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Single Crystals of V-Amylose Inclusion Complexes

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Summary: Lamellar single crystals of V-amylose have been prepared from dilute solutions in the presence of various complexing agents (isopropanol, thymol, linalool, terpineol, α-naphthol and quinoline). They were characterized by means of electron microscopy imaging and electron crystallography. Crystals of the $V_{isopropanol}$-type were more frequently obtained even with complexing molecules with different sizes and chemical structures. α-naphthol and quinoline formed crystals involving 8-fold amylose helices. Flower-like "pseudo-spherocrystals" of lamellar units were observed when the crystallization was carried out at higher amylose concentration.

Keywords: electron crystallography; inclusion complexes; single crystals; V-amylose

Introduction

Amylose is a linear homopolymer of α(1,4)-D-glucose which can be either extracted from native starch[1] or synthesized *in vitro* by enzymes such as amylosucrase[2] and phosphorylase.[3] It can be crystallized from solution by cooling or addition of a precipitant. The resulting morphology (aggregates, gels, spherulites, lamellar crystals, etc.) and allomorphic type depend on the nature of the solvent and parameters such as molecular weight, concentration or temperature.[1]

Short amylose chains, with a degree of polymerization (DP) typically ranging from 12 to 30, can be crystallized from dilute solutions by cooling or using a slow diffusion of acetone vapors at 60 °C, yielding crystals corresponding to the so-called B or A allomorph, respectively. In both cases, the structural models involve 6-fold left-handed amylose *double* helices. In the A structure, the double helices are packed in a monoclinic unit cell containing 4 water molecules.[4] In the B structure, double helices are packed in a hexagonal unit cell containing 36 water molecules.[5]

When crystallized from dilute solutions (0.5–1 g/L) in the presence of a large variety of complexing agents (alcohols, lipids, aroma compounds, etc.),[6] amylose also forms V-type lamellar clathrates.[7–10] Due to the similarity between X-ray powder diffraction patterns of V-amylose compounds and cyclodextrins, models based on amylose *single* helices mimicking the packing of cyclodextrins were proposed to describe V-type structures.[11] Due to the possible rotation around the osidic bond, helices containing 6, 7 and 8 glucose units per turn can be built.[9] Their central cavity is hydrophobic and can host small molecules. In the crystals, the guest molecules can be entrapped inside and/or in-between amylose helices. Whatever the DP of amylose, the helical axis is perpendicular to the crystal base. As the chain length can be much longer than the thickness of the crystal (8–10 nm), chain folding takes place at the surface, favored by a so-called "flip" between two adjacent glucosyl moieties which results in a reversal of molecular trajectory.[12]

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Depending on the complexing agent, micrometer-sized lamellar crystals with distinct shapes and base-plane electron diffraction patterns have been described. So far, five families are known. V_H amylose crystals are prepared in the presence of ethanol or fatty acids. They are hexagonal-shaped and have hexagonal electron diffraction diagrams.[13] A first family of rectangular crystals is prepared by addition of *n*-butanol or *n*-pentanol.[9,14] A second family, under the generic name of $V_{isopropanol}$, corresponds to crystals formed in the presence of isopropanol[9,15] or a large number of complexing agents.[10,16] The crystals from both families have orthorhombic unit cells. $V_{glycerol}$ crystals are square and their diffraction diagrams exhibit a near tetragonal symmetry.[17] Square lamellae with tetragonal diffraction patterns are also obtained when amylose is crystallized in the presence of α-naphthol or quinoline.[9,10] Whereas a molecular model based on 6-fold single helices has been calculated for V_H crystals,[13] only hypothetic models, based on 6- or 7-fold helices have been proposed, depending on the authors, for complexes from the $V_{butanol}$, $V_{isopronanol}$ and $V_{glycerol}$ families.[10,14,15,17,18] $V_{\alpha\text{-naphthol}}$ crystals have been thought to contain 8-fold helices.[10,19]

The knowledge of the crystalline structure of V-amylose complexes is important in order to understand how the guest molecules that have been entrapped in the unit cell during crystallization can be released. This property can find applications in pharmacology and in the food industry, for instance.[20,21] During the course of several recent studies carried out at CERMAV, we have used enzymatically-synthesized amylose with a well-defined DP to prepare model crystals whose morphology and structure were characterized using the resources of electron microscopy imaging and electron crystallography.

