# Single crystals of amylose with a low degree of polymerization

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# Single Crystals of Amylose with a Low Degree of Polymerization

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*SUMMARY*

*Single crystals of amylose with a low degree of polymerization were prepared from dilute solution in water or mixtures of water and ethanol. Depending on the concentration of ethanol used, three different polymorphs resulted. From pure water amylose B was obtained and, respectively, from 15% (v/v) ethanol, amylose A, and from 40% (v/v) ethanol, V amylose. The crystals were studied by electron diffraction after quench-freezing and the crystallographic parameters were compared with those already reported in the literature.*

## INTRODUCTION

In reviewing the structure and morphology of crystalline polysaccharides, the frequent occurrence of polymorphism for any given substance is striking. This is particularly the case with amylose, where fibre

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diffraction analysis has revealed three different polymorphs called A, B and V amylose (Katz & Van Itallie, 1930; Sarko, 1974; French & Murphy, 1977; Duprat *et al.*, 1980; Sarko and Zugenmaier, 1980).

The A and B polymorphs are commonly found in native starches; the A polymorph occurs frequently in cereal starches while the B polymorph is largely found in tuber starches. A C polymorph intermediate between A and B is also found, typically in bean or root starches. The V polymorph is seldom encountered in nature (Senti, 1967). On the other hand, it is often found in recrystallized amyloses where it encompasses a family of crystalline structures which are characterized by helical amylose chains complexed with small molecules such as water, iodine, alcohol or DMSO (Bear, 1942; Rundle & Edwards, 1943, Rundle, 1947; Zobel *et al.*, 1967).

Each of the polymorphs of amylose has been examined with a view to determining the three-dimensional structure using X-ray fibre-diffraction data. It is currently believed that the V crystals are composed of single helical amylose chains (Winter & Sarko, 1974; Rappenecker & Zugenmaier, 1981). For the A and B crystals, a double helical chain structure has been proposed (Wu & Sarko, 1978*a*, *b*; Sarko & Zugenmaier, 1980), but this has been questioned by other authors who favour a single amylose helix for these two polymorphs (Brant, 1976; Cleven *et al.*, 1978; Van den Berg, 1981). A further aspect of the crystallization behaviour of amylose is the formation of single crystals that are suitable for electron microscopy from dilute solution. Until now this behaviour has only been observed for V amylose but has nevertheless led to an important series of morphological and crystallographic observations for amylose and its complexes (Saint John Manley, 1964; Yamashita, 1965; Yamashita & Hirai, 1966; Bittiger & Husemann, 1969; Yamashita & Monobe, 1971; Yamashita *et al.*, 1973; Booy *et al.*, 1979).

Amylose can be crystallized from solution in the A and B forms but until now, in spite of numerous trials in different laboratories, it has not proved possible to obtain single crystals of the A and B polymorphs. A crystallographic and morphological study of such crystals should prove invaluable in furthering the detailed knowledge of the structures of the A and B forms and their implications for the morphology and structure of starch. In fact the mechanism for the enzymic degradation of starch, and for its gelatinization and lintnerization

would be ideally studied with such single crystals by electron microscopy and other ultrastructural techniques.

This paper describes an approach to preparing and characterizing single crystals of the A and B polymorphs of amylose. Under certain conditions V amylose crystals were also obtained and these results are included. Following the general crystallization techniques developed in our laboratories, experiments were performed using amylose with a low degree of polymerization which was obtained either by fractionation or by biosynthesis *in vitro* (Pfannemuller, 1968).

## EXPERIMENTAL

### Preparation of single crystals of amylose A

Amylose with a $\overline{DP}$ of 15 was obtained by mild hydrolysis of potato starch as described by Duprat *et al.* (1980). A suspension of this sample was dispersed in water ($\leqslant 1\%$ w/v) and heated inside a small autoclave at 130°C for 30 min, after which the amylose had dissolved. The autoclave was allowed to cool and the solution was filtered. Pure ethanol (15 ml) was then added to the clear filtrate (85 ml) and the mixture was maintained at 60°C while crystallization occurred in the form of a colloidal precipitate. This was collected by centrifugation and washed repeatedly with a mixture of water and ethanol (85/15 v/v) by successive centrifugation. Drops of the crystals in suspension were then mounted conventionally on carbon-coated grids for electron microscopy.

