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Hydrolysis of A- and B-type crystalline polymorphs of starch by α-amylase, β-amylase and glucoamylase 1

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The hydrolysis of A-type and B-type spherulitic polycrystalline amylose (degree of polymerisation ~ 20) by glucoamylase 1 (*Aspergillus niger*), by β-amylase (sweet potato) and by α-amylase (*Aspergillus oryzae*) has been examined by differential scanning calorimetry, X-ray powder diffraction and microscopy (polarised light and scanning electron). B-type structure is hydrolysed more slowly than A-type by all enzymes probably due to surface area effects. A-type shows no discernible spherulitic structure after partial hydrolysis, whereas the B-type spherulites retain a large amount of visible spherical structure. Glucoamylase 1-treated B-type crystals retain their positive birefringence, appear smooth, exhibit a 40% decrease in melting energy and retain crystallinity. On the other hand, α- and β-amylase treatment of B-type spherulites results in spherulites that are not birefringent, show extensive small pits (β-amylase) or several very large pits (α-amylase), exhibit an 83% (β-amylase) and 87% (α-amylase) decrease in melting energy and lose a substantial amount of crystallinity. A-type crystals show no change in melting energy of the solid material after partial hydrolysis by any enzyme, and fully retain crystallinity of the remaining material. The results demonstrate that the pattern of enzyme attack depends on the crystal type, and that each enzyme has a specific mode of attack on B-type amylose even in chemically homogeneous spherulites of a single crystalline type.

NOTATION

DP Degree of polymerisation
DSC Differential scanning calorimetry
T_m Melting temperature

1 INTRODUCTION

The rate of hydrolysis of starch granules depends strongly on the botanical source (Gallant *et al.*, 1972; Fuwa *et al.*, 1980; Cone & Wolters, 1990). For example, rice or wheat starch is degraded over 6 times more rapidly than banana starch, and over 20 times faster than potato starch, by pig pancreatic α-amylase (EC 3.2.1.1., α-1,4-glucan-4-glucanohydrolase) (Fuwa *et al.*, 1980). In general, cereal starches are much more readily hydrolysed by α-amylase than are potato starches, which is often attributed to a higher content of crystalline structure in potato starch (Gallant *et al.*, 1972) and to granule size (Ring *et al.*, 1988). In addition, α-amylase is thought to initially hydrolyse amorphous regions of the starch granule (Marsden & Gray, 1986; Franco *et al.*, 1988). On the basis of this information, hydrolysis by α-amylase is often used to determine the physical structure of starch granules (Gallant *et al.*, 1972; Manners, 1989).

Glucoamylase 1 (EC 3.2.1.3, 1,4-α-D-glucan glucohydrolase) from *Aspergillus niger* (Svensson *et al.*, 1983) and β-amylase (EC 3.2.1.2, 1,4-α-D-glucan maltohydrolase) from sweet potato are both *exo*-acting enzymes, unlike α-amylase. Glucoamylase 1 produces glucose from starch and is able to hydrolyse α1,6 and α1,3 in addition to α1,4 linkages. β-Amylase, on the other hand, produces maltose from starch, and is only able to hydrolyse α1,4 linkages. The action of α-amylase,

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glucoamylase 1 and β-amylase on native starch granules is therefore strongly affected by the granule structure. In particular, the number of α1,6 branch points (i.e. amylose/amylopectin ratio and the type of amylodextrin), the type and arrangement of crystal structures, the average molecular weights of the components and the existence of other material (lipid, protein) will also play a role in determining the rate of hydrolysis by a given enzyme. All of these factors make it difficult to define if granule hydrolysis is due to enzyme specificity, binding affinity, molecular and physical structure of the granule, or to a combination of factors. In order to increase the understanding of the action of amylases on granular starch, A- and B-type spherulitic polycrystalline amylose were prepared with an average DP of 20 (Ring *et al.*, 1987) and we examined and compared the hydrolysis by α-amylase, β-amylase and glucoamylase 1. The results show that each enzyme has a characteristic degradation pattern even though there is no chemical variability of the starch component and there is only one predominant crystal type.

