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A method for the study of the enzymic hydrolysis of starch granules*

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ABSTRACT

A method for the study of the enzymic hydrolysis of starch granules is described. A solution of alpha-amylase was pumped through a bed of normal or waxy-maize starch in a column, and the eluate was passed through a column of ion-exchanger that retained the enzyme but not the solubilised dextrins which could be analysed further by gel-permeation chromatography. The material released first from the waxy-maize starch granules contained comparatively large proportions of dextrins of d.p. >3000. Subsequently, maily dextrins were produced from each type of starch with d.p. 50–200 similar to those produced from the gelatinised starches. Dextrins with d.p. ~6 were also solubilised in the later stages. The content and the lengths of amylose-like chains within the normal maize granules decreased notably when large concentrations of enzyme were used. These chains were probably not released from the granules and the reaction seemed to be correlated with the enzyme adsorbed on the granules, whereas the solubilised material originated from the amylopectin component.

INTRODUCTION

The most important sources of starch are cereals and tubers where it is found in semicrystalline granules. The shape and size of these granules can differ widely depending on the source of the plant. Amylopectin, which usually constitutes 70–80% of the granules, comprises chains of (1→4)-linked α-D-glucopyranosyl residues inter-connected by (1→6) linkages in clusters[1-6]. There are three categories of chains: (*a*) short with average lengths (c.l.) of 11–25 (refs. 7–12), which are the major chains and build up the crystalline regions of the starch granule[13-16]; (*b*) long with c.l. 40–70, which are thought to inter-connect individual clusters[5]; and (*c*) extremely long with c.l. >100, small proportions of which have been reported[17-19].

The minor component of starch, amylose, has a d.p. in the range 600–6000 (refs. 10, 17, 20 and 21) and may contain a few branches[10,17,20-23]. It has been shown[24] that the shortest side chain in the branched amylose of maize has a c.l. of 6. Some strains of plants, especially of maize[25], rice[26], and barley[27,28], possess so-called waxy endosperms in which the starch granules have a markedly reduced content of amylose.

Hydrolysis of starch granules by alpha-amylase involves an enzyme in solution

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acting on a solid substrate. Thus, the surface area accessible to the enzyme and the efficiency of the adsorption of the enzyme onto this surface are critical kinetic parameters[29-31]. Alpha-amylases of various origin attack starch granules with different efficiencies[32,33]. The susceptibility of starch granules of different origin to alpha-amylolysis also varies widely. Generally, cereal-starch granules are susceptible, whereas potato-starch granules are resistant to hydrolysis[32-34]. "Pinholes" arise on the surface during the hydrolysis of many granules, *e.g.* from maize[35] and many other cereals[35-37] so that both exo-corrosion on the surface and endo-corrosion at preferential sites within are possible[35].

Many studies of the solubilisation of starch granules have been reported[32,34,36,38-41], but little is known about the products that are released from the granules by the alpha-amylases. Because of the relatively low rate at which the starch is solubilised, it is necessary to use high concentrations of enzyme and/or long times of incubation in order to effect detectable degradation of the granules. The starch components or fragments released into the solution are hydrolysed rapidly further and only the resulting small fragments can be analysed. A method by which degradation of the starch granule can be monitored, and in which the further degradation of the solubilised dextrins is largely inhibited, is now presented and exemplified with starches from maize and waxy-maize (low content of amylose).

EXPERIMENTAL

Starch granules of commercial flours from normal maize (NMS) and waxy-maize (WMS, amylopectin, Sigma) were defatted by extraction (Soxhlet) with hot aqueous 85% methanol overnight, then dried with acetone.

The activity (11 U/mg) of the alpha-amylase of *B. subtilis* [(1→4)-α-D-glucan glucanohydrolase, EC 3.2.1.1; Koch–Light] was measured at 25° in 0.001M sodium acetate buffer (pH 6.5) containing 0.001M NaCl, using soluble starch (Merck) as the substrate at 5 mg/mL. The reducing power was determined with the Nelson reagent[42], using D-glucose as the standard. One unit (U) of alpha-amylase activity is defined as that which produces 1 μmol of reducing groups per min. Isoamylase from *Pseudomonas amyloderamosa* (glycogen 6-glucanohydrolase, EC 3.2.1.68) was obtained from Hayashibara Shoji Inc.

