10] and acid hydrolysis.[11,12] Electron diffraction achieved on partially hydrolyzed ("lintnerized") potato starch granules have confirmed the radial organization of the amylose segments within the corresponding hydrolyzed granules.[11,12] But so far, there have been no reports of electron diffraction analysis of native unhydrolyzed starch granules. In any case, the complete diffraction mapping of a granule structure is difficult with serial sectioning as electron diffraction diagrams which present a high ratio of inelastic scattering are poor on this type of sample. On the other hand, well-resolved oriented electron diffractograms were recorded on minute starch fragments resulting from granule crushing.[13]

The present work was undertaken to see whether the crystalline details of the ultrastructure of individual starch granules could be revealed with the use of the microfocus beamline at the European Synchrotron Research Facility (ESRF). Samples of potato starch from Sigma and wheat starch from Roquette were dispersed in a 0.15% (w/w) solution of polyacrylic acid (Aldrich Chemie) in water. Then 3 mm electron microscopy copper grids with reference lettering (H2 TAAB 200 mesh) were excised in such a way that square windows of five by five grid bars were removed from their center. These grids were coated with a membrane of collodion, and drops of starch suspensions were deposited and allowed to dry. Photomicrographs of the grids holding the starch granules were enlarged to give a map of the samples. The grids were then mounted at the eucentric position of a goniometer head which was positioned on a precise *x*/*y* stage of the microfocus beamline ED13 at ESRF, Grenoble, France. The experimental setup involved an ellipsoidal mirror and a tapered glass capillary producing a monochromatic (λ = 0.0948 nm) X-ray beam of 2 μm full width at half height (fwhh) at the exit of the capillary. The flux was about 10^{10} photons/second/μm^2. A 10 μm platinum aperture at the exit of the capillary was used to reduce the background from the optics.[14] The sample could be placed at about 0.5 mm from the capillary exit, which resulted in a slight increase of the tails of the beam profile as determined by a knife-edge scan.[15] All recordings were achieved with an image intensified CCD camera with video frequency readout, online digitalization, and 16 bit frame accumulation. The CCD camera was positioned 50 mm from the sample.

Residual diffuse scattering upstream from the sample was modulated by the copper grid and resulted in a magnified image of the grid grating at the position of the CCD camera. This allowed an identification of the location of individual grains on the grid by comparison with the optical image. Individual grains, located in the central part of the grid−showing no modulated background from the copper grid−could thus be scanned. Mesh scans were achieved with either 5 or 10 μm step width. Each diagram consisted of 400 successive frames

* To whom all correspondence should be addressed.
[†] Institut National de la Recherche Agronomique.
[‡] European Synchrotron Radiation Facility.
[§] Centre de Recherches sur les Macromolécules Végétales.

S0024-9297(97)00136-8 CCC: $14.00

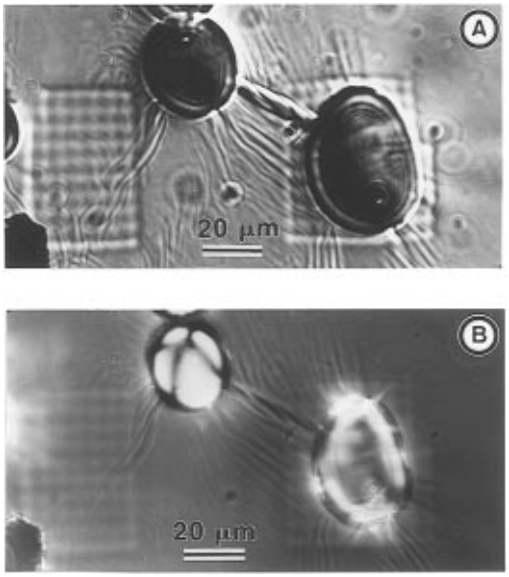

Figure 1. Optical micrograph in Nomarski contrast (A) and polarized light (B) of a typical sample of potato starch after step irradiation with an about 2 μm X-ray beam, each step consisting of a 16 s exposure. The grid was scanned with 5 μm steps: this is clearly visualized in the left part of the picture in an area where there was no granule. The imprint of the beam in the sample is associated with loss of birefringence and beam damage of the supporting collodion film.

for an accumulated time in the beam of 16 s. This time was chosen as it corresponds roughly to the lifetime of the diffraction diagram of the specimens in the beam. After the experiments, photomicrographs of the grids were taken again, both in Nomarski interferential contrast (Figure 1A) and in polarized light with crossed nicols (Figure 1B). The areas hit by the X-ray beam were identified by the black spots created by the X-ray beam, either in the collodion supporting film or in the starch granules themselves. In the latter case, these areas corresponded also to a disappearance of the birefringence (Figure 1B).