Experimental Part

Preparation of the Crystals

Commercial amylose (DP 100, purchased from Hayashibara Chemicals, Japan) or synthetic amylose (DP 100) was dispersed in water (0.5–20 g/L). The solutions were submitted to nitrogen bubbling for 20 min, sealed in a vial, heated for 30 min at 150 °C by immersion in an oil bath, cooled down to 60–90 °C and filtered through a 0.2 μm pre-heated filter. Pre-heated solutions of complexing agents (isopropanol, linalool, thymol, terpineol, quinoline, purchased from Sigma) were added and, after gentle shaking, the mixtures cooled down slowly in a Dewar bottle containing hot water. α-naphthol, a solid insoluble in water at room temperature, was added to the initial amylose solution in a 1:3 (wt/wt) proportion with respect to the amylose. The mixture was heated to 150 °C, filtered, maintained at 95 °C for 2 h and cooled down to room temperature. Crystallization occurred overnight. In some cases, a recrystallization treatments were applied to the samples, consisting in heating the solution until the turbidity disappeared (*i.e.*, the crystals dissolved) and immediately allowing it cooling down again. The crystals were rinsed by sedimentation or very slow centrifugation in water saturated with the corresponding complexing agent or in a 50/50 (v/v) mixture of water and isopropanol.

Transmission Electron Microscopy (TEM)

Drops of dilute crystal suspensions were deposited onto glow-discharge carbon-coated copper grids and after 1 min, the liquid in excess was blotted with filter paper. The grids were then mounted in a Gatan 626 cryoholder, quench-frozen in liquid nitrogen, transferred in the microscope and cooled down to −180 °C. The specimens were observed with a Philips CM200 'Cryo' microscope operating at 80 kV for imaging and 200 kV for diffraction and lattice imaging. Images were recorded on Kodak SO163 films and diffraction patterns on Fujifilm imaging plates, read with a Fujifilm BAS-1800II Bio-imaging Analyzer.

Scanning Electron Microscopy (SEM)

Drops of dilute crystal suspensions were allowed to dry onto copper stubs. The specimens were coated with Au/Pd and observed in secondary electron mode with a JEOL JSM-6100 microscope operating at 8 kV. Images were digitally recorded using the ADDA II acquisition system from SIS.

Results and Discussion

Rectangular lamellar crystals were prepared by crystallizing DP 100 synthetic amylose (0.5–1 g/L) with isopropanol (Fig. 1a), linalool (Fig. 1c), thymol (Fig. 1d) and terpineol (Fig. 1e). In the first three cases, the crystals were organized in flower-like groups of several unit lamellae. In the case of terpineol, the crystals have developed at the periphery of large and thick aggregates, as if the growth of lamellae occurred as a secondary process. While our complexes with isopropanol were similar in shape to those prepared by Buléon *et al.*,[15] they were generally longer and wider. We observed crystals that reached several μm in length and up to 1–2 μm in width, with a very well defined rectangular shape. As the DP of the amylose used in both studies was 100 (commercial for Buléon *et al.* and synthetic in our case), we assumed that this higher "perfection" resulted form the perfect linearity of our synthetic chains. The base-plane electron diffraction pattern of $V_{\text{isopropanol}}$ single crystals (Fig. 1b) corresponds to an orthorhombic unit cell with parameters $a = 2.83$ nm, $b = 2.95$ nm and $\alpha = \beta = 90°$,[22] in agreement with the values reported by Buléon *et al.*[15] A c parameter of 0.80 nm was determined by Buléon *et al.* by calibrating diffraction patterns recorded from crystals rotated around the a^* or b^* axis.[15] The diffraction diagrams (not shown) and unit cell parameters of our crystals prepared with linalool, thymol and terpineol were similar, as were those from complexes with carvacrol, fenchone, menthone and geraniol, as reported in other studies.[10,16]