2 EXPERIMENTAL

2.1 Enzymes

Glucoamylase 1 from *Aspergillus niger* was purified by affinity chromatography on granular starch (Subbaramaiah & Sharma, 1988) from a crude enzyme preparation (6100 U/ml). Crude Taka-amylase (α-amylase from *Aspergillus oryzae*) was precipitated twice with 80% $(NH_4)_2SO_4$, dialysed against 0·05 M tris/Cl pH 7·5 and then purified on a DEAE-Sepharose ion-exchange column (Fukada *et al.*, 1987). β-Amylase (type 1-B) from sweet potato (980 U/mg) was not purified further. All enzymes were purchased from Sigma Chem. Co. (Poole, Dorset, UK).

The enzymes were all >95% homogeneous as determined by sodium dodecylsulphate-polyacrylamide gel electrophoresis on a 10% acrylamide gel (Laemmli, 1970). Contaminating α-amylase was measured using the amylase detection kit from Randox Labs Ltd (Crumlin, Co. Antrim, UK). Glucoamylase 1 contained less than 0·006% of α-amylase activity. β-Amylase contained no detectable α-amylase activity. Protein concentration was measured using the Coomassie Blue reagent from Pierce Europe BV (Chester, UK). The specific activities on potato starch amylose in solution (1%, w/v) at 25 °C were: glucoamylase 1, 15·6 U mg^{-1}; α-amylase, 106 U mg^{-1}; β-amylase, 356 U mg^{-1}. One unit (U) is the amount of enzyme required to yield the equivalent of 1 μmol of product per minute under the conditions specified. The buffers used were: glucoamylase 1, 5mM sodium acetate, pH 3·6; β-amylase, 5mM sodium malate, pH 6·1; Taka-amylase, 5mM sodium malate, pH 6·9.

2.2 Starch

Maltooligosaccharides (average DP = 20) were produced by lintnerisation of potato starch (Robin *et al.*, 1974). A- and B-type spherulities were crystallised from 30% ethanol and H_2O respectively (Ring *et al.*, 1987). Both crystal types were dialysed against NaN_3 (0·02%, w/v), repeatedly dialysed against water and used immediately.

2.3 Enzyme assays

The extent of hydrolysis by glucoamylase 1 was measured by a glucose oxidase/peroxidase linked assay (Lloyd & Whelan, 1969). Hydrolysis by α- and β-amylase was measured by estimation of reducing groups using dinitrosalicylic acid (Rick & Stegbauer, 1974). In 1·5 ml Eppendorf tubes, enzyme and starch were gently mixed by rotation in a thermostatically controlled incubator (25 °C) at 17 rpm. Controls containing no enzyme were treated in the same way. At the desired time, the reaction was stopped by cooling to 4 °C and allowing the spherulites to settle. The solid material was gently washed repeatedly with water, and used in calorimetry, X-ray and microscopy experiments. The supernatant from the assay mixture was assayed for either glucose or for reducing groups. For glucose production, the % hydrolysis was defined by:

$$\frac{[\text{glucose}]_{t=\text{end}}/20}{[\text{amylose (DP = 20)}]_{t=0}} \times 100\%$$

For measurement of reducing groups, the % hydrolysis was calculated by:

$$\frac{[\text{reducing groups}]_{t=\text{end}}}{[\text{reducing groups of equivalent amount of completely hydrolysed amylose (DP = 20) in solution}]_{t=0}} \times 100\%$$

The buffers used were as stated above for hydrolysis of soluble substrates.

2.4 Calorimetry

DSC was carried out using a Perkin Elmer (Beaconsfield, Bucks, UK) DSC-2B calibrated using naphthyl ethyl ether and indium. Samples of spherulitic material were dispersed in excess water in the ratio 1 : 10 and aliquots (~ 10 mg) were accurately weighed into aluminium sample pans and sealed. Each was analysed against a reference pan containing air, over the temperature range 280–380 K at a heating rate of 10 K min^{-1}. The differential heat input was logged by computer for later analysis. Weight of dry material was determined after DSC by puncturing the pans, drying in a vacuum at 70 °C over P_2O_5, and reweighing.

