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Single crystals of V amylose complexed with isopropanol and acetone

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Single crystals of amylose complexed with isopropanol or acetone were prepared by adding these precipitants to a metastable aqueous solution of amylose. With both precipitants, similar micrometre sized platelet crystals were obtained. They gave indistinguishable electron diffraction diagrams which could be indexed in an orthorhombic unit cell, with $a = 28.26 \text{ \AA}$, $b = 29.30 \text{ \AA}$, $c = 8.01 \text{ \AA}$ and in a space group $P2_12_12_1$ or $P2_12_12$. Within the unit cell, the amylose chains are organized in antiparallel pairs of parallel 6_1 amylose helices occupying 70% of the cell content, the remaining 30% consisting of isopropanol/acetone and water, with an estimate of 10 isopropanol/acetone molecules for 52 water molecules per unit cell. If the crystals are suspended in pure isopropanol at various temperatures or in pure methanol at room temperature, they undergo a de-solvation process that ultimately converts them into V_H amylose. De-solvation with isopropanol left the crystals intact whereas with methanol, they became cracked during the shrinkage. An explanation is proposed for such difference.

Keywords: Amylose; single crystals; electron diffraction; crystalline complex

Introduction

The crystalline polymorphism of starch and that of its linear constituent, amylose, is usually taken as one of the best examples to illustrate the different ways in which a given polysaccharide can crystallize depending upon the experimental conditions. This versatility is well demonstrated when one studies the crystallization of amylose from an aqueous solution to which various amounts of a precipitant are added¹. If only water is present, amylose crystallizes as double helices in the highly hydrated B allomorph^{2,3}. The addition of a small percentage of a precipitant, e.g. ethanol or acetone, to the solution leads to the crystallization of the A allomorph where the amylose chains are still crystallized as double helices but in a different arrangement. In that case, they accommodate only a small amount of water of crystallization^{1,3}. If then a larger amount of precipitant is added, amylose will no longer crystallize in double helices but will adopt a single helical conformation. One then obtains the crystalline V_H (V_{Hydrate}) amylose which leads to the V_A ($V_{\text{Anhydrous}}$) upon dehydration^{4,5}.

Following several studies including recent ones, substantially detailed information has been gained concerning the molecular structure of A and B amylose crystals⁶⁻⁹. In the case of V amylose, the knowledge is less precise. V amylose is a generic name that encompasses a wealth of crystalline amyloses where single helices are co-crystallized with inclusion substances such as water,

iodine, DMSO, fatty acids, alcohols, etc. Several V amylose structures have been carefully determined by X-ray analysis on fibre diagrams. For these structures, namely V_H , V_A , V_{DMSO} , V_{iodine} , the chain conformation was precisely established¹⁰. It consists in a left-handed six residue (6_1) helix with a rise per monomer h comprised between 1.32 and 1.36 Å. In projection perpendicular to the chain axis, such V structures of amylose are very reminiscent of those found for the six members α cyclodextrin for which a wealth of high resolution diffraction data are available. With other V amylose structures, and in particular where only fragmental diffraction information is available, several authors have proposed the existence of 7_1 ¹¹⁻¹⁵ and 8_1 helices¹⁶⁻¹⁸, which would mimic the structures of the 7 and 8 members β and α cyclodextrins. To date however, it seems that the 7_1 and 8_1 V amylose helices need to be confirmed and in particular to be established with the same precision and certainty as the well documented 6_1 case.

Many crystalline V amyloses and in particular those where the 7_1 and 8_1 conformations have been proposed are not easily amenable to a good X-ray analysis due to the difficulty in preparing crystalline fibres of these complexes. On the other hand, for several of them, it is possible to prepare single crystals susceptible to yield electron diffraction diagrams of high quality provided that one can preserve the volatile complexing agents within the vacuum of the electron microscope. As we have successfully mastered the recording of electron diffraction of such solvated biopolymer crystals^{19,20}, this work was initiated to obtain more diffraction data on the crystalline complex of amylose with isopropanol. This complex was

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Figure 1 Typical preparation of single crystals of V amylose complexed with isopropanol, shadowed with W/Ta

Figure 2 X-ray powder diagram recorded on a preparation of single crystals of V amylose complexed with isopropanol. The diagram was obtained from crystals still moist from their mother liquor and sealed in a capillary

selected since it has been tentatively assigned to the 7_1 amylose family^{12,13}. However, upon drying, the crystals were shown to yield a typical V_H electron diffractogram indicative of the 6_5 amylose helix¹². Such drying behaviour, if it can be confirmed would be remarkable for a crystalline polysaccharide. So far, in most cases studied in detail (dextran^{21,22}, mannan^{23,24}, amylose^{4,5}, etc.), the conformation of the polymer was found to be identical or almost identical in the solvated (or hydrated) and de-solvated (or dried) form.