Figure 2 corresponds to a typical experiment where a potato starch granule was scanned with 10 μm steps. A total of 11 X-ray diffractograms presenting the features of B starch were obtained. Some of them, diagrams 2, 5, 6, and 9, could be clearly identified as B starch fiber diagrams (Figure 3) similar to that obtained by Wu and Sarko[16] from amylose fibers. Other diagrams, 3, 4, and 7, corresponded to B starch powder diagrams. Finally the four remaining diagrams, namely 1, 8, 10, and 11, were strongly arced. In the four fiber diagrams, 2, 5, 6, and 9, two independent layer lines are clearly resolved and a third is only guessed. The indexation of these patterns indicates without ambiguity that they are of the B starch type with the fiber axis oriented toward the center of the granule (direction arrowed in Figure 2C). In the equator of these four patterns, one distinguishes clearly a strong arc at a *d* spacing of 1.6 nm, together with other rather strong reflections at 0.79, 0.60, 0.53, and 0.45 nm (Figure 3A,B). In the first layer line, a strong arc is also seen at 0.52 nm, together with weaker ones at 0.63 and 0.40 nm.

The position of the patterns 2, 5, 6, and 9 with respect to the starch granule in Figure 2 indicates that it is on the edge of the granule that one obtains the best radial orientation of the polymer chains. On the other hand, diagrams 3, 4, and 7 corresponding to the center of the granule are unoriented powder patterns. As for pattern

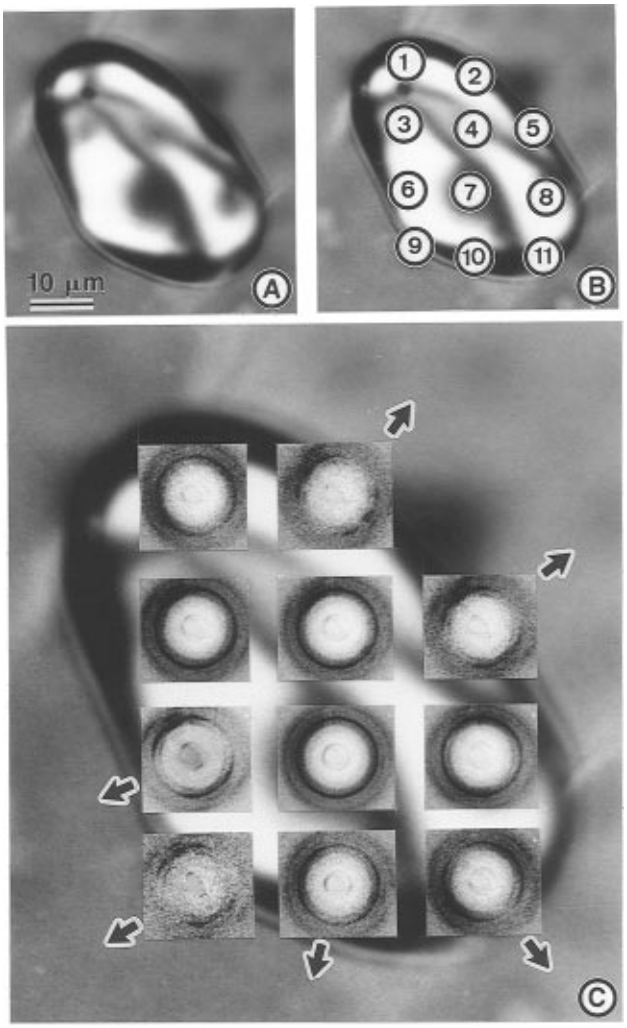

Figure 2. Scanning of an individual potato starch granule: (A) micrograph in polarized light; (B) identification of the areas scanned with the X-ray beam; (C) series of X-ray diffraction diagrams collected from different areas studied. The steps were 10 μm and the irradiation time for the recording of each diagram was 16 s. The irradiated area had a diameter of about 2 μm.