2.5 Electron microscopy

Samples were prepared and examined using an Emscope 2000 Sputter-cryo system attached to a Philips 501B scanning electron microscope. The spherulites were spread in a thin layer on a copper microscope holder, frozen by plunging the holder into slushed liquid nitrogen (77 K) and transferred to the microscope stage. Excess moisture was sublimed off the specimen surface by raising the temperature to 191 K for 18 min. The sublimed specimen was coated with a layer of gold (~20 nm) and examined at 103 K.

2.6 X-ray powder diffraction

Wide angle X-ray patterns were recorded photographically using a flat plate camera flushed with helium, using an X-ray wavelength of 0·154 nm (CuKα line). The reflections on the photograph were calibrated by photographing the specimens with or without a calcite standard. Exposure time was typically 2·5 h using a fine focus X-ray tube (Philips Scientific, Cambridge, UK). Powdered specimens were contained in thin capillary tubes. The intensities of the reflections were estimated as strong, weak, etc., by visual inspection. Interplanar spacings (*d* spacings) were calculated from the reflection angles using Bragg's equation, assuming a first order reflection.

3 RESULTS

3.1 Hydrolysis of A-type spherulites

3.1.1 Rate of hydrolysis

Table 1 shows the average rates of enzymic hydrolysis of A-type spherulites. The values are for comparative purposes, and do not imply a linear time course over 158 h. Glucoamylase 1 exhibited the highest apparent rate of hydrolysis and also showed a relatively high activity compared to its rate of hydrolysis of soluble amylose (DP ~ 20). Relative to its intrinsic activity on soluble substrates, β-amylase showed the lowest rate of hydrolysis. Subsequent analyses were performed on partially hydrolysed spherulites from this experiment (extent of hydrolysis as shown in Table 1).

3.1.2 Crystallinity

Spherulites crystallised from 30% ethanol showed a highly crystalline X-ray diffraction pattern (Fig. 1). The four strong reflections can be assigned to the 0·578, 0·517, 0·486 and 0·378 nm reflections characteristic of A-type starch (Zobel, 1988). Additional medium and weak reflections can be assigned to the 0·330 and 0·288 nm reflections of A-type starch. The pattern also contains weak reflections corresponding to *d*-spacings of 1·58, 0·89 and 0·794 nm. The presence of such reflections could indicate a C-type structure (Zobel, 1988) or the presence of a small fraction of B-type spherulites. The strong B-type reflection at 0·516 nm would be obscured by the A-type reflection at 0·517 nm. After partial hydrolysis with glucoamylase 1, β-amylase, or α-amylase, the X-ray patterns remained highly crystalline with no marked increase in amorphous background. The characteristic A-type reflections remained, but the weak reflections at 1·58 and 0·794 nm disappeared. This does suggest a small amount of B-type crystalline material which is hydrolysed preferentially. Despite the substantial hydrolysis the X-ray patterns of the enzyme treated material remain highly crystalline.

Table 1. Extent of hydrolysis of A- and B-type spherulitic amylose. A- or B-Type (80 mg ml^{-1}) were incubated with enzyme (40 μg ml^{-1}) for 24 h and 158 h respectively at 25°C

Enzyme	Hydrolysis (%)	Ratio of spherulitic hydrolysis to hydrolysis in solution
A-type		
Glucoamylase 1	53	0.26 : 1
β-Amylase	37	0.008 : 1
α-Amylase	42	0.03 : 1
B-type		
Glucoamylase 1	19	0.014 : 1
β-Amylase	20	0.0006 : 1
α-Amylase	36	0.004 : 1

3.1.3 Microscopy of A-type spherulites

Freshly prepared A-type spherulites were birefringent spheres (Table 2). After hydrolysis by each of the three enzymes, however, no spherulitic structure was observed. Only structureless solid material could be seen. Electron microscopy showed that before enzyme treatment, A-type spherulites possessed an uneven surface. Thus each of the amylases is able to break up the A-type spherulites into smaller crystalline fragments, and hence increase the surface area available for hydrolysis.