The goal of this study was thus to clarify the case of V amylose-isopropanol complex by recording diffraction

Table 1 Indexation and observed intensities of a powder X-ray diffraction pattern recorded on a preparation of single crystals of V amylose complexed with isopropanol

hkl	d_{obs}	I_{rel}
210	12.68	30
220	10.15	15
310, 001	8.94	10
410, 121, 311, 330	6.81	80
141, 401, 520, 411, 331	5.27	15
530, 350, 060, 051, 600	4.87	50
260, 511, 450, 251, 620, 540	4.48	100
461, 271, 711, 641, 740	3.57	10

information on its solvated crystals and studying their de-solvation. As during this work it was discovered that acetone could be substituted for isopropanol in the crystallization solution to give crystals that looked and diffracted identically to those of isopropanol, the experiments were duplicated with both precipitants.

Experimental

Preparation of the amylose crystals complexed with acetone and isopropanol

Solutions of 0.1% (w/v) amylose were prepared by dissolving DP 100 amylose (amylose B from Nakarai Chemical Ltd) in 1.2N NaOH. The solutions were dialysed against distilled water until neutrality was reached. Some solutions were then brought to 60°C and a percentage of preheated isopropanol was added to obtain a crystallization mixture having a water-to-isopropanol ratio of 65/35 (v/v). Other solutions were mixed with acetone. In that case, the temperature was 50°C and the water-to-acetone ratio was 50/50 (v/v). In both cases, crystals started to grow immediately following the addition of the precipitant. The crystals which sedimented slowly were washed by successive decantation over a period of 2–3 days, always using fresh solutions containing the same proportion of solvent and

Figure 3 Low dose electron micrograph recorded under frozen wet conditions of a preparation of V amylose crystals complexed with isopropanol. *Inset:* electron diffractogram of the circled area, properly oriented with respect to the image

precipitant. Care was taken to maintain the temperature at 60°C for the crystals grown in isopropanol and 50°C for those grown in acetone. The last step of decantation was achieved by using fresh solutions containing 60% of precipitant in water (v/v). At this stage, the crystalline suspension, which consisted exclusively of platelet crystals, was stable enough to be cooled and stored at room temperature.

De-solvation of the crystals

The suspended crystals were subjected to several de-solvating treatments of increasing severity in order to remove part of the solvent from within the crystalline lattice. A first and rather mild treatment involved the transfer by centrifugation of the crystals into pure isopropanol and holding them under such conditions at room temperature for several days. In a more severe treatment, a suspension of these crystals in pure isopropanol was sealed into thick walled capillaries and positioned inside an autoclave which was brought to 160°C and maintained at this temperature for 6 h. The third and most drastic de-solvating treatment followed the method of Yamashita *et al.*¹² in which the initial crystals were centrifuged, resuspended into pure dry methanol in which they were stored.

Electron microscopy and electron diffraction experiments

The crystals were deposited on carbon coated grids. For imaging purpose, these grids were shadowed with W/Ta and the pictures recorded with a Philips EM 400T transmission electron microscope operated at 80 kV. Electron diffractograms were obtained using the quench freezing technique described elsewhere¹⁹. In that case, an accelerating voltage of 120 kV was selected and the diagrams were recorded on never observed crystals, while using minimum dose techniques. Calibration of the electron diffractograms was achieved either with gold or cubic ice standards.

The best diagrams were digitized using an Optronics rotating drum scanning microphotometer with a 25 μm aperture. The diffraction intensities were obtained after background noise removal from the digitized films using the EDFILMS program developed by D. P. Miller *et al.*²⁵

X-ray diffraction data

Suspension of the crystals in their mother liquor were centrifuged and their solvent removed. The crystals were then allowed to dry for a few minutes. When still moist, they were inserted into thin walled capillaries which were quickly sealed. X-ray powder diagrams were recorded using a vacuum flat film Wahrens X-ray camera mounted on a Siemens Kristalloflex generator operated with Cu Kα radiation.

Density measurements

The crystals were equilibrated by immersing them in toluene and slowly adding CCl₄. The density of the mixture was measured at the equilibration point.