### Preparation of single crystals of amylose B

Amylose with a $\overline{DP}$ of 35 obtained by biosynthesis *in vitro* was kindly supplied by Dr B. Pfannemuller. Batches of a dispersed suspension (5 ml, 0·05% w/v) of this amylose were heated to 100°C where almost total solution occurred. These were then filtered into thick-walled glass ampoules which were sealed and maintained at 160°C for 15 min to erase any memory of previous crystal forms. The ampoules were then cooled to 4°C and maintained at this temperature for periods ranging from 6 months to 2 years. The amylose crystallized slowly in the form

of a rather coarse precipitate. The ampoules were then heated slowly to 117°C and left at this temperature for 10 min. Subsequently they were cooled to room temperature and recrystallization occurred in 4–5 days. The ampoules were then opened and the precipitate was washed repeatedly by centrifugation and mounted on carbon-coated grids, as before, for electron microscopy.

### Preparation of single crystals of V amylose

Amylose with a $\overline{DP}$ of 35 was prepared by mild hydrolysis of wrinkled-pea starch using the method of Colonna *et al.* (1982) and 10 mg of this were dissolved in 10 ml of 4% (w/v) NaOH, filtered, and dialysed at room temperature against a mixture of water and ethanol (60/40 v/v). After 1 day a flocculent crystalline precipitate was present which was centrifuged and repeatedly washed in a 60/40 v/v mixture of water and ethanol. The crystals were then mounted as previously described for electron microscopy.

### Electron microscopy

Transmission electron microscopy was carried out with a Philips EM 400 T electron microscope. For imaging, the prepared grids were first shadowed with a tungsten–tantalum alloy using a Balzers electron gun; micrographs were recorded at 80 kV. For diffraction, the prepared grids were first positioned for 24 h inside a desiccator where a relative humidity of 95% was maintained by a large excess of saturated zinc sulphate solution. Grids were then rapidly mounted in the specimen cooling holder, quench-frozen in liquid nitrogen and inserted into the microscope and examined in the frozen, hydrated state (Booy *et al.*, 1979). Electron diffraction patterns were recorded at 120 kV using minimal exposure techniques. For calibration purposes, some crystals were also examined supported on a gold-coated carbon film.

### X-Ray diffraction

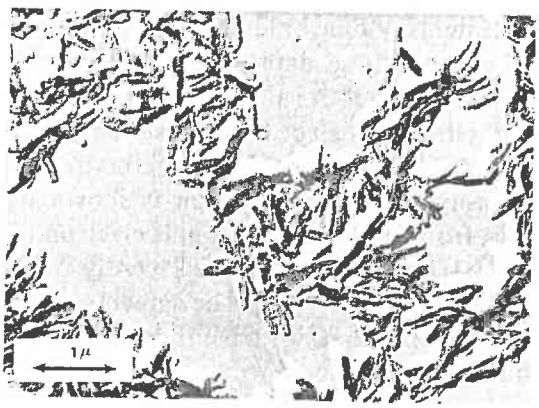
X-ray powder diffraction was carried out with a CGR X-ray generator operating at 45 kV and 30 mA equipped with a Guinier monochromator and a scintillation counter. When only a very small sample was

available either a holder specially designed for examining micro-samples with the counter outfit or a flat-film camera was used.

## RESULTS

### A Amylose crystals

Crystals of A amylose grow in a rosette-like fashion with an individual rectangular platelet crystal forming a branch of the rosette. When the rosette-like structures are disturbed, the platelet crystals are loosened and can be examined by transmission electron microscopy. Figure 1(A)




A


B C D

Fig. 1. (A) Transmission electron micrograph of A amylose crystals shadowed with tungsten-tantalum. (B–D) Typical electron diffraction patterns obtained from individual platelet crystals as in (A) but unshadowed and examined in the frozen hydrated state.

shows a preparation from the shaken crystals where quite thick platelets with lateral dimensions of 0·3 × 0·3 nm can be seen. The remarkable homogeneity of the crystals probably results from the low polydispersity of the $\overline{DP}$ 15 amylose used.

Electron diffraction data are only obtained after quench-freezing and examination of the crystals in the frozen, hydrated state when sharp spots result. However, due to the random orientation of the crystals on the supporting film, a variety of diffraction patterns is recorded. This is illustrated in Figs 1(B)–(D) where the patterns most frequently obtained are shown. At first sight, these patterns seem uncorrelated as they originate from tilted crystals. To clarify this it is necessary to tilt and rotate each crystal, sequentially recording the electron diffraction patterns (Chanzy *et al.*, 1981). Unfortunately, because our specimen cooling holder has no rotation facility, we are not yet able to exploit these data and thus can neither establish the point group nor the unit cell. As an alternative, powder diffraction data can be obtained either by electron diffraction (again in the frozen, hydrated state), or by conventional X-ray methods.