3.1.4 Calorimetry

Untreated A-type spherulites showed a characteristic (Whittam *et al.*, 1990) melting curve (Fig. 2(a)) with T_m = 361 K (Table 3). A small shoulder at ~344 K was also seen. This shoulder remains after partial hydrolysis, suggesting that it is not B-type, since the B-type powder diffraction pattern disappears after partial hydrolysis (above). After partial hydrolysis by glucoamylase 1 or β-amylase, there was no clear shift in the melting temperature, and no clear peaks could be seen. The curve suggests that only some components of the A-type spherulites were hydrolysed. In contrast, after

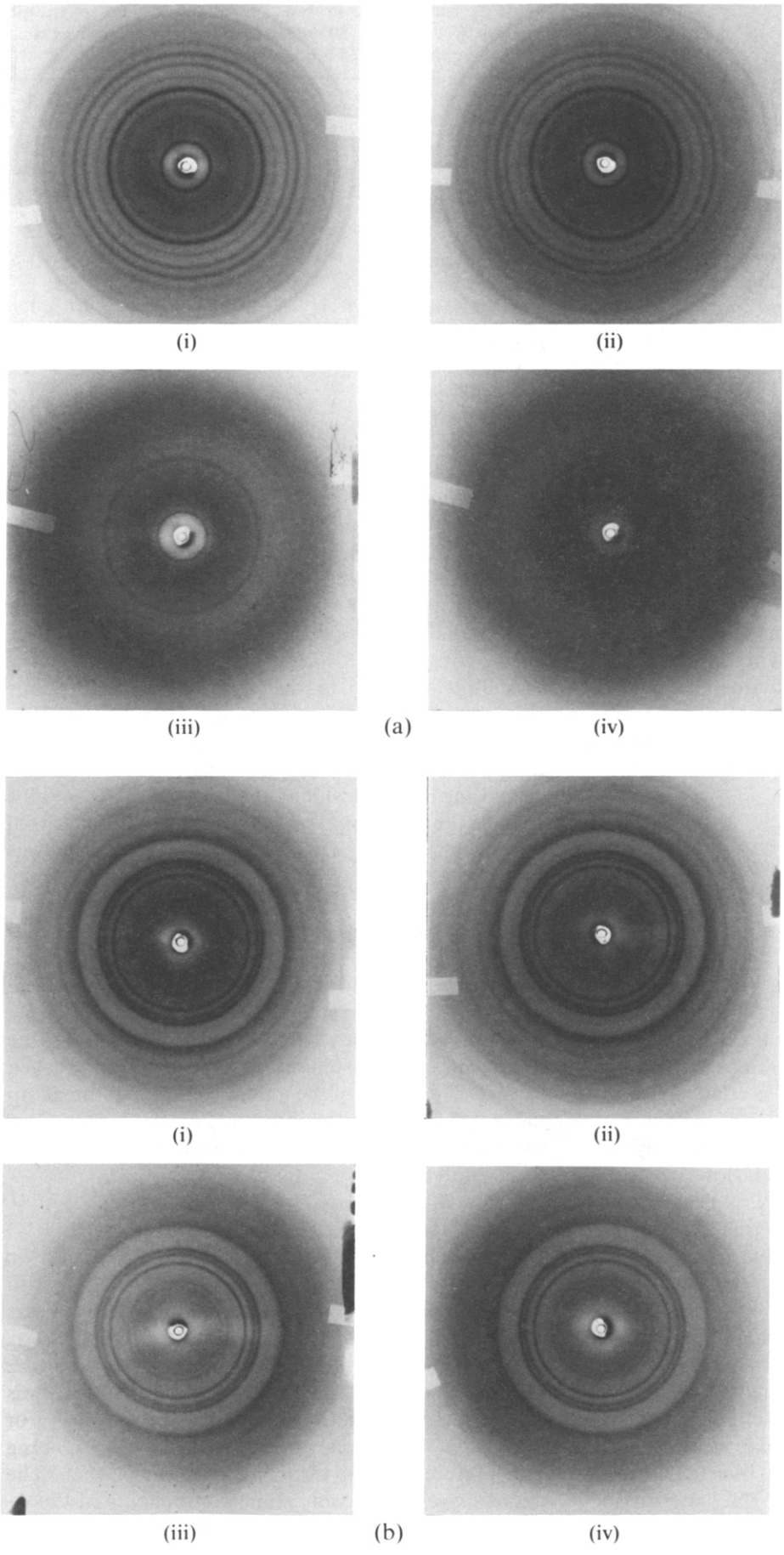

Fig. 1. X-ray powder diffraction patterns of A-type (a) and B-type (b) spherulitic amylose after hydrolysis. (i) No enzyme; (ii) glucoamylase 1-treated; (iii) β-amylase-treated; (iv) α-amylase-treated. The exposure time for all A-type and untreated B-type was 2·25 h. Other exposure times for B-type were (ii) 2·5 h (iii) 4 h and (iv) 3·5 h.

Table 2. Description of the appearance of A- and B-type spherulitic amylose by light microscopy viewed under crossed-polarisers

Enzyme	A-Type		B-Type	
	Appearance	Birefringent	Appearance	Birefringent
None	Whole, spherulitic	Yes	Whole, spherulitic	Yes
Glucoamylase 1	No structure	No	Whole, spherulitic	Yes
β-Amylase	No structure	No	Some breakdown, but many spherulites remaining	No
α-Amylase	No structure	No	Some breakdown, but many spherulites remaining	No

partial hydrolysis with α-amylase, the curve was similar to untreated material, although T_m was decreased (355 K). After partial hydrolysis by each enzyme the melting enthalpies were unchanged. This demonstrates that the remaining fragments contain the same proportion of crystalline material both before and after partial hydrolysis.

3.2 Hydrolysis of B-type spherulites

3.2.1 Rate of hydrolysis

B-type spherulites were apparently hydrolysed more slowly than A-type. Relative to its intrinsic activity on soluble starch, β-amylase showed the lowest rate of hydrolysis. α-Amylase exhibited the highest rate on B-type spherulites. Subsequent analyses were performed on partially hydrolysed spherulites from this experiment (extent of hydrolysis as shown in Table 1).

3.2.2 Crystallinity

