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Published in:
Carbohydrate Polymers

DOI:
[10.1016/j.carbpol.2016.08.032](https://doi.org/10.1016/j.carbpol.2016.08.032)

Julkaistu: 01/01/2016

Document Version
Peer reviewed version

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Please cite the original version:

Galvis, L., Bertinetto, C. G., Putaux, J. L., Montesanti, N., & Vuorinen, T. (2016). Crystallite orientation maps in starch granules from polarized Raman spectroscopy (PRS) data. *Carbohydrate Polymers*, 154, 70-76. <https://doi.org/10.1016/j.carbpol.2016.08.032>

Crystallite orientation maps in starch granules from polarized

Raman spectroscopy (PRS) data

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Abstract

In this work, polarized Raman spectroscopy (PRS) was used to determine orientation maps of crystallites present in *Phajus grandifolius* starch granules based on the anisotropic response of the glycosidic Raman band at 865 cm^{-1} . The response of this band was preliminarily evaluated using model A-amylose crystals as standard. The A-amylose crystals oriented "in plane" showed a maximal intensity ratio of ~ 3.0 for bands $865/1343\text{ cm}^{-1}$ when the polarization of the laser was along the chain axis of the crystal, *i.e.*, parallel to the axis of the amylose double helices, and a minimal of ~ 0.25 when perpendicular. The orientation maps of *Phajus grandifolius* starch granules showed two distinct regions: one isotropic and the other with a highly anisotropic response. The origin of the difference might be changes in both organization/concentration and orientation of the crystallites across the starch granules.

Keywords: polarized Raman spectroscopy, starch granules, amylose single crystals

Chemical compounds studied in this article: Amylose (PubChem CID: 53477771); Amylopectin (PubChem CID: 439207)

Abbreviation:

DP: degree of polymerization, CARS: coherent anti-Stokes Raman scattering, FWHM: Full width at half maximum, SR: synchrotron radiation.

1. Introduction

The most accepted ultrastructural model of starch granules consists of concentric alternating semicrystalline and amorphous growth rings whose thickness ranges from 100 to 400 nm depending on the botanical source. These rings are believed to contain "blocklets" with amylopectin as the main component (Gallant, et al., 1997, Pérez and Bertoft, 2010). Native starch granules are composed of mostly linear amylose (20-30 wt %), branched amylopectin (70-80 wt %) and other minor components (lipids and proteins) (Tester, et al., 2004). Amylopectin is believed to be the framework of the semicrystallinity of the starch granules as the short branches of its clustered architecture self-organize into double helices that associate into lamellar crystallites (Buléon, et al., 2007). Three types of X-ray diffraction patterns can be recorded from hydrated starch granule powders that depend on the botanical source. A and B types correspond to different allomorphs whereas C-type is a mixture of A and B. Although amylose is only partly involved in the crystallites and is mostly amorphous in the granules, after solubilization in water at high temperature, it can be recrystallized into the same A and B allomorphs, that are both constituted of parallel 6-fold double helices organized into monoclinic and hexagonal unit cells, respectively, and associated to different numbers of water molecules (Lourdin, et al., 2015).

The molecular organization in native starch granules has been studied using various imaging techniques. Polarized light optical microscopy has been used to describe the starch granules as distorted spherulites with positive birefringence and radial molecular orientation (French, 1984). Transmission electron microscopy (TEM) images and electron diffraction patterns of ultrathin sections of resin-embedded starch granules confirmed that the crystallites were oriented perpendicular to the growth rings and to the granule surface (Helbert and Chanzy, 1996, Oostergetel and van Bruggen, 1993). More recently, molecular orientation and

crystallinity maps have been produced by analyzing local diffraction patterns recorded by scanning single starch granules with synchrotron X-ray microbeams (Bulon, et al., 1997, Bulon, et al., 1998, Gebhardt, et al., 2007, Lemke, et al., 2004, Waigh, et al., 1997). Atomic force microscopy (AFM) has also been used to image the internal organization in sectioned starch granules (Ridout, et al., 2004, Ridout, et al., 2002). Other techniques like coherent anti-Stokes Raman spectroscopy (CARS) and second-harmonic generation (SHG) have investigated the structure and alignment of crystallites in potato starch granules (Cisek, et al., 2014, Slepko, et al., 2010, Zhuo, et al., 2010).