Square single crystals were prepared by crystallizing DP 100 synthetic amylose (0.5 g/L) with α-naphthol (Fig. 2a). Base-plane electron diffraction patterns revealed an exceptional crystallinity up to a 0.13 nm resolution (Fig. 2b). The diagram had a tetragonal symmetry and the corresponding unit cell parameters were $a = b = 2.32$ nm and $\alpha = \beta = 90°$,[22] in agreement with previous results.[10,19] A c parameter of 0.79 nm along the helical axis was determined by calibrating diffraction patterns recorded from crystals rotated around the

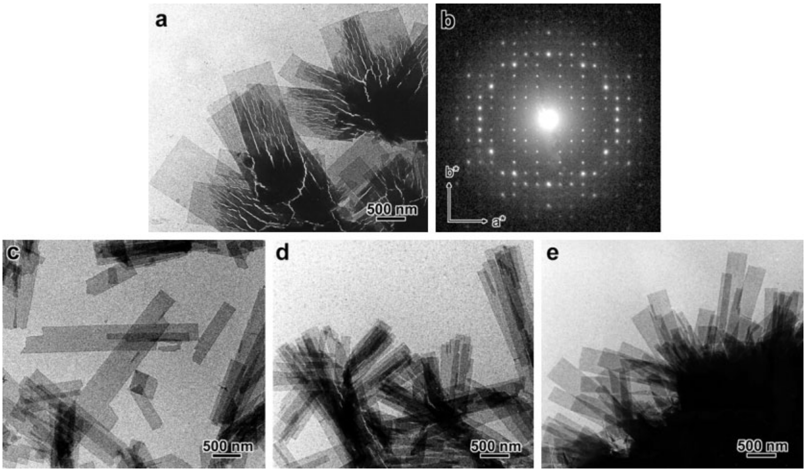

Figure 1.
TEM micrographs of lamellar crystals of synthetic amylose complexed with isopropanol (a), linalool (c), thymol (d) and terpineol (e). In (c), the flower-like groups of crystals were damaged during rinsing, resulting in isolated lamellae. b) Base-plane electron diffraction pattern of a V-amylose single crystal prepared with isopropanol.

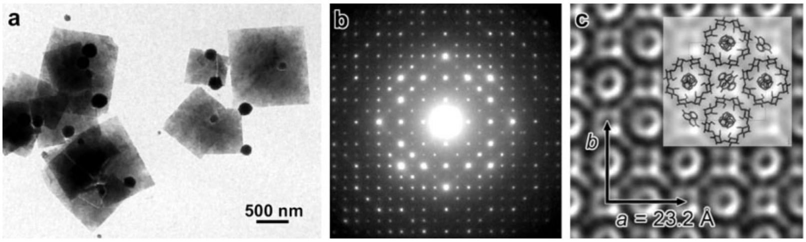

Figure 2.
Single crystals of DP 100 synthetic amylose complexed with α-naphthol: a) TEM image of lamellar crystals in plan view; b) base-plane electron diffraction pattern of one crystal; c) translational average of a lattice image of the crystal recorded along the helical axis. Inset: projection of a preliminary molecular model, indicating the position of α-naphthol molecules inside and in-between the 8-fold single helices.

a^* or b^* axis.[22] In addition, TEM lattice images were successfully recorded, containing structural information up to a resolution of 0.39 nm. As described in details by Cardoso *et al.*,[23] translational and rotational real-space averaging procedures were used, providing projection images of the tetragonal lattice of amylose helices. Confronted with preliminary molecular models (Fig. 2c), these images allowed validating for the first time the existence of 8-fold single helices in V-amylose crystals, as previously proposed by Yamashita and Monobe.[19] Work is in progress to build an improved molecular model of the $V_{\alpha\text{-naphthol}}$ crystal by combining conformational and packing analyses together with a structure refinement based on the set of 3D diffraction data collected during our study.