Untreated spherulites crystallised from water show a highly crystalline X-ray powder diffraction pattern. The reflections in the pattern can be assigned to the strong 0·516, medium 1·58, and weak 0·89, 0·794, 0·454, 0·400 and 0·390 nm reflections characteristic of B-type starch. After partial hydrolysis by glucoamylase 1, the X-ray pattern remains highly crystalline and B-type. In contrast, the X-ray patterns obtained after partial hydrolysis with β-amylase and α-amylase show a B-type crystalline pattern superimposed upon an amorphous background.

3.2.3 Microscopy of B-type spherulites

Untreated B-type spherulites exhibited a characteristic 'maltese cross' pattern (Ring *et al.*, 1987) under crossed polarisers (Table 2). After partial hydrolysis by glucoamylase 1, the appearance was unchanged. After partial hydrolysis by β-amylase and by α-amylase, spherical structures made up most of the material, but no birefringence was seen. Electron microscopy of untreated B-type spherulites showed a spherical structure with a smooth surface (Fig. 3(i)). The structure is very similar after partial hydrolysis by glucoamylase 1 (Fig. 3(ii)), indicating that this enzyme acts on the surface of the B-type spherulites. After partial hydrolysis by β-amylase (Fig. 3(iii)), the B-type spherulites contained numerous small pits which may extend through the entire structure. Partial hydrolysis by α-amylase (Fig. 3(iv)) produced structures with a small number of very large pits. These results show that β-amylase and α-amylase must disrupt the ordering of the crystallites resulting in retention of the spherical appearance and crystallinity but loss of birefringence.

3.2.4 Calorimetry

Untreated B-type spherulites show an endothermic peak (Fig. 2(b)) with T_m and melting enthalpy characteristic for this material (Ring *et al.*, 1987). There was a shoulder at ~353 K, which may be due to a small amount of A-type, although this was not detected by X-ray diffraction. After partial hydrolysis by glucoamylase 1, the 344 K peak shifted to 341 K, and the shoulder became resolved into a peak at 355 K. The melting enthalpy decreased by 40%. In contrast, partial hydrolysis with β-amylase or α-amylase results in a much larger decrease in transition enthalpy, and a peak (without shoulder) at 343–344 K. Clearly, the component with T_m = 355 K is completely hydrolysed and some of the component with T_m = 343–344 K remains. The decrease in melting enthalpies demonstrates a loss of crystalline material.