Polarized Raman spectroscopy (PRS) is a vibrational spectroscopic technique that provides both compositional and orientation information of the molecules in materials. Raman spectroscopy is based on the analysis of the inelastic scattering of light interacting with molecules in which the frequency shift between the incident and the scattered light is associated to specific vibration modes of a chemical bond. In addition, PRS can be used to determine the molecular orientation within the sample by measuring the anisotropic Raman response of certain chemical bonds at different polarization of the incident radiation (Tanaka and Young, 2006). PRS has been employed to produce 3D orientation maps in complex biological structures like human osteon lamella (Schrof, et al., 2014), to study the chemistry inside wheat kernels (Philippe, et al., 2006, Piot, et al., 2002) and analyze the chemical and structural changes associated to barley malting (Galvis, et al., 2015).

In the present work, we experimentally assessed the anisotropic PRS response of model A-amylose single crystals to interpret orientation effects of crystallites on the Raman spectra of native starch granules. We also adapted a PRS mapping procedure originally used to obtain orientation maps of collagenous materials to map crystallites in *Phajus grandifolius* starch granules based on the anisotropic response of the glycosidic Raman band at 865 cm⁻¹.

2. Experimental Section

2.1. A-amylose single crystals

As described in details in a previous paper (Montesanti, et al., 2010), a fraction of amylose (degree of polymerization DP ranging from 16 to 21) enzymatically synthesized *in vitro* from sucrose by the *Neisseria polysaccharea* amylosucrase (Potocki-Veronese, et al., 2005) was dispersed in water and solubilized in sealed vials at 150 °C for 15 min. The solutions were cooled down to 60 °C and the crystallization was triggered by diffusion of acetone vapors. The resulting A-type single crystals were kept in the water-acetone mother liquor and stored at 4 °C.

2.2. Native starch granules

Starch granules from the pseudo-bulbs of the *Phajus grandifolius* orchid purchased in Brazil were extracted and purified according to the protocol described by Chanzy et al. (2006). The pseudo-bulbs were grated into a powder that was sieved through 200 µm bolting cloth. After decantation, the starch sediment was washed by centrifugation in water. The granules were finally stored at 4 °C in a 20/80 (v/v) ethanol/water mixture.

2.3. Polarized light optical microscopy and scanning electron microscopy

The native starch granules and amylose single crystals were observed in suspension between crossed nicols with a Zeiss Axiophot 2 optical microscope equipped with a SIS ColorView 12 CCD camera. In addition, after drying on freshly cleaved mica and metal coating with Au/Pd, secondary electron images of the specimens were recorded using a FEI Quanta 250 scanning electron microscope operating at 2 kV.

2.4. Polarized Raman spectroscopy and imaging

A continuous excitation laser beam was focused down to a micrometer-sized spot on starch granules through a confocal Raman microscope (WITec alpha 300 Ulm, Germany) equipped with a piezoscanner. A frequency doubled Nd:YAG, 532 nm linear polarized excitation laser (~20 mW) was used in combination with a 100× (Nikon, NA : 0.90) microscope objective. The polarization angle of the laser was rotated using a half-wave plate in the optical pathway. The spectra were acquired using a CCD camera (Andor Newton DU970-BV, Andor Technology plc, Belfast, UK) behind a grating (600 g mm⁻¹) spectrograph with a spectral resolution of 2-3 cm⁻¹. For the anisotropic response of the glycosidic band at 865 cm⁻¹, spectra from seven A-amylose crystals were acquired, integrating the signal during 1 s. For mapping, the single crystals were scanned with 1 μm steps and an integration time of 1 s. *P. grandifolius* starch granules were scanned with 2 μm steps, integrating the signal for 0.4 s.