The trace of a typical X-ray diagram is shown in Fig. 2 and the principal spacings from both the X-ray and electron diffraction studies are listed in Table 1 together with some recently published X-ray data for A amylose (Wu & Sarko, 1978*a*). The agreement between the data is quite good and confirms that we have indeed obtained single crystals of the A polymorph of amylose. We are unfortunately unable to confirm the symmetry and unit cell parameters that Wu & Sarko derived because of the limitations imposed by our specimen cooling holder.

## B Amylose crystals

A typical sample of the B amylose preparation is shown in Fig. 3. It consists of a lamellar precipitate without any clearly defined geometrical shape. Electron diffraction from this sample in the frozen hydrated state results in sharp hexagonal diffraction patterns as shown in the inset in Fig. 3. The pattern contains seven independent reflections which may be indexed as the (hk0) plane of a hexagonal system with $a = b = 1{\cdot}8$ nm and $\gamma = 120°$.

Table 2 compares the data from the pattern shown in the inset in Fig. 3 with the data for B amylose obtained from X-ray fibre-diffrac-

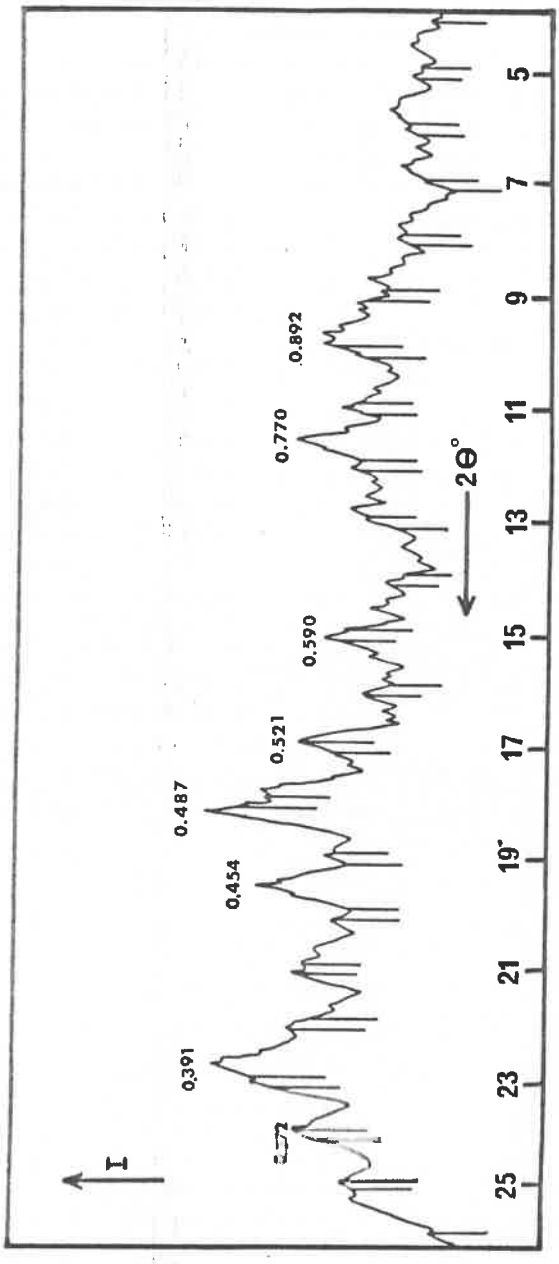



Fig. 2. X-ray diffractometer trace from a powder sample of A amylose crystals.

**TABLE 1**
A Amylose Crystals

| *X-ray powder diagram (this study)* | | *Electron diffraction powder diagram (this study) d spacings in nm* | *X-ray analysis (Wu & Sarko, 1978a)* | | |
|---|---|---|---|---|---|
| *d spacings in nm* | *Intensity*[a] | | *d spacings in nm* | *Miller indices (hkl)* | *Intensity*[a] |
| 0·892 | M | | 0·888 | 020 | M |
| | | | 0·786 | 101 | M |
| 0·770 | M | | | | |
| | | 0·680[b] | | | |
| 0·590 | M | 0·590 | 0·589 | 030 | S |
| 0·521 | M | 0·525 | 0·520 | 201 | S |
| 0·487 | S | | 0·487 | 220 (211) | S |
| 0·454 | M | 0·446 | 0·446 | 040 | W |
| 0·391 | S | 0·386 | 0·389 | 231 | S |
| 0·372 | M | 0·372 | 0·372 | 310 (132) | S |

[a] S = strong; M = medium; W = weak.
[b] Probably originates from traces of V amylose.