Results

The crystals and their diffraction features

The crystals of V amylose prepared either with isopropanol or acetone had identical shapes. They consisted of a series of superimposed rectangular platelets radiating from a centre (*Figure 1*). Each platelet was around 2–3 μm long, 0.5 μm wide and about 100 Å thick. Preparations of these crystals were subjected to X-ray analysis. They gave sharp X-ray powder diagrams (*Figure 2* and *Table 1*) that differed markedly from other V-amylose X-ray diagrams. Both diagrams corresponding to acetone or isopropanol were indistinguishable.

When studied by electron diffraction under frozen wet conditions, an isolated platelet yielded a sharp spot diagram (*Figure 3*) that could be indexed along a regular net consisting of rectangular meshes. This diagram contains around 300 spots symmetrically distributed in 4

Figure 4 Series of electron diffraction diagrams recorded under frozen wet conditions on single crystals of V amylose complexed with acetone and rotated about a^* and b^* . Significant diagrams are obtained for rotations of 45° and 30° about b^* , 45° and 36° about a^* . In all cases, diagrams rotated clockwise are identical to those rotated anticlockwise

quadrants with symmetry axes a^* and b^* aligned along and perpendicular to the long side of the platelet. As along the a^* and b^* axes, only even order reflections are present, a p_{gg} symmetry is suggested for the diagram and therefore for the corresponding projection of the crystalline structure. This symmetry as well as the overall three-dimensional symmetry was confirmed by rotating a crystal about its long and short axes (Figure 4) and recording the corresponding diagrams. In the present case, two equivalent diagrams were systematically obtained when a crystal was successively rotated clockwise and anticlockwise by an angle of the same value. This indicates that the third reciprocal cell

parameter c^* is perpendicular to the a^*b^* plane. Therefore, the crystals belong to an orthorhombic space group either $P2_12_12_1$ or $P2_12_12$ (an ambiguity remains along c which is either a 2_1 or a 2 axis). Upon calibration of the tilted and untilted diagrams, the unit cell parameters can be determined as:

$$a = 28.26 \text{ \AA}, \quad b = 29.30 \text{ \AA}, \quad c = 8.01 \text{ \AA}, \\ \alpha = \beta = \gamma = 90^\circ$$

The $hk0$ electron diffraction diagrams of the V amylose isopropanol crystals were digitized (Figure 5) and the intensities of the various spots were computed (Table 2).

Figure 5 Schematic representation of a digitized electron diffractogram recorded from a crystal of V amylose complexed with acetone. A: $hk0$ level with contours of equal intensity before background removal. B: identical to A but in a pseudo 3D representation. C: $hk0$ level with contours of equal intensity after background removal. D: identical to C but in pseudo 3D representation

There were about 60 reflections of significant intensities in one quadrant.

De-solvation of the crystals

An interesting property of the crystals of V amylose-isopropanol or V amylose-acetone was their susceptibility to de-solvation which could be achieved in several ways. The de-solvation could be conducted in pure isopropanol or pure acetone. In both cases (Figure 6), the crystals maintained their shape and did not show any significant shrinkage or cracks during the experiment. In diffraction however, the effect of de-solvation was significant as both the symmetry of the diffraction diagrams as well as the cell parameters displayed substantial modifications. When, for instance, the crystals were left for several days in isopropanol at room temperature, a well resolved pseudo-hexagonal diffraction pattern (Figure 6C) was obtained. Its cell parameters (presented in Table 3 under the name of $V_{\text{intermediate}}$) indicate an overall shrinkage of the unit cell projection ab as a slight increase of a from 28.19 to 29.35 Å does not compensate the decrease in b that drops from 29.4 to 25.5 Å. Further de-solvation was achieved by autoclaving

the crystals in pure isopropanol at 160°C. At that temperature, the unit cell slowly shrunk (e.g. giving the intermediate diagram in Figure 6D) to yield the hexagonal V_H in the ultimate case (Figure 6E). In that case, the hexagonal V_H unit cell²⁶ was oriented with its a axis along the long axis of the platelet.

If the de-solvation is achieved in pure methanol, following the description of Yamashita *et al.*¹², the treatment was much more drastic as fully de-solvated crystals were readily obtained within hours, even at room temperature. In addition, the crystals displayed substantial shrinkage defects denoted by a series of cracks running parallel to the short dimension of the platelets (Figure 7). When analysing the diffraction diagram presented in Figure 7 and comparing it with the one shown in Figure 6E, one sees that both patterns are identical and correspond to V_H amylose. Their orientation with respect to the platelet is however different, as a 90° rotation is found between the pattern shown in Figure 6E and that in Figure 7. This indicates a substantial difference in the de-solvating mechanism when pure isopropanol is substituted by methanol for de-solvation. This must be correlated with the occurrence of cracks only in crystals treated with methanol.