2.5. Standard deviation analysis of spectra in A-amylose single crystals

The evaluation of anisotropic response of the different Raman bands in a single A-amylose crystal at different polarization angles in a single spot was performed as follows. The spectra were first pre-processed by removing cosmic ray peaks, smoothing (using a Whittaker smoother, 2nd degree polynomial, smoothness parameter = 5) and subtracting a constant background. Subsequently, the mean spectrum, the standard deviation spectrum (i.e. the standard deviation for each wavenumber) and the coefficient of variation spectrum (i.e. the latter spectrum divided by the former) were calculated from these pre-processed spectra. The coefficient of variation was employed to identify which bands had particularly high or particularly low anisotropic response (see below in “Results and Discussion” Section). The calculations were performed using Matlab ®, version 8.2 R2013b (MathWorks Inc., Natick, MA, USA).

2.6. Starch granule mapping

The orientation mapping of ordered structures in native starch granules was performed by collecting multiple Raman images at thirteen polarization angles of the laser on the same focal plane located deep in the sample. Subsequently, in each spectrum, the ratio of the intensity of the glycosidic band at 865 cm⁻¹ by that of the 1343 cm⁻¹ band was calculated. The intensity ratio vs the polarization angle of the laser was fitted with a non-linear least squares method using a graphical interface application (Matlab 7.5 MathWorks Inc., Natick, MA, USA). This application collects the band ratio intensity response at different polarization angles and fits them to a sinusoidal model by using a non-linear least squares method.

$$I = D(1 + E(\cos 2(x - F))) \quad \text{Equation 1}$$

I corresponds to the intensity ratio, D is the mean intensity ratio, E the level of anisotropy (amplitude of the fitting), x the polarization angle of the laser (in radians), and F the phase shift. The fitting parameters were plotted as vectors, whose length represents E , and the direction corresponds to the calculated fitted angle of crystallites in the considered starch granule. The color code in plots corresponds to the range of intensity ratio in starch granules.

3. Results and Discussion

3.1. Morphology and molecular organization in the model single crystals and native starch granules

Model A-type amylose crystals have been used to solve the crystal structure of this specific allomorph. Expanding from previous results from Hsien-Chih and Sarko (1978), Imberty et al. (1988) collected X-ray powder diffraction and single crystal electron diffraction patterns and proposed a monoclinic unit cell that contains a compact packing of left-handed 6-fold

double helices as well as a number of water molecules. This model has been partly revised by Popov et al. (2009) from datasets collected by synchrotron X-ray microdiffraction of single crystals. In particular, the results showed that the double helices were distorted due to the presence of pockets of water molecules. A complete morphological and crystallographic description of A-type amylose single crystals have been subsequently proposed by Montesanti et al. (2010) and Putaux et al. (2011) from scanning and transmission electron microscopy images combined with tilt series of electron diffraction patterns. As seen in **Figure 2a**, A-amylose single crystals have an asymmetrical acicular shape. The sharp end is where the crystal started to axially grow while the opposite apical end is flat. All amylose double helices are parallel to the *c*-axis of the monoclinic unit cell and oriented parallel to the long axis of the crystal, which explains the single polarization color in optical micrographs (**Figure 2b**). In addition, the *c*-axis is oriented opposite to the growth direction and the distal end of the crystal only exposes the reducing ends of the constituting double helices (Putaux, et al., 2011). During our PRS analysis, due to their specific shape, the A-type single crystals always lay on their largest face, which also means that the double helices were oriented perpendicular to the observation direction (**Figure 1b**).

It has to be noted that contrary to the well-documented A-type crystals, no large B-type single crystals have been reported in the literature. Although this allomorph is frequently observed in native starch granules, spherulites (Buléon, et al., 2007) and, more recently, axialites (Putaux, et al., 2006), and although the preparation of thin lamellar single crystals has been reported once (Buléon, et al., 1984), the *in vitro* formation of large B-type single crystals is still elusive. Consequently, we could not use any B-type single crystal model specimen.