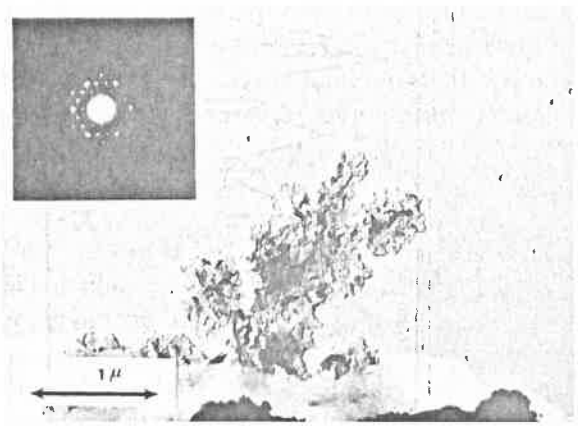



**Fig. 3.** Transmission electron micrograph of B amylose crystals shadowed with tungsten-tantalum; in the upper left corner typical electron diffraction pattern obtained from fragments of crystals unshadowed and examined in the frozen hydrated state.

**TABLE 2**
B Amylose Crystals

| *Electron diffraction diagram (this work)* | | *X-ray analysis (Wu & Sarko, 1978b)* | | |
|---|---|---|---|---|
| *d spacings in nm* | *Relative intensity*[a] | *d spacings in nm* | *Relative intensity*[a] | *Miller indices (hkl)* |
| 1·560 | M | 1·614 | M | 100 |
| 0·780 | M | 0·797 | M | 200 |
| 0·589 | M | 0·600 | M | 120 |
| 0·520 | S | 0·534 | S | 300 |
| 0·447 | M | 0·459 | S | 220 |
| 0·337 | M | 0·347 | M | 140 |
| 0·263 | M | 0·269 | M | 600 |

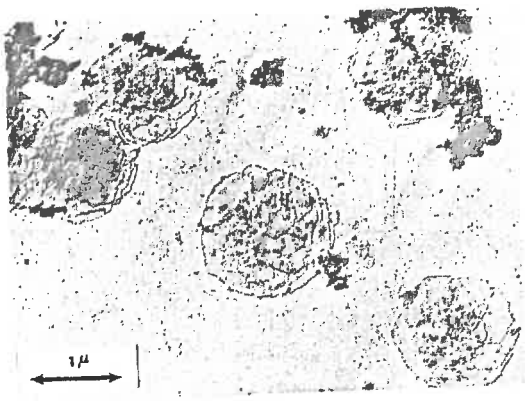
[a] S = strong; M = medium.

tion by Wu & Sarko (1978*b*). The agreement between the intensities and the *d*-spacings is quite good, although the unit cell size in this study is $a = b = 1{\cdot}8$ nm compared with 1·85 nm in Wu & Sarko's study. This small difference probably results from the fact that in our study the crystals may not have been fully hydrated in spite of the freezing technique employed. However, we can conclude with certainty that the crystals presented in Fig. 3 are indeed of the B polymorph of amylose.

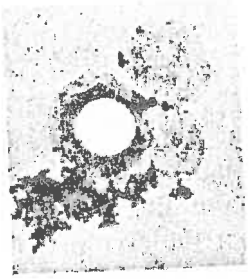
### V Amylose crystals

As shown in Fig. 4(A), the V amylose crystals prepared here have a uniform and clear morphology and consist of individual platelets with lateral dimensions of about 1 μm that thicken by the formation of multiple screw dislocations.

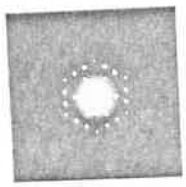
In the frozen hydrated state each crystal gives a well-defined diffraction pattern. Figure 4(C) is an example emanating from the region marked by a circle in Fig. 4(B). The pattern again has hexagonal symmetry and may be indexed with $a = b = 1{\cdot}37$ nm; it may also be indexed in terms of the larger orthorhombic unit cell that is normally considered for V amylose with $a = 1{\cdot}37$ nm, $b = 2{\cdot}37$ nm and $\gamma = 90°$.