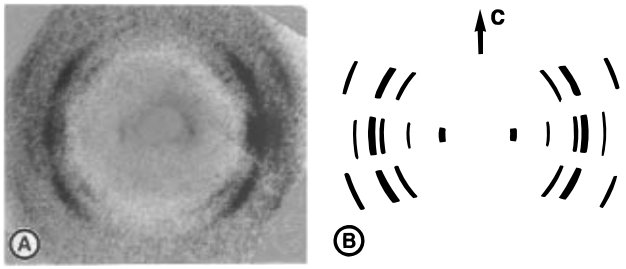

Figure 3. (A) Typical fiber diffraction diagram obtained on an area of about 2 μm diameter and located at the edge of a potato starch granule, with its center along a vertical line and (B) the corresponding schematic representation showing the main reflections recorded.

1, which does not show much orientation either, it corresponds to the origin of growth of the granule, commonly called the *hilum*, that is known to be poorly organized. Diagrams 8, 10, and 11 are less oriented. In diagrams 10 and 11, one recognizes nevertheless a general radial orientation (arrowed) but less pronounced than in diagrams 2, 5, 6, and 9. There are also some discrepancies with these general observations as sometimes we found that the edges of some granules were

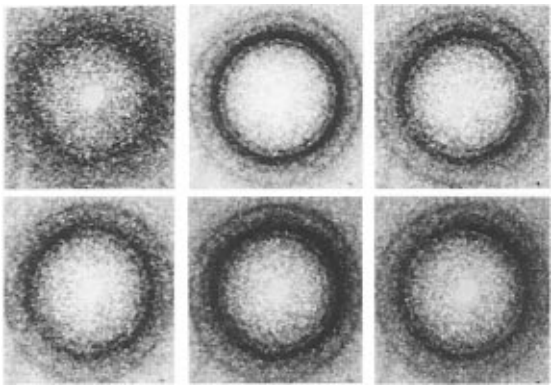

Figure 4. Typical diffraction diagrams obtained by systematic scanning of a single A-type starch granule. These diagrams are part of complete scans of several A-type granules showing orientation neither on the edges nor in the center of the granules.

unoriented or even oriented with the polymer chains in a somewhat tangential orientation.

Figure 4 shows some typical diagrams obtained from a 5 μm mapping of a wheat starch granule. As opposed to the potato starch granules, the wheat starch granules showed almost no orientation in the center or their edges. All the diagrams could be identified easily as being essentially A starch powder patterns.

When the micro-diffraction data obtained in this study are compared with those resulting from electron diffraction analysis on the starch section,[11,12] the synchrotron technique appears to be superior for several reasons. The difference in absorption cross sections implies that X-rays, in contrast to electrons, penetrate easily the 1−100 μm thickness of any starch granule and, therefore, one does not need to slice thin sections as in the case when electrons are used. Also, since the crystallinity of starch is low, there is a high percentage of inelastically scattered electrons in the starch electron diffraction diffractograms. These inelastic electrons, which dominate the elastic ones in the low-angle region of the diagram, eclipse completely all useful information in this part of the diagrams. With electron diffraction, we could never resolve the strong equatorial diffraction spacing at 1.6 nm in B starch even under cryomicroscopy conditions. This diffraction is however clearly visible in all the X-ray patterns recorded in the present synchrotron experiment. In fact, this diffraction line is one of the most important in B starch patterns as its intensity is directly related to the number of water molecules in the B starch crystals.[17] In terms of beam damage, the synchrotron experiment seems to be more favorable than that with electrons. We have estimated that the lethal dose for a B starch electron diffraction pattern recorded at an accelerating voltage of 200 kV and on a specimen kept at liquid nitrogen temperature was around 1electron/nm^3 on 100 nm thick sections.[18] In the present experiments, with starch granules of an average thickness of 50 μm and an X-ray beam diameter of about 2 μm, the patterns were recorded at room temperature with an accumulated dose of 5 photons/nm^3. This dose corresponds roughly to the lethal dose beyond which no diffraction could be observed. The use of liquid nitrogen for cooling the sample is expected to increase further the lifetime of the diffraction patterns. For spider dragline silk, which has a lethal dose toward X-ray similar to that of a starch granules the increase in lethal dose was observed to be more than an order of magnitude upon cooling.[19] Thus, the microfocus synchrotron experiment, which allows one to record more meaningful local diffraction patterns on relatively thick starch granules and under less damaging conditions, appears to be far better than a corresponding electron diffraction analysis which not only requires thin sectioning but also leads to less resolved diffraction patterns.