Adsorption of alpha-amylase and hydrolysis of starch granules. — To washed starch granules (50 mg) was added a solution of alpha-amylase (5 mL) diluted in 0.001M sodium acetate buffer (pH 6.5, containing 0.001M NaCl) to concentrations in the range 0.003–30 U/mL. The mixtures were shaken continuously at 25° in order to prevent sedimentation. After a suitable interval, each sample was filtered under vacuum through a Millipore filter (0.45 μm). The starch granules were washed immediately with water and then with ethanol, dried with acetone, and used for further analysis as described below.

In order to determine the residual enzyme activity, soluble starch (10 mg/mL) was added to an equal volume of the filtrate. The activity was then calculated from the

increase in reducing power. The amount of enzyme adsorbed onto the starch granules was estimated from the difference between the original and residual enzyme activity.

The filtrate was also used to determine the solubilisation of the granules by assaying the total carbohydrate content with the phenol – sulphuric acid reagent[43].

In order to measure the molecular weight distribution in the hydrolysates of the starch granules, the reaction was interrupted by the addition of 5M KOH (0.5 mL). After stirring for 2 days, the residue of granular starch had dissolved and an aliquot (0.5 mL) of the solution was submitted to gel-permeation chromatography.

Solubilisation of starch granules in a column. — A slurry of starch granules (2.5 g) in the saline-containing acetate buffer (pH 6.5) was added to a column (diameter, 4.6 cm) to give an ~2-mm layer that was covered with a Millipore filter (0.45 μm). The column was eluted at 25° from the bottom with buffer (400 mL), using a peristaltic pump, and into a column (2.5 × 25 cm) containing DEAE Sepharose Fast Flow (Pharmacia) ion-exchanger. The inter-column volume was 0.25 mL. A solution of alpha-amylase in the buffer at a concentration in the range 0.003–0.3 U/mL was passed through the layer of starch granules at either 0.33 or 1.0 mL/min. The enzyme was retained in the ion-exchange column, whereas the soluble dextrins were eluted and collected (11.2-mL fractions. The carbohydrate in the fractions was determined with the phenol–sulphuric acid reagent and those containing collectively 25 mg (representing 0–1%, 1–2%, *etc.*, of solubilised carbohydrates) were combined and concentrated to 3.3 mg/mL. To an aliquot (1 mL) of each sample was added 5M KOH (0.1 mL) before gel-permeation chromatography.

The residual starch granules were collected, washed, and dried. The alpha-amylase bound to the ion-exchanger was eluted with acetate buffer containing M NaCl (400 mL) and the ion-exchanger was regenerated with buffer containing 0.001M NaCl.

Debranching experiments. — An aqueous suspension of starch granules, before and after treatment with alpha-amylase (5 mg in 0.65 mL), was gelatinised and debranched with isoamylase as described[44].

Alpha-amylolysis of gelatinised starch. — A suspension of starch granules (30 mg) in water (3 mL) was gelatinised by boiling for 30 min, cooled to 25°, and treated with an equal volume of a solution of alpha-amylase (0.06 U/mL) in 0.001M acetate buffer (pH 6.5). At intervals, aliquots (1 mL) were treated with 5M KOH (0.1 mL) in order to stop the reaction.

Some samples of soluble dextrins were also treated with alpha-amylase, then concentrated to 3.7 mg/mL, and aliquots (0.9 mL) were treated with the enzyme (0.1 mL, 0.03–3.0 U/mL) for 6 min.

Gel-permeation chromatography. — Solutions (0.5 mL) of samples diluted in KOH were eluted from a column (1.5 × 90 cm) of Sepharose CL 6B (Pharmacia) with 0.5M KOH at 1 mL/min. Fractions (1 mL) were analysed for carbohydrates, using the phenol–sulphuric acid reagent. The column was calibrated[45] with alpha-dextrins of known d.p.

RESULTS

The adsorption of the alpha-amylase of *B. subtilis* onto the two granular maize starches is compared in Fig. 1. The enzyme was adsorbed only partly onto the normal maize starch (NMS) when small concentrations (0.003–0.03 U/mL) were used. After ~ 30 min of reaction, an adsorption maximum was obtained, whereafter the enzyme partly dissociated from the granules (Fig. 1a). With higher concentrations (0.3–3 U/mL), nearly 100% of the enzyme was adsorbed within 10 min and remained adsorbed up to at least 90 min. With high concentrations (30 U/mL), the granules seemed to be saturated, so that ~ 50% of the enzyme remained unadsorbed. The enzyme was adsorbed more firmly onto the waxy-maize starch (WMS, Fig. 1b), so that, even with low concentrations, nearly 100% adsorption occurred, although, with the lowest concentration tested, the rate of adsorption was low. The use of high concentrations of enzyme saturated the granules.