Figure 6 A: Low dose electron micrograph of a single crystal of amylose complexed with isopropanol, observed under frozen wet conditions. B: Its electron diffraction diagram properly oriented with respect to the crystal and recorded also under frozen wet conditions. C: Typical diagram recorded after equilibrating the crystals for 1 week in pure isopropanol. The diagram, which was recorded under frozen wet conditions, is properly oriented with respect to the long axis of the crystal. This diagram is printed at the same camera length as that in Figure 6B. D and E: as in Figure 6C but after annealing the crystals for 6 h at 160°C in pure isopropanol. E was calibrated and identified as corresponding to V_H amylose. Its orientation indicates that the a axis of V_H amylose is along the long dimension of the crystal

Table 2 List of observed intensities after background removal obtained from an electron diffractogram of a single crystal of V amylose complexed with isopropanol

hkl	I_{obs}	hkl	I_{obs}
400	11	150	18
600	59	250	15
800	25	350	66
410	14	450	29
510	12	550	1
610	85	650	5
710	2	750	3
810	8	850	3
320	9	060	59
420	5	160	28
520	29	260	41
620	59	360	17
720	13	460	5
330	11	560	6
430	2	660	1
530	74	760	3
630	1	860	8
730	12	270	2
830	5	370	20
040	11	470	30
140	26	570	5
240	2	670	8
340	21	080	25
440	5	180	10
540	30	280	7
640	8	380	5
740	29	190	5
840	1	290	4
		390	2
		490	4
		590	2

Discussion

The results presented in this study are somewhat different from those reported by Yamashita *et al.*^{12,13} for their crystals of amylose-isopropanol complex. In particular, our results give the value of 8.01 Å for the fibre repeat of the complex. This value is slightly different from the 7.8 Å reported by these authors¹³. Our value results from the calibration of a series of electron diffraction patterns followed by a cell refinement on tilted and un-tilted crystals, whereas theirs was obtained from the measuring of pseudo fibre diagrams recorded by the X-ray analysis of crystalline mats. Our measurements which were refined from a series of spot patterns are basically more accurate than those reported in the X-ray analysis on strongly arced diagrams¹³. For this reason, we favour our value of

Table 3 Lattice parameters for the various crystalline V amylose complexes discussed in this study

	a (Å)	b (Å)	c (Å)	Unit cell volume (Å ³)
$V_{\text{isopropanol/acetone}}$	28.26	29.32	8.01	6636
$V_{\text{intermediate}}^a$	29.35	25.51	8.03	6012
V_{H}^b	27.30	23.70	8.05	5208
V_{A}^c	25.94	22.46	7.91	4608

^a The c value of the $V_{\text{intermediate}}$ was not measured but taken as an average between that of $V_{\text{isopropanol}}$ and V_{H} .

^b The parameters correspond to a 4 chains orthorhombic unit cell deduced from that in Refs 4, 26–28. The unit cell is oriented with respect to that of $V_{\text{isopropanol/acetone}}$ as in Figures 6 and 8A.

^c The parameters correspond to a 4-chain orthorhombic unit cell deduced from that in Ref. 5.

Figure 7 Low dose electron micrograph recorded under frozen wet conditions on a preparation of V amylose crystals complexed with isopropanol and suspended in pure methanol for a few hours at room temperature. Note the number of cracks perpendicular to the long edge of the crystal. Inset: electron diffractogram with proper orientation of the circled area. The diagram was identified and calibrated as corresponding to V_{H} amylose. Its orientation indicates that the a axis of the hexagonal²⁶ V_{H} amylose is perpendicular to the long axis of the circled crystal.

Figure 8 Schematic representation of the de-solvation of crystals of V amylose complexed with acetone or isopropanol. A illustrates a schematized crystal that was first suspended in pure isopropanol at room temperature for a few days and then annealed in isopropanol at 160°C for 6 h. *Inset* shows the packing of the amylose helices in the de-solvated crystal. B: shows that de-solvation which led to a substantial cracking of the crystals was obtained by suspending the crystals in pure methanol at room temperature for a few hours. *Inset* shows the packing of the amylose helices in the de-solvated crystal

8.01 Å for the fibre repeat of the complex. Such value matches closely that of the fibre repeat found frequently for V amylose complexes such as V_H amylose, $V_{H \text{ iodine}}$, $V_{H \text{ butanol}}$, etc. In all these cases, the 6_5 conformation is well established for amylose¹⁰. Therefore, in the present amylose complexes, it is likely that the amylose chains are also in the 6_5 and not in the 7_1 conformation. This hypothesis seems a logical concept since the $V_{\text{isopropanol/acetone}}$ crystals convert readily into the 6_5 V_H amylose. In the present case, it is indeed expected that amylose should behave as most crystalline polysaccharides studied so far in their solvated and de-solvated states: the de-solvation or drying is usually accompanied only by a shrinkage of the unit cell parameters that are perpendicular to the chain axis while this parameter and consequently the chain conformation remain unperturbed.