4 DISCUSSION

The rate of hydrolysis of non-solution starch is dependent on the accessible surface area (Leloup *et al.*,

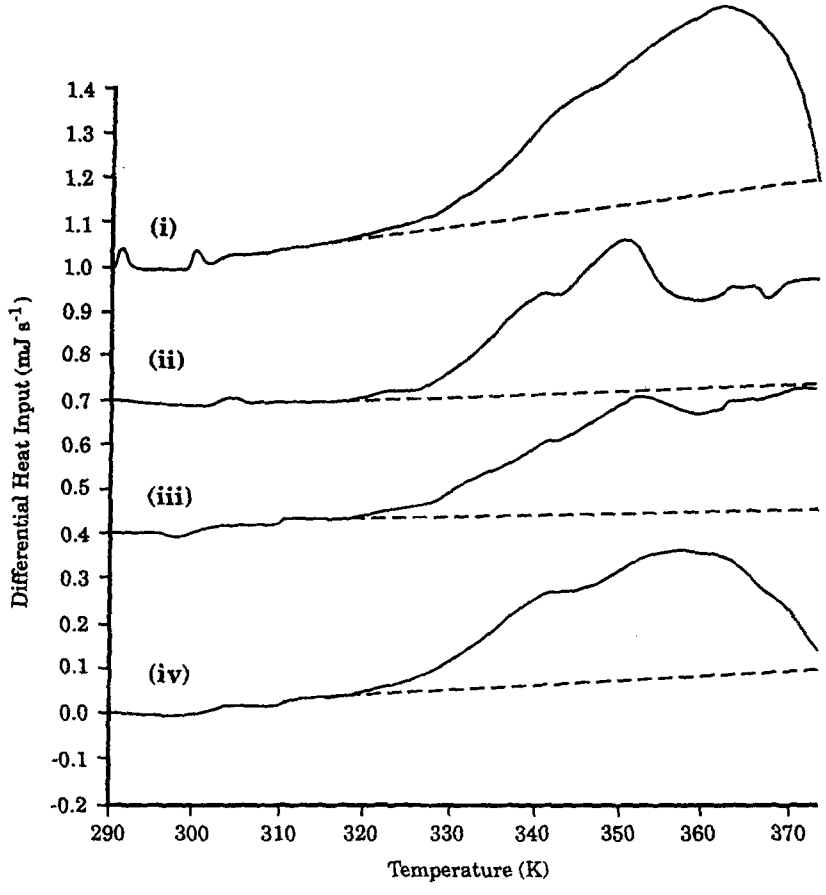

Fig. 2. Differential scanning calorimetry traces of A-type (a) and B-type (b) spherulitic amylose after hydrolysis. (i) No enzyme; (ii) glucoamylase 1-treated; (iii) β-amylase-treated; (iv) α-amylase-treated.

Table 3. Melting energy and melting temperature of A- and B-type spherulitic amylose after enzyme hydrolysis

Enzyme	A-Type		B-Type	
	T_m (K)	Melting energy (Jg^{-1})	T_m (K)	Melting energy (Jg^{-1})
None	361	43	344	45
Glucoamylase 1	349	43	341	27
β-Amylase	351	45	344	7·7
α-Amylase	355	35	343	5·7

1991). The electron microscope has revealed that the surface of A-type spherulites is uneven, yielding a greater surface/volume ratio than for smooth B-type spherulites. Since all of the hydrolytic amylases broke the A-type spherulites into small pieces, this dramatically increases the surface area, and hence the rate of hydrolysis. Thus kinetic constants for hydrolysis of A-type and B-type starch as a fraction of surface area remain to be measured, provided accessible surface area can be estimated without concomitant hydrolysis. Gallant *et al.* (1972) reported that B-type structures within starch granules were attacked more rapidly than A-type, even though potato starch (with a high content of B-type) is hydrolysed much more slowly than wheat starch (with a high content of A-type). It is probable that accessible surface area is initially one of the most important parameters for determining rate of hydrolysis since potato starch granules are larger than wheat starch granules.