The rate of solubilisation of granular WMS was somewhat higher than that for NMS, with the exception of the highest concentration of enzyme used for which the rate

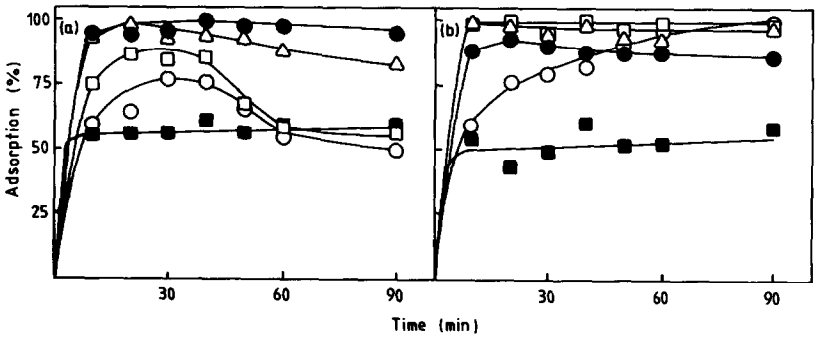

Fig. 1. Adsorption of alpha-amylase onto granules of (a) NMS and (b) WMS with 0.003 (o), 0.03 (□), 0.3 (△), 3 (●), and 30 U/mL (■).

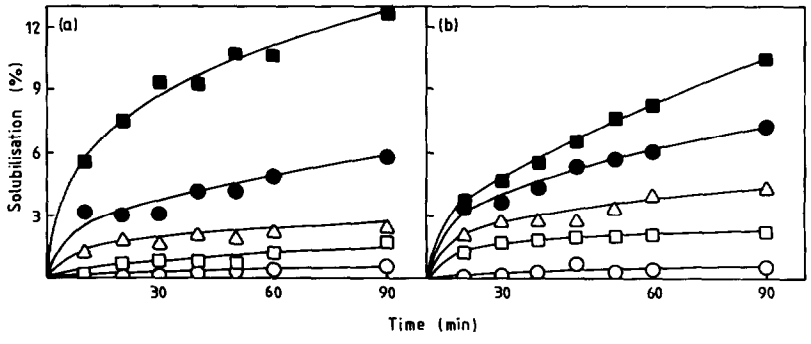

Fig. 2. Solubilisation of granules of (a) NMS and (b) WMS with 0.003 (o), 0.03 (□), 0.3 (△), 3 (●), and 30 U of alpha-amylase/mL (■).

of solubilisation was higher with NMS (Fig. 2). The solubilisation proceeded in two stages with an initial rapid rate followed by a lower rate. The differences between the two starches were caused primarily by the rapid initial rate of solubilisation, whereas the second stages were similar.

Gel-permeation chromatography of the hydrolysates was carried out for some of the samples in which the higher concentrations of enzyme were used. With 3 U/mL, ~ 4.5% of the NMS granules were solubilised after incubation for 50 min and ~ 6% after 90 min. The products were eluted almost completely at a volume corresponding to that for maltohexaose (Fig. 3a). At 30 U/mL, similar chromatograms were obtained (Fig. 3b). In the higher molecular weight range (d.p. >50), the composition was almost identical to that of the original NMS granules. This material probably remained within the granules, as shown from the chromatograms of the residual granules (example given