The exceptionally large native starch granules from *P. grandifolius* have been used by Kreger (1951) to publish the first fiber X-ray diffraction diagrams of B-starch from oriented regions of single granules. The morphology and molecular organization in such granules have been recently extensively described by Chanzy et al. (2006). The granules are generally flat and elongated, with a length ranging from 50 to 200 μm and a thickness of about 20 μm (**Figure 2c**). Most granules have a triangular shape and polarized light micrographs reveal typical extinction Maltese crosses originating from the granule hilum (**Figure 2d**). However, the hilum is unusually eccentric in the granule. The growth rings are thus asymmetrically distributed and the rather uniform polarization color up to the distal end of the granule suggests that the chain orientation is very high (**Figure 2e**). This was confirmed by scanning individual granules with a narrow beam of synchrotron X-ray and producing molecular orientation maps with a resolution of 5 μm (Chanzy, et al., 2006).

3.2. Analysis of PRS spectra of model A-amylose single crystals

Wellner et al. (2011) showed that the response of the glycosidic Raman band at 865 cm^{-1} on starch granules exhibits a high spatial variation that cannot be explained only by variations in the degree of crystallinity (molecular packing). The authors suggested that the local molecular orientation in ordered structures also influenced such variation. In order to evaluate this point, we analyzed the anisotropic response of the amylose Raman bands in a single spot over an A-amylose single crystal at thirteen different polarization angles of the laser (**Figure 3**). In general, the intensity of all the Raman bands changed but the maximum variation was observed at the 865 cm^{-1} band. This band is assigned to stretching vibrations of glycosidic band/ring breathing in unbranched chains (Liu, et al., 2004). The Raman band at 1343 cm^{-1} , assigned to C–O–H bending, showed only a small variation and was used as "internal standard" to calculate the intensity ratio of bands 865 / 1343. The Raman spectra of

A-type single crystals lying on the (a,c) crystal plane at $\theta = 0$ and 90° of the global coordinate system are shown in **Figure 4**. Due to the uniaxiality of the system, it is possible that the larger component of the global Raman tensor of this vibration is located along the amylose double helices, as suggested by Wellner et al. (2011).

A more detailed analysis of the anisotropic response of the A structure is shown in **Figure 5**, where the 865 / 1343 intensity ratio for A-amylose crystals oriented at $\theta = 90^\circ$ is shown for thirteen polarizations of the laser. The maximum response was obtained when the laser polarization was oriented along the c -axis of the crystal.

3.3. Orientation maps of native starch granules

A preliminary orientation mapping test was carried out on A-type single crystals (**Figure 6a**). As expected, the vectors in the map are uniformly oriented along the c -axis of the crystal ($\theta \approx -30^\circ$) in the (x,y) plane of the global coordinate system. The intensity ratio for a particular spot (white circle) is shown in **Figure 6b**. Since A and B allomorphs share structural similarities (both are composed of 6-fold left-handed double helices, oriented along the c -axis of uniaxial crystals), their global Raman tensor of the C–O–C vibration at 865 cm^{-1} is also assumed to be similar. This structural similarity is also responsible for the close values reported for the theoretical intrinsic birefringence (Δn) of A and B allomorphs (Shogren, 2009). As a consequence, we have used the anisotropic response of C–O–C in A-amylose crystals to analyze orientation maps of B-type *P. grandifolius* starch granules.

The orientation map of the crystallites in a *P. grandifolius* starch granule obtained by PRS is shown in **Figure 7a** where the vector direction corresponds to the average direction of the ordered elements and its length to parameter E (*i.e.*, the level of anisotropy) from Equation 1. This map shows regions with isotropic response represented by short vectors and lower mean intensity (yellow color) close to the hilum and other with high anisotropic response

characterized by long vectors and higher mean intensity (red color) at the distal end. The intensity variation at different polarization angles of the laser for regions 1, 2 and 3 is shown in **Figure 7b**.