The partial hydrolysis of highly crystalline B-type spherulites by α- and β-amylase was associated with disruption of the ordered crystallites as shown by a loss of birefringence, and a reduction in crystalline material as shown by a dramatic decrease in enthalpy and changes in the X-ray powder diffraction patterns. This disruption may be brought about directly by the enzymes themselves or indirectly by increasing hydration of the crystallites. The crystallinity of the A-type

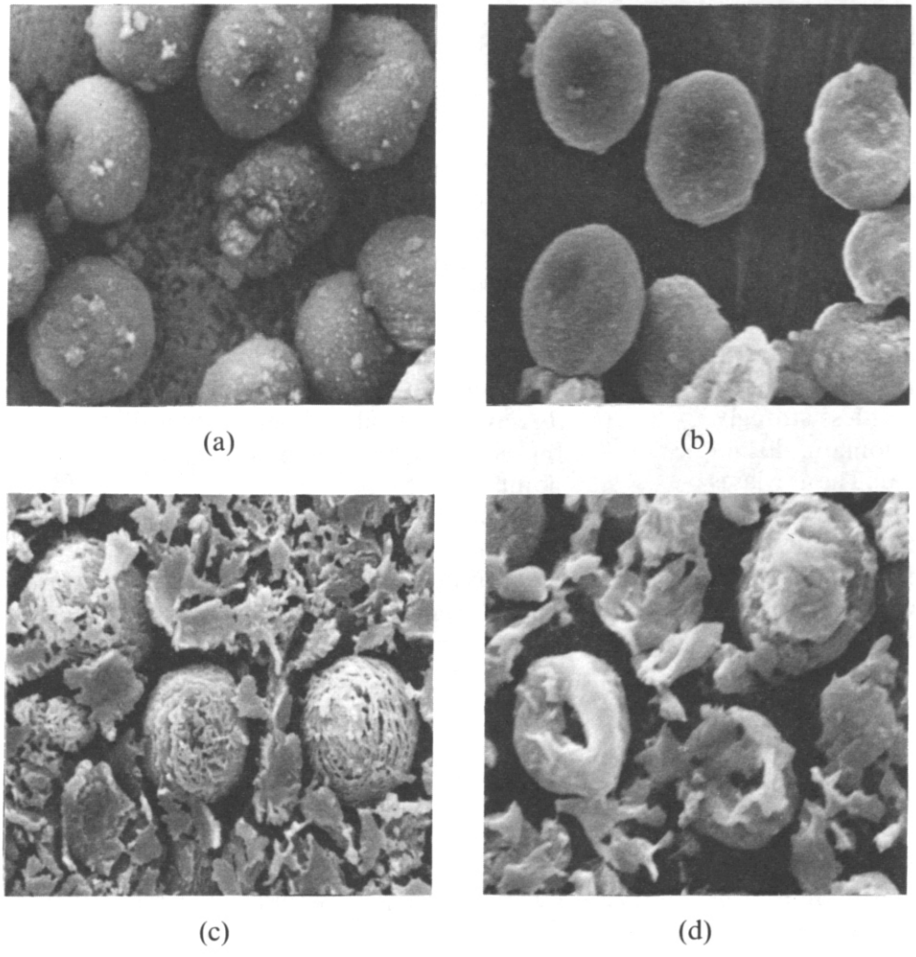

Fig. 3. Scanning electron micrographs of B-type spherulitic amylose after hydrolysis. (a) No enzyme; (b) glucoamylase 1-treated; (c) β-amylase treated; (d) α-amylase-treated. Original magnification ×2500.

spherulites was not disrupted. It has been reported that hydrolysis occurs predominantly in the amorphous region of the granule (Franco *et al.*, 1988). Our results support this for hydrolysis of A-type, but not for the hydrolysis of B-type by α- or β-amylase. It is important to note, however, that in the starch granule, β-amylase action would be rapidly blocked by α1,6 linkages, whereas α-amylase would not.