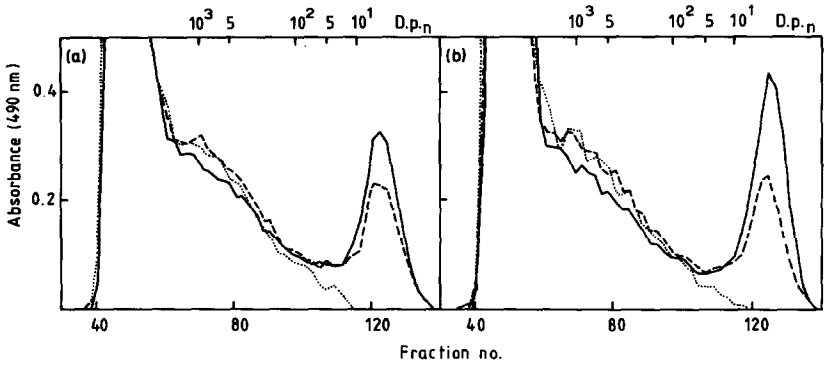

Fig. 3. Fractionation on Sepharose CL 6B before and after solubilisation of NMS: (a) native granules (·····) and the hydrolysates after treatment with alpha-amylase (3 U/mL) for 50 (-----) and 90 min (——); (b) granules after treatment with 30 U of alpha-amylase/mL for 90 min (·····) and the hydrolysates after treatment with alpha-amylase (30 U/mL) for 10 (------) and 90 min (——).

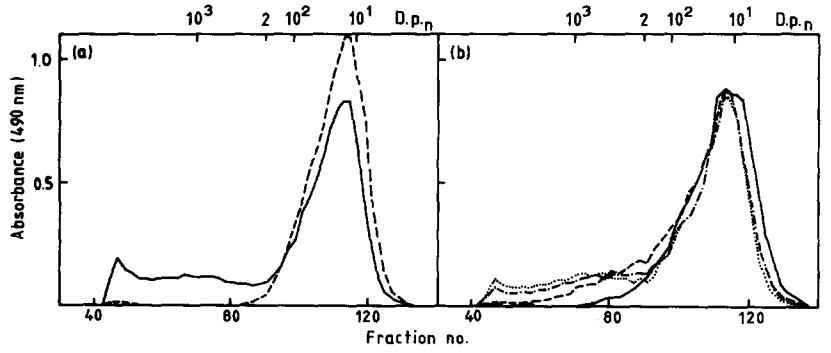

Fig. 4. Distribution of the unit chains after debranching and fractionation on Sepharose CL 6B of (a) native NMS (——) and WMS (-----); and (b) residual NMS granules after alpha-amylase treatment with 3 U/mL for 50 (·····) and 90 min (-·-·-), and with 30 U/mL for 10 (------) and 90 min (——).

TABLE I

Solubilisation and apparent content of amylose of native and alpha-amylase-treated starch granules.

Type of treatment	*Enzyme conc. (U/mL)*	*Hydrolysis time (min)*	*Flow rate (mL/min)*	*Solubilisation (%)*	*Apparent amylose*[a] (%)
Native WMS	–	–	–	–	0.8
Native NMS	–	–	–	–	25.1
Batch[b]	3	50	–	4.2	23.4
Batch	3	90	–	5.8	20.8
Batch	30	10	–	5.6	15.2
Batch	30	90	–	12.7	3.7
Column[c]	0.03	577	0.33	3.9	25.2
Column	0.03	4276	0.33	9.4	25.4
Column	0.03	1523	1.0	3.4	25.0
Column	0.3	1411	1.0	8.3	23.6

[a] Chains with c.l. >200. [b] NMS granules treated with alpha-amylase in a test tube. [c] NMS granules treated with alpha-amylase in a column.

in Fig. 3b) which had a molecular weight distribution almost identical to that of the original granules. However, when the starch components of the granule residues were debranched with isoamylase, it was observed that the carbohydrate chains with c.l. >200 had been reduced in length (Fig. 4). These chains comprised the amylose component[46] of the NMS granules and were practically absent from the WMS granules (Fig. 4a and Table I). The changes within the granules were more marked when the higher concentrations of enzyme were used (Fig. 4b and Table I), *e.g.*, when the granules had been treated for 90 min with 3 U/mL of alpha-amylase and ~6% of the material had been solubilised, ~21% of apparent amylose remained, whereas, when the granules were treated with 30 U/mL for 10 min and the same degree of solubilisation was reached, both the content and the average length of the apparent amylose chains were less.

The products solubilised from WMS granules (Fig. 5) were similar to those obtained from NMS. A slight increase of the carbohydrate material was detected in the higher molecular weight range (d.p. 50–2000). Most of this material probably remained within the granules because the granular-starch residues also contained increased amounts of carbohydrates of this size (Fig. 5b). The granules treated with alpha-amylase had unit-chain distributions identical to that of the untreated WMS granules shown in Fig. 4a.