If the 6_5 conformation is accepted for the amylose chains in the isopropanol/acetone complex of density 1.42, the unit cell must contain 4 amylose chains. Within the unit cell, and according to the crystal's symmetry, the amylose chains are organized in antiparallel pairs of parallel 6_5 amylose helices. These 4 chains correspond to roughly 70% of the cell weight, the remaining 30% consisting of water and isopropanol/acetone. Some idea concerning the composition of the solvent within the crystals may be deduced from the de-solvation study and its concurrent unit cell shrinkage.

When the crystals are equilibrated in pure isopropanol, at room temperature, it is likely that only exchangeable water is removed from the cell content. As deduced from the volumes displayed in Table 3, this is accompanied by a cell shrinkage of 623 Å³, which must correspond roughly to the departure of 20 water molecules (a water molecule of density 1 occupies a volume of 30 Å³).

In the next step, consisting of autoclaving the crystals at 160°C for 6 h in pure isopropanol, one goes to V_H amylose where the only remaining solvent is water^{4,16}. Therefore, this step must correspond essentially to a departure of isopropanol/acetone. It is accompanied by a shrinkage of 800 Å³, which corresponds approximately to the departure of 10 isopropanol/acetone molecules per unit cell, assuming a density of around 1 for crystalline isopropanol/acetone. Since V_H amylose contains 8 water molecules per amylose chain^{4,16}, i.e. 32 for a 4-chain unit cell, one may conclude that the fully solvated crystalline complex contained 32 + 20 = 52 water and 10 isopropanol/acetone molecules for 4 amylose chains (13 water and 2.5 isopropanol/acetone per chain). This leads to a calculated density for the fully solvated crystal of 1.32 which is not too far from the observed value of 1.42 g/cm³.

The two modes of de-solvation for the crystals and in particular the occurrence of cracks in one case and not in the other, must be correlated with the change in packing of the amylose chains when one goes from the fully

solvated to the de-solvated state. In the fully solvated case, the amylose helices are separated by labile solvent layers, whereas in the de-solvated one, the helices come in close contact to give a close packed hexagonal arrangement, with the amylose helices 13.65 Å apart. One can represent the V_H close packed arrangement with an orthorhombic 4-chain unit cell with $a=27.30$ Å, $b=23.70$ Å, $c=8.03$ Å. When orienting this cell with respect to a platelet (Figure 8), there are basically two ways in which the unit cell can be positioned, namely with its a axis parallel (Figure 8A) or perpendicular (Figure 8B) to the long axis of the rectangular platelet. In the former case, the cell parameter a along the platelet axis has shrunk by only 1 Å in going from the fully solvated complex to V_H amylose. In the latter case, this shrinkage is much more severe, as it reaches 4.5 Å per unit cell. This dramatic difference, borne by the full length of the platelet induces overwhelming strains, hence the observed cracking perpendicular to the platelet axis, seen in Figure 7 and schematically drawn in Figure 8B.

One may wonder why the crystals did not present any cracking parallel to the platelet axis in the case of Figure 6 since in this case the b unit cell is shrinking by 5.6 Å during the de-solvation process. Such absence must be correlated with the fact that the crystals dimensions are only of 0.5 µm along b . This corresponds to less than 200 unit cells. Obviously the overall de-solvation strains, distributed along such a small number of cells, are not enough to bring the crystals beyond their yielding point.

Several questions remain concerning the present amylose isopropanol/acetone complex. The most interesting is to know exactly the role of isopropanol or acetone during the complex formation. Here, since the complex could be de-solvated to V_H amylose where only water is believed to be present within the helix cavity^{4,26,27}, we have assumed that the alcohol or acetone was not inserted within the helix cavity. However, such insertion may be present and until the crystal structure determination of the complex is solved to a good resolution, the question will remain unanswered. This structure determination involves the positioning of two antiparallel pairs of parallel amylose helices, together with that of more than 60 molecules of solvent within the unit cell. It is presently under way by using at the same time classic polymer crystallography techniques applied to single crystals, together with high resolution electron microscopy, using lattice imaging of the crystals observed under frozen wet conditions.

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