3–4 μm-wide square crystals were also prepared by crystallizing synthetic amylose (0.5 g/L) in the presence of quinoline (Fig. 3a). These large crystals seem to be constituted of superimposed 50–100 nm square units but the monolamellar periphery yielded well-resolved electron diffraction patterns similar to those recorded from $V_{\alpha\text{-naphthol}}$ complexes (Fig. 3b). For this reason, quinoline appears to be the second known molecule that promotes the formation of 8-fold amylose helices. So far, we have been unsuccessful in finding a third complexing agent with the same property. Molecules that presented analogies with α-naphthol, in terms of molecular shape or chemical structure, were tested but they did not produce the expected result. Quinolinol and quinoxaline, for instance, did not promote crystallization, while decahydro-1-naphthol resulted in the formation of $V_{\text{isopropanol}}$-type crystals. One of our ideas is to search for molecules which are known to form complexes with γ-cyclodextrins (constituted by 8 glucosyl units).

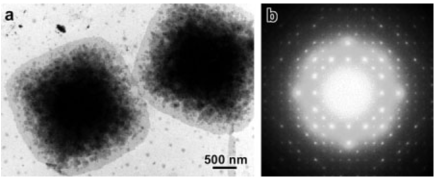

Figure 3.
Inclusion complexes of DP 100 synthetic amylose complexed with quinoline: a) TEM image of lamellar crystals in plan view; b) base-plane electron diffraction pattern of one crystal (recorded on Kodak SO163 film).

The crystallization protocols were modified in an attempt to form larger $V_{\alpha\text{-naphthol}}$ single crystals that would be used for X-ray crystallography. In a first set of experiments, we varied the annealing/cooling conditions. Figure 4a and 4b are SEM images of complexes prepared with synthetic amylose (1 g/L) and α-naphthol. On the one hand, the suspension was left in the warm oil bath to cool down slowly overnight. If the crystals were slightly larger than those shown in Figure 2a, they generally grew on top of each other and formed aggregates of a few units (Fig. 4a). The slow cooling thus promoted heterogeneous nucleation and growth on previously formed crystals. On the other hand, the