The rate of solubilisation was dependent on the concentration of the enzyme, in a manner that was comparable to the batch-wise experiments when the starch granules were packed into a column (Fig. 6). Again, the rate was initially high and was followed by a slower stage, but there were no obvious difference between the two starches. When low rates of flow were used and the enzyme was in contact with the granules for longer periods of time, more solubilised carbohydrates were eluted in each fraction than when higher rates were used.

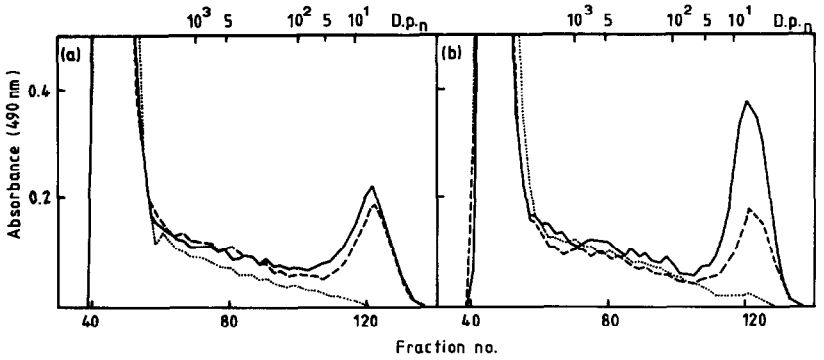

Fig. 5. Fractionation on Sepharose CL 6B before and after solubilisation of WMS: (a) native granules (·····) and the hydrolysates after treatment with alpha-amylase (3 U/mL) for 50 (------) and 90 min (——); (b) granules after treatment with 30 U of alpha-amylase/mL for 90 min (·····) and the hydrolysates after treatment with alpha-amylase (30 U/mL) for 10 (------) and 90 min (——).

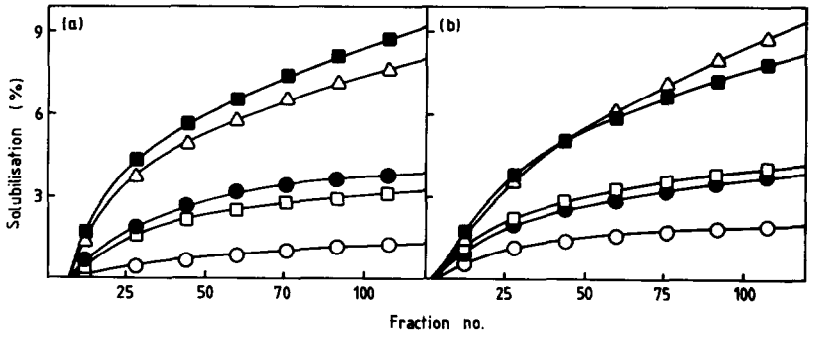

Fig. 6. Solubilisation of granules of (a) NMS and (b) WMS in a column with 0.003 (o), 0.03 (□), and 0.3 U of alpha-amylase per mL (△) pumped through the starch bed with a flow rate of 0.33 (dark symbols) or 1.0 mL/min (light symbols).

The molecular weight distributions of the products collected at different stages of the solubilisation of NMS granules are shown in Fig. 7. The composition of the first 1% of the solubilised carbohydrates was dependent on the concentration of the enzyme and on the rate of flow. With the most dilute enzyme solution (0.003 U/mL), almost identical complex mixtures of dextrins that covered the whole range of the Sepharose gel were obtained with each flow rate (0.33 and 1.0 mL/min). Dextrins within a d.p. range of 50–500 (fractions **c** and **d**) preponderated in the mixtures together with small molecules of d.p. ~6. A similar distribution of dextrins was obtained also with 0.03 U/mL of alpha-amylase and a lower flow rate, but the higher rate increased the amounts of fractions **d** and the dextrins with d.p. ~6, and decreased the yield of dextrins with high d.p., so that the material eluted at the void volume (fraction **a**) disappeared and only traces of dextrins with d.p. 500–2000 (fraction **b**) remained.