The variations observed in the anisotropic response over starch granules (vector length) with changes in mean intensity ratios can be interpreted in two ways. First, the variation can correspond to simultaneous changes in starch density and degree of order. In the map of **Figure 7b**, the yellow regions at the proximal end of the granule would have a lower density and degree of order while starch would be more densely packed and organized in the red regions. The lower starch density exhibited in the region close to the hilum has also been reported on potato starch granules using coherent anti-Stokes Raman scattering (CARS) (Slepkov, et al., 2010). The lack of orientation in the yellow area is in agreement with the lower degree of organization observed around the hilum. The periphery of the granules revealed also lower anisotropy. However, higher fitting errors are present in this area due to the rounded shape of the starch granules.

A second interpretation is based on the assumption that the Raman band at 865 cm^{-1} has a cylindrical symmetry, due to the helical conformation of amylopectin branches (Tanaka and Young, 2006). In this case, the modulation in intensity (color) and level of anisotropy (vector length) are due to changes in the orientation of ordered structures from "out of plane" in the proximal end (yellow regions) to "in plane" orientation (red regions) in the distal part of the granule. Moreover, considering the radial orientation of amylopectin molecules in starch granules, the modulation observed in Raman maps also depends on the position of the focal plane within the granule from where the Raman data are actually collected. The observed modulation resembles that of the amide I vibration in alpha helices of polypeptide chains that,

in idealized cases, possesses a tensor with cylindrical symmetry (Galvis, et al., 2013, Tsuboi and Thomas JR, 1997).

In general, the PRS resolution is lower than 1 μm . In our setup, the estimated lateral resolution is $\sim 0.36 \mu\text{m}$. As a comparison, infrared microscopy has a diffraction-limited spatial resolution of about 10 μm . Most published orientation maps obtained by synchrotron X-ray microdiffraction on starch granules have lateral resolutions of 4-5 μm (Chanzy, et al., 2006, Lemke, et al., 2004) and the beam was transmitted through the whole granule so the diffraction signal was integrated over a larger volume along the beam direction. More recently, Riekkel et al. (2010) collected microdiffraction map on *P. grandifolius* and *Canna edulis* granules with 1 and 0.5 μm raster steps, respectively. The authors explained that the main limiting factor was the propagation of radiation damage beyond the beam tracks, resulting in a significant loss of crystallinity.

Confocal Raman microscopes focus the laser beam into a so-called "confocal illumination volume". However, when focusing below the surface of starch granules immersed in air, the rays are refracted, shifting the focal point deeper in the sample (Everall, 2000). As example, when focusing at nominal depth of 0.5 μm inside of an homogeneous material with refractive index 1.53 similar to starch granules (Wolf, et al., 1962), one illuminates 0.6 to 2 μm below the surface with a depth resolution of $\pm 0.65 \mu\text{m}$. For a nominal depth of 1 μm the resolution degrades to $\pm 1.31 \mu\text{m}$. As the confocal volume is function of the lateral and depth resolutions in a 3D Gaussian shape approximation of the laser beam (Rüttinger, et al., 2008) the confocal volume increases when focusing deeper in the sample. It is worth noting that the collected Raman signal corresponds to the interaction of the laser light with several semicrystalline and amorphous growing rings, for this reason focusing deeper increases the number of growing

rings probed and makes the crystallite orientation evaluation less accurate (the calculations are detailed in the Appendix A).

Another consequence of the averaged nature of the Raman signal is that the orientation evaluation is influenced by the position of the probed volume over the starch granule. In this sense, due to its radial structure, the orientation evaluations in regions close to the hilum are less accurate than far from it. In order to obtain reliable orientation information from Raman mapping is important to focus the laser beam close to the surface of the sample and use immersion objective lenses to reduce the refraction in the interface of the mounting medium/sample. Additionally, a basic requirement to perform Raman mapping on sample is a flat surface. In our case, we successfully collected maps on *P. grandifolius* granules without any specific sample preparation due to their rather flat shape and low surface roughness. However, better results may be achieved if blocks of resin-embedded granules were sectioned in a microtome in order to obtain very flat and smooth surfaces.