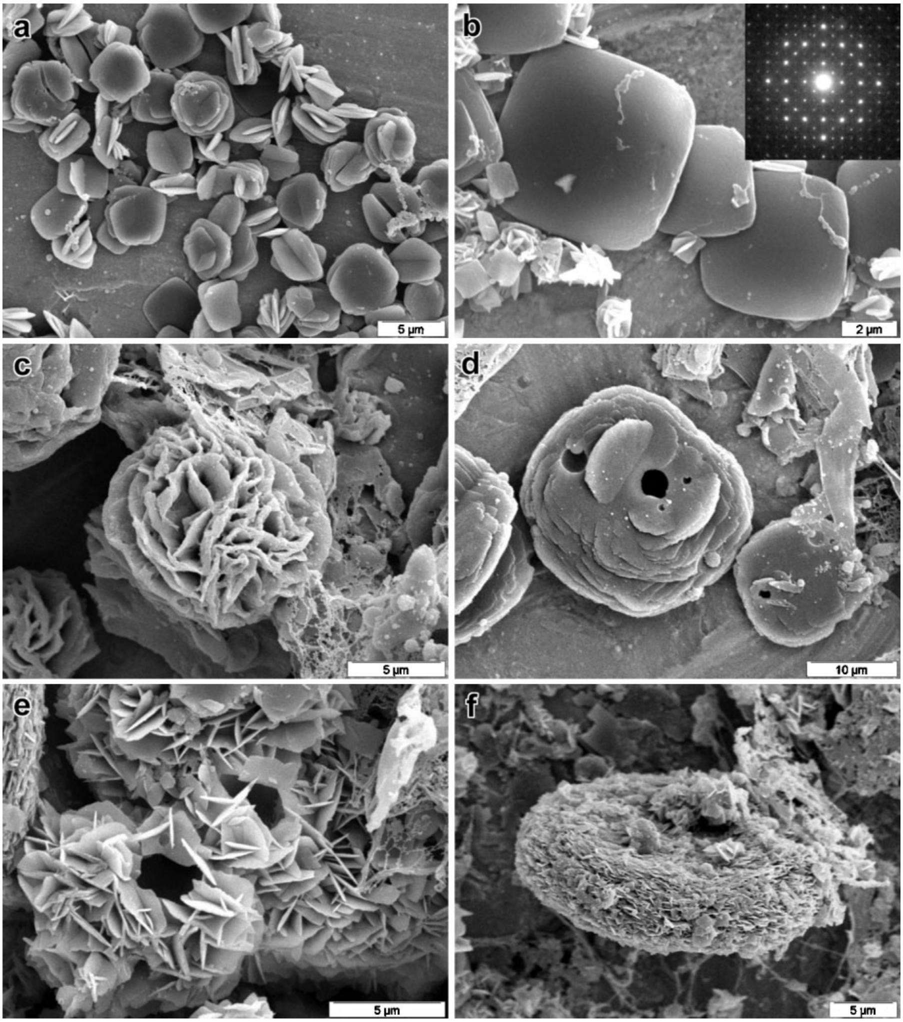

Figure 4.

SEM images of single crystals and pseudo-spherocrystals prepared by crystallizing synthetic amylose with α-naphthol: a) DP 100 synthetic amylose, 1 g/L, slow overnight cooling; b) DP 100 synthetic amylose, 1 g/L, 3 d at 60 °C. Inset: base-plane electron diffraction pattern of one small cushion-shaped crystal; c) DP 100 amylose from Hayashibara Chemicals, 20 g/L, recrystallization; d) DP 100 synthetic amylose, 20 g/L, first crystallization; e,f) DP 100 synthetic amylose, 20 g/L, recrystallization.

solution was left at 60 °C for 3 d and cooled down. Two families of objects were observed (Fig. 4b): groups of 1–2 µm-large lamellar crystals and 5–8 µm-large cushion-shaped complexes exhibiting single crystal electron diffraction patterns (recorded from the thinnest crystals–see inset in Fig. 4b). The growth of these unusually large single crystals was clearly promoted by the extended annealing treatment and the coexistence of complexes with two distinct size distributions may suggest that Ostwald ripening was involved.

A second set of experiments was carried out at a higher amylose concentration (20 g/L) while trying to prevent the formation of spherulites (usually prepared at 50–100 g/L). The crystallization of commercial DP 100 amylose resulted in shapeless aggregates. A recrystallization step was thus carried out, resulting in flower-like "pseudo-spherocrystals" with "petals" constituted by lamellar crystals (Fig. 4c). The first crystallization of DP 100 synthetic amylose produced 10–20 μm lenticular stacks of thin lamellae that possibly grew through a screw dislocation mechanism (Fig. 4d). The effect of a recrystallization step was spectacular and two types of objects were observed: spheroidal 3D aggregates of interlocked lamellae organized in a sand rose fashion (Fig. 4e), and peculiar 20 μm donut-shaped objects corresponding to dense stacks of very thin lamellar units (Fig. 4f).

Another experiment was carried out to form complexes with quinoline using a 2 g/L amylose solution. As seen in Figure 5, we mostly observed flower-like spheroidal objects with lamellar "petals", similar to those obtained with α-naphthol using a 20 g/L solution of commercial amylose (Fig. 4c).