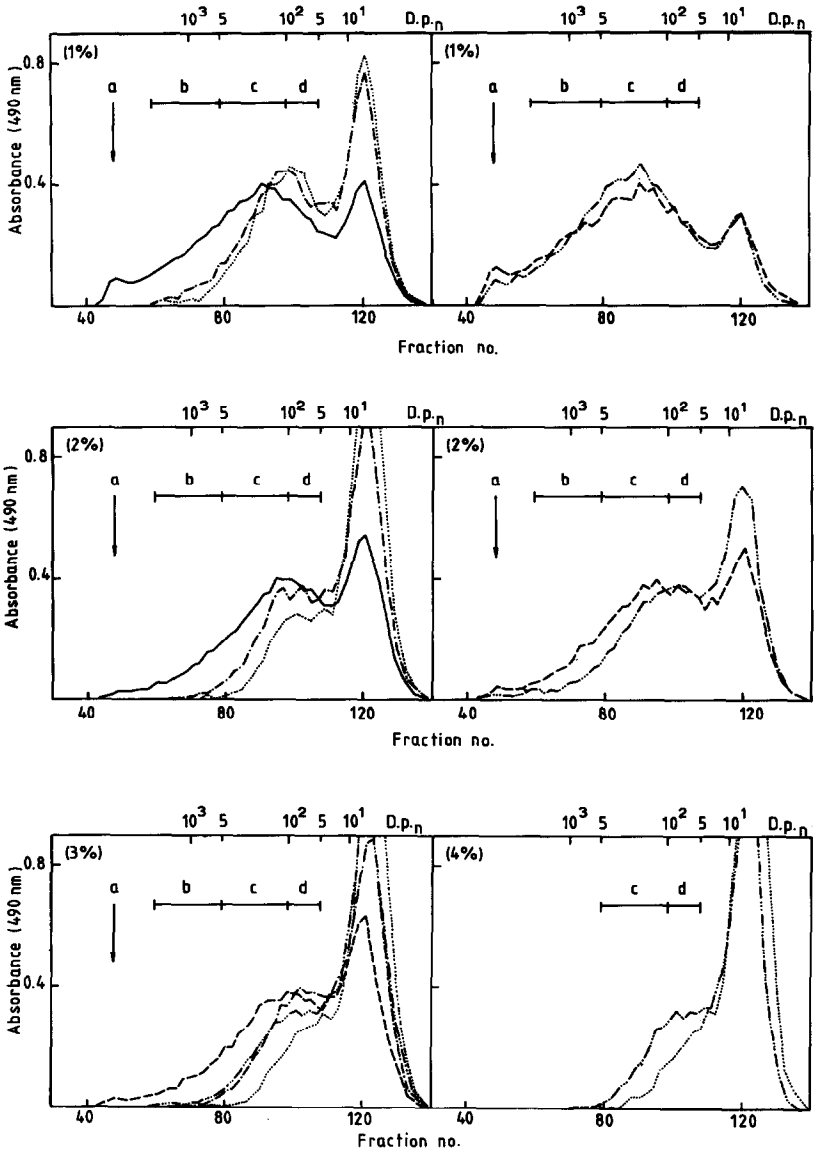

Fig. 7. Fractionation on Sepharose CL 6B of the dextrins collected at different stages of the solubilisation of NMS granules in a column with alpha-amylase concentrations and flow rates as follows: 0.003 U/mL and 1.0 mL/min, ——; 0.003 U/mL and 0.33 mL/min, -----; 0.03 U/mL and 1.0 mL/min, -·-·-; 0.03 U/mL and 0.33 mL/min, -···-; and 0.3 U/mL and 1.0 mL/min, ·····: **a–d** show areas of fractions with different molecular weights.