The results presented in this study open the way to a systematic investigation of starch ultrastructure where important parameters such as polymorphism, distribution, and size of crystalline regions together with the orientation of the polymer chains can be examined at the individual granule level. Future experiments are planned to scan less-known starch granules such as those of amylose-rich and C types. Also, the effect of acid and enzymatic hydrolysis will be investigated. The knowledge of the crystalline microstructure of starch is indeed central to the understanding of its susceptibility toward acid, enzymes, or chemicals that are essential for starch derivatization. The crystallization process of starch during its biogenesis is completely unknown, and very large discrepancies are observed in the susceptibility of starch to hydrolysis, depending on the botanical origin of the samples and their structural features. The microfocus synchrotron mapping approach may have a real impact on important food and nonfood applications of starch such as the genetic tailoring of starch and the optimization of hydrolysis processes in nutrition and the manufacture of glucose products.

References and Notes

(1) French, D. In *Starch, Chemistry and Technology*; Whistler, R. L., BeMiller, J. N., Paschalls, E. F., Eds.; Academic Press: New-York, 1984; pp 183−247.
(2) Jane, J.-L.; Kasemsuwan, T.; Leas, S.; Zobel, H.; Robyt, F. *Starch/Staerke* **1994**, *46*, 121.
(3) Gaillard, T.; Bowler, P. In *Starch: Properties and Potential*; Gaillard, T., Ed.; Critical Report on Applied Chemistry, 13; J. Wiley and Sons: New York, 1987; pp 55−79.
(4) French, D. *J. Jpn. Soc. Starch Sci.* **1972**, *19*, 8.
(5) Leloup, V.; Colonna P.; Buléon, A. *Eur. J. Clin. Nutr.* **1992**, *46*, S17.
(6) Katz, J. R. *Z. Phys. Chem. Abt. A.* **1930**, *150*, 37.
(7) Zobel, H. F. *Starch/Staerke* **1988**, *40*, 1.
(8) Colonna, P.; Buléon, A.; Mercier, C. *J. Food Sci.* **1981**, *46*, 88.
(9) Kreger, D. R. *Biochem. Biophys. Acta* **1951,** *6*, 406.
(10) Planchot, V.; Colonna, P.; Gallant, D. J.; Bouchet, B. *J. Cereal Sci.* **1995**, *21*, 163.
(11) Chanzy, H.; Vuong, R.; Jésior, J-C. *Starch/Staerke* **1990**, *48*, 377.
(12) Helbert, W.; Chanzy, H. *Starch/Staerke* **1996**, *48*, 185.
(13) Oostergetel, G. T.; Van Bruggen, E. F. J. *Carbohydr. Polym.* **1993,** *21*, 7.
(14) Engström, P.; Riekel, C. *Rev. Sci. Instrum.* **1996**, 67, 4061.
(15) Engström, P., Personal communication.
(16) Wu, H.; Sarko, A. *Carbohydr. Res.*, **1978,** *61*, 7.
(17) Bulèon, A.; Bizot, H.; Delage, M.-M.; Multon, J.-L. *Starch/Staerke* **1982**, *34*, 362.
(18) Helbert, W.; Chanzy, H. Unpublished results.
(19) Bram, A.; Bränden, C. I.; Craig, C.; Snigireva, I.; Riekel, C. *J. Appl. Crystallorg.*, in press.

MA970136Q