The pitting by α-amylase on B-type spherulites has also been observed on most types of starch granule (Fuwa *et al.*, 1980). However, the action of glucoamylase 1 on wheat starch granules is dependent on surface protein. In the presence of surface proteins carried through from purification of the starch granule, glucoamylase 1 formed pits on the granule surface. In the absence of surface protein, however, glucoamylase 1 hydrolyses only the surface of the granule, resulting in smaller but intact and unpitted spherical structures (Greenwell *et al.*, 1985). Plant β-amylase is very poor at hydrolysing intact starch granules, although some microbial β-amylases are able to do so (Ueda & Marshall, 1980). However, β-amylase produced substantial pitting of the surface of B-type spherulites and hydrolysed both A- and B-type structures. Consequently, crystallinity *per se* does not inhibit β-amylase action on starch granules; its action is limited by the existence of α1,6 branches which are absent in A- and B-type spherulites.

The results raise interesting questions on the relationship between enzyme molecular structure/mechanism of action and the observed modes of attack. A comparison with cellulases is often drawn. *Endo*glucanases and cellobiohydrolases only hydrolyse microcrystalline cellulose if they are able to bind tightly to the substrate. This is usually facilitated by an additional, discrete binding domain on the protein which is spatially and functionally separate from the catalytic site (Knowles *et al.*, 1987). These cellulases also mechanically disperse the cellulose by penetrating into microcracks and allowing water to diffuse in and break intermolecular hydrogen bonds. Cellulases which bind less strongly, and which do not have the binding domain, do not penetrate into microcracks (Klyosov, 1990). The amylases reported here appear to behave differently. Glucoamylase 1 possesses a binding domain in addition to the catalytic domain which can be proteolytically cleaved in functional form (Belshaw & Williamson, 1990). α-Amylase from *A. oryzae* consists only of a catalytic domain (Matsuura *et al.*, 1984). β-Amylase has no binding domain, but is a tetrameric protein with one catalytic site per oligomer (Thoma *et al.*, 1971). By analogy with cellulases, it might be expected that glucoamylase 1 would burrow into the B-type spherulites and disrupt the mechanical structure, facilitated by the binding site. However, even though glucoamylase 1 binds more tightly to B-type spherulites than α- or β-amylase (G. Williamson, unpublished results), it only acts on the surface of B-type spherulites. The α- and β-amylases do not possess a binding site, but burrow into the spherulite, causing disruption of the individual crystallites, loss of birefringence and a change from a highly crystalline structure to a less crystalline one. Furthermore, the mode of action is not dependent on the chemical nature of the hydrolysis: α-amylase (an *endo* acting enzyme) and β-amylase (*exo*) both pit the spherulite surface; glucoamylase 1 (*exo*) does not.

On A-type, it appears that all three enzymes mechanically disrupt the spherulites since control samples treated identically in the absence of enzyme retained their spherulitic structure. The surface structure of A-type spherulites is floriated and it is probable that amylases attack in the many crevices between the extrusions where the enzyme concentration is highest. This would lead to rapid break-up of the spherulite with a low concomitant extent of hydrolysis. Once broken into smaller pieces, the increase in surface area would substantially increase the rate of hydrolysis.

In order to further resolve the mechanism of action of these enzymes on starch, it will be necessary (i) to quantify the rate of hydrolysis of A- and B-type crystals as a function of surface area, (ii) to examine enzyme action on single amylose crystals, and (iii) to determine the effect of synergy on the hydrolysis of these substrates.

5 CONCLUSIONS

(a) After partial hydrolysis of spherulitic, polycrystalline material by α- and β-amylase, the remaining material was analysed; A-type retained its crystallinity but B-type showed a reduction in the level of crystallinity.
(b) A- and B-type spherulites retained their crystallinity after partial hydrolysis with glucoamylase 1.
(c) A-Type spherulites were disrupted by amylases, leading to an increase in surface area and consequently in the rate of hydrolysis.
(d) β-Amylase was able to attack crystalline starch but is inhibited in the granule by α1,6 branch points.
(e) Hydrolysis of B-type spherulites by glucoamylase 1 occurred at the surface of the spherulite. Hydrolysis by α- or β-amylase yielded heavily pitted structures, with substantially decreased melting enthalpy but unchanged T_m.

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