The results of these experiments have shown that the optimization of the annealing/cooling conditions should be preferred in order to increase the size of single crystals and that low amylose concentrations remain mandatory. The tendency to form crystalline lamellae was strong, even when higher amylose concentrations were used. Lamellar or lenticular organizations of crystals were often promoted during the first crystallization whereas a recrystallization step generally allowed the formation of 3D architectures. However, this was achieved at the expense of single crystal size since a higher amylose concentration promoted the heterogeneous nucleation of smaller crystals.

Conclusion

In the last 40 years, it has been shown that V-amylose lamellar inclusion complexes with different morphologies and structures could be prepared using a variety of complexing molecules. However, despite the progress in electron crystallography, a limited number of crystal structures have been solved and several structural models proposed for V-amylose complexes are still hypothetical. The formation of V-amylose complexes is an important issue, in particular for food applications. It is thus important to carry out detailed structural analyses in order to: i) understand why the $V_{\text{isopropanol}}$ type is the most commonly formed complex despite the diversity of size and chemical structure of the complexing agents available; ii) predict if a given molecule will promote the formation of inclusion complexes with amylose and which type of crystal structure will result.

However, before achieving this goals, efforts will have to be made on three aspects:

1) prepare better single crystals by using fractions of linear synthetic amylose and optimizing the crystallization protocols, in order to prepare large and thin mono-

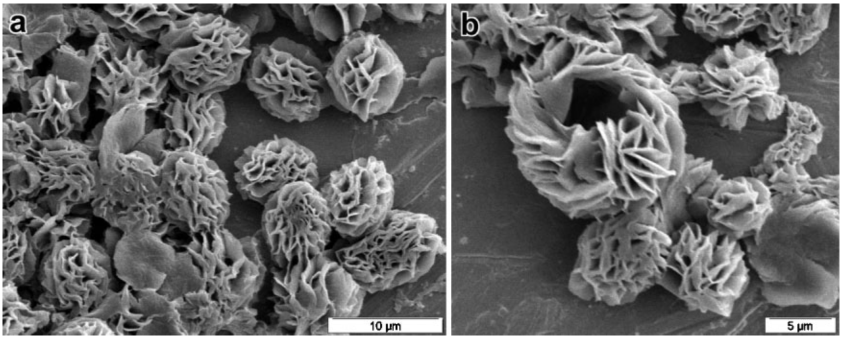

Figure 5.
SEM images of pseudo-spherocrystals prepared by crystallizing synthetic amylose (2 g/L) with quinoline.

lamellar single crystals from each class of V-type structure for electron diffraction, and even larger and bulkier single crystals for X-ray crystallography.

2) collect good 3D diffraction datasets from single crystals. In particular, imaging plates were extremely helpful as their high sensitivity and linearity allowed recording electron diffraction patterns under very low radiation doses and measuring undistorted diffracted intensities. In addition, thanks to the recent progress in producing micron- and sub-micron-sized synchrotron radiation beams, X-ray crystallography on small polymer crystals can be envisaged. Such an experiment was recently carried out on the ID13 beamline at ESRF and a diffraction dataset was successfully collected from 10 μm-long needle-like single crystals of double helical A-amylose, at 100 K, using a 10 μm beam.[24]
3) carry out detailed molecular modeling to determine amylose helicity and packing as well as the exact location of guest molecules inside and/or in-between helices. In particular, it is of major importance to validate the existence of 6- or 7-fold single helices in $V_{butanol}$, $V_{isopropanol}$ and $V_{glycerol}$ crystals. The quantitative analysis of electron diffraction should provide reliable data to refine molecular models.

Finally, another exciting prospect is to use cycloamylose molecules constituted by a number of glucose units significantly larger than that of cyclodextrins.[25] Using X-ray crystallography, it has recently been shown that macrocyles containing 26 glucose residues could fold into two antiparallel V-amylose-like single helices[26] and complex undecanoic acid and dodecanol.[27] If some of the reagents used in our studies can be successfully complexed with large cycloamylose molecules, it may then be possible to grow near mm-sized single crystals suitable for X-ray microdiffraction and refine the crystal structure with an improved resolution.

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