Finally, the average orientation of the ordered elements shown in the Raman maps can be considered as projections on the (x,y) plane but no information about rotation value in positive or negative "out of plane" directions can be directly obtained by PRS as opposed to other optical microscopy techniques such as SHG imaging where information about the tilt of the double helices at different depths inside the starch granules could be obtained (Zhuo, et al., 2010)

4. Conclusions

We have studied the anisotropic response of the 865 cm^{-1} Raman band assigned to C–O–C stretching / ring breathing on model A-amylose single crystals made of parallel double helices lying "in plane" with respect to the incident laser beam. The maximum response of this band

was obtained when the laser polarization was oriented along the *c*-axis of the crystals. Using these results, we have produced orientation maps of ordered regions in *P. grandifolius* native starch granules. These maps showed low organized areas around starch hilum and more organized and radially oriented towards the edge of granule. Changes in the anisotropic response of this band from isotropic to highly anisotropic response and in the mean intensity of the 865 cm⁻¹ Raman band across starch granules were observed, which can be interpreted as changes in organization / density and orientation across the starch granule.

Acknowledgment

The authors thank P.-C. Escalier and G. Véronèse (Laboratoire d'Ingénierie des Systèmes Biologiques des Procédés, Université de Toulouse, INSA-UPS-INP, INRA UMR792, CNRS UMR5504, France) who supervised the preparation of the fraction of amylose biosynthesized by amylosucrase, and C. Lancelon-Pin (CERMAV) for SEM observations on the equipment of the NanoBio-ICMG Electron Microscopy Platform (Grenoble, France).

The financial contribution by the Academy of Finland within the “Microtools” project (project n. 13138411) is gratefully acknowledged.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/xxxxxxxxxxxxxxxxxxx>

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435 **Figure captions**

436 **Figure 1. a)** Raman setup used in the present work. The laser excites the sample in the z-
437 direction at a particular polarization angle in the (x,y) plane. The resulting Raman radiation is
438 collected in the z-direction for all possible polarization directions of the Raman scattered
439 light. **b)** Sketch of the double helix organization in the (x,y) plane of an A-amylose single
440 crystal. The amylose double helices are represented by yellow cylinders oriented along the c-
441 axis of the crystal.

442 **Figure 2.** Morphology and molecular orientation in the studied samples: **a,b)** A-type single
443 crystals, **c-e)** *Phajus grandifolius* native starch granules. a and c are secondary electron SEM
444 images, while **b, d** and **e** are polarized light micrographs. A λ compensator was used in **b** and
445 **e** to generate polarization colors.

446 **Figure 3.** Average spectra of an A-amylose crystal lying on its (a,c) crystal plane (equivalent
447 to the (x,z) plane in the global coordinate system) for thirteen polarization angles (continuous
448 line) and variation coefficient at different Raman shifts (dashed blue line). The maximum
449 variation corresponds to the band located at $\sim 865\text{ cm}^{-1}$ assigned to the ν_s C–O–C/ring
450 breathing.

451 **Figure 4. a)** Raman anisotropic response of A-amylose crystals oriented "in plane" at $\theta = 0^\circ$
452 and 90° . The maximum anisotropic response of the glycosidic band at 865 cm^{-1} was observed
453 when the polarization angle of the laser lied along the c-axis.

454 **Figure 5.** Anisotropic response of the intensity ratios of the Raman bands 865 / 1343 for A-
455 amylose crystals oriented at $\theta = 90^\circ$.

456 **Figure 6. a)** Orientation map of molecules present in the A-amylose crystals shown inside the
457 white rectangle in the inset micrograph. The analysis is based on the intensity ratio 865 /
458 1343. **b)** Anisotropic response over the white circle on the A-amylose crystal. The maximum
459 response was obtained when the laser polarization lied along the c-axis of the crystal.

460 **Figure 7. a)** Orientation map of crystallites present in starch granules of *Phajus grandifolius*
461 (2 μm step). The vector length in the map is proportional to the level of anisotropy E in

462 Equation 1. The color code corresponds to the mean band ratio $865 / 1343$. **b)** Anisotropic
463 response of the band ratio in regions A, B and C of the starch granule outlined in **a** (black
464 circles).
465

466 **Figure 1**

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b

469 **Figure 2**

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472 **Figure 3**

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476 **Figure 4**

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Figure 6

485 **Figure 7**