A



B



C

**Fig. 4.**. (A) Transmission electron micrograph of V amylose crystals shadowed with tungsten-tantalum. (B) Transmission electron micrograph of a cluster of unshadowed V amylose crystals in a frozen hydrated state. (C) Electron diffraction pattern (correctly oriented) recorded from the circled area of Fig. 4(B).

The results are listed in Table 3 and compared with the X-ray data of Zobel *et al.* (1967). The agreement is excellent and demonstrates that the crystals presented here are of the $V_H$ type that is already well documented in the literature.

## DISCUSSION

The results presented here illustrate clearly the diverse nature of amylose in crystallizing from dilute solution. By merely modifying the

**TABLE 3**
V Amylose Crystals

| *Electron diffraction diagram (this work)* | | *X-ray analysis (Zobel et al., 1967)* | | |
|---|---|---|---|---|
| *d spacings in nm* | *Relative intensity*[a] | *d spacings in nm* | *Relative intensity*[a] | *Miller indices (hkl)* |
| 1·181 | M | 1·185 | S | 110 |
| 0·684 | S | 0·683 | VS | 200 |
| 0·446 | S | 0·448 | VS | 240 |
| 0·392 | M | 0·393 | M | 060 |
| 0·326 | M | 0·326 | W | 420 |

[a] VS = very strong; S = strong; M = medium; W = weak.

proportions of water and ethanol in the mother liquor one of three different crystalline polymorphs may be obtained. From pure water the B polymorph is obtained, while with only a small proportion of ethanol, the A polymorph results. When the proportion of ethanol is increased to roughly 50% the V polymorph is obtained.

Even though water and ethanol are listed as poor solvents or even precipitants for amylose, small changes in their relative concentrations must induce modifications in the conformation adopted by the amylose molecules in solution. This is witnessed by the three different types of crystal which can be grown when the alcohol percentage of the crystallization solution is increased. A substantial amount of ethanol stabilizes single helices of amylose which crystallize accordingly in the mono-helical V form. On the other hand, with solutions where water is either the dominant or the only solvent the two conformations leading to A and B amylose are stabilized. In these two cases our present data cannot confirm the occurrence or the absence of a pairing of the amylose chains in solution leading to the eventual formation of double helices. The evidence of such pairing, which could be investigated by classical solution techniques (such as NMR and viscometry), is important to gain an understanding of the structure of native starch granules which occur in either the A or the B form depending on their origin. In the light of the data presented here it would also be most interesting to compare the conditions of biosynthesis to see whether

native polymorphism is also due to compositional changes in the biocrystallization media during starch biosynthesis.

Of the three polymorphs, only the V amylose crystals behave as typical chain folded polymer single crystals. They can be prepared with any molecular weight amylose and always yield the same type of crystal – 10 nm thick hexagonal platelets where the planar faces have grown perpendicular to the helical amylose chain axis. Similar V amylose crystals have already been described by Yamashita *et al.* (1973), but the use of our freezing technique to obtain electron diffraction data enables us to assign them unambiguously to the $V_H$ form of amylose. This implies that no ethanol is involved in the crystal structure and thus the hexagonal structure (in projection) is stabilized only by water molecules. With other alcohols such as *n*-butanol or *iso*-propanol it is probable that alcohol molecules are incorporated in the crystal lattice (Booy *et al.*, 1979), and this leads to different crystal morphologies (Yamashita *et al.*, 1973).

The preparation and characterization of single crystals of A and B amylose are presented here for the first time. Previous attempts to obtain B amylose crystals had given only a polycrystalline precipitate (Pfannemuller & Bauercarnap, 1977). Our success is certainly due to the use of fractions of amylose with a low degree of polymerization. In that case, the crystals are made of short molecular stems and the crystallization does not require a chain-folding mechanism.

Both the A and B polymorphs are found in native starch granules. Until now, the native granules have been studied only by X-ray diffraction and, with one exception (Kreger, 1951), only by powder methods. The present work shows that with the electron microscope oriented diffraction data may be obtained from micron-sized crystals of amylose. A logical extension of this work is to investigate native starch granules by electron diffraction using ultra-thin sections or fragmented starch granules. Such work is currently in progress in our laboratories.

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