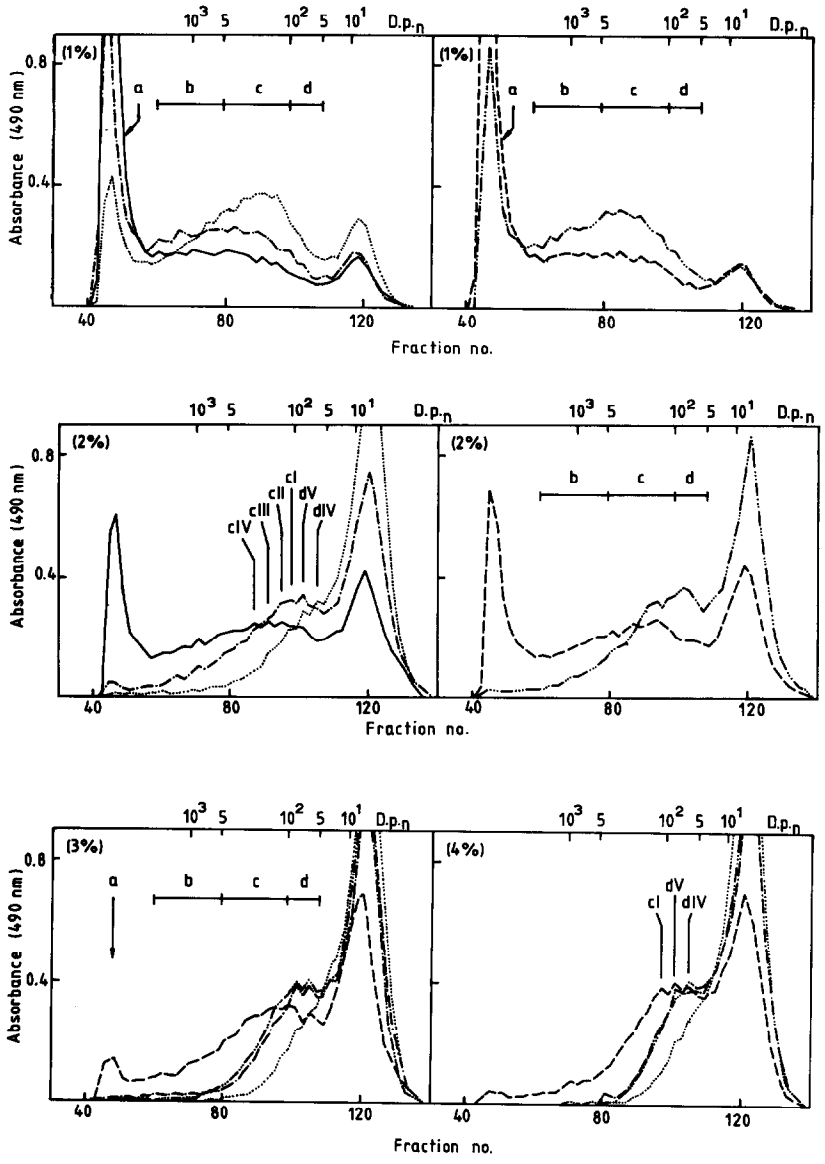

Fig. 8. Fractionation on Sepharose CL 6B of the dextrins collected at different stages of the solubilisation of WMS granules in a column with alpha-amylase concentrations and flow rates as follows: 0.003 U/mL and 1.0 mL/min, ——; 0.003 U/mL and 0.33 mL/min, -----; 0.03 U/mL and 1.0 mL/min, -·-·-; 0.03 U/mL and 0.33 mL/min, ·-·-; and 0.3 U/mL and 1.0 mL/min, ·····: **a–d** show areas of fractions with different molecular weights, and dIV–cIV show the position of individual dextrins.

The molecular weight distribution of the mixtures of dextrins changed as the hydrolysis of the granules proceeded. In the second 1% of the carbohydrates collected (Fig. 7), decreased amounts of fraction **a** were solubilised and both the proportions and sizes of the dextrins in fractions **b** and **c** had decreased. During the two following stages, the decrease continued so that, in the fourth 1% of solubilised material, fraction **d** preponderated among dextrins with d.p. >50 and those with d.p. ~6 constituted the major product. After this stage, however, only minor changes in the carbohydrate composition occurred, so that, when 8% of the granules had been solubilised, the gel-permeation chromatograms (not shown) were similar to those obtained at 4% solubilisation.

In the initial stages of the solubilisation of WMS, considerably more of the high molecular weight material was released from the granules (Fig. 8), but the composition was dependent more on the concentration of enzyme used than on the flow rate. With the most dilute solution, ~ 50% of the dextrins in the first 1% of the solubilised material had d.p. >3000. When more enzyme was used, increased amounts of fractions **b–d** were obtained and, with 0.3 U/mL of enzyme, fraction **c** preponderated. A shift towards lower d.p. occurred during the following stages of the solubilisation, so that the compositions of the third and fourth 1% were similar to those obtained with the NMS granules. At later stages (up to 13% of solubilisation with 0.3 U/mL), no more changes in the carbohydrate composition were detected (not shown).

The susceptibility of the material solubilised from the granular WMS to further hydrolysis was tested by reaction with alpha-amylase for 6 min. The high molecular weight material obtained as the first 1% of granule hydrolysis with 0.003 U/mL and a flow rate of 1 mL/min remained unchanged when it was treated further with the same concentration of enzyme, but it was hydrolysed with higher concentrations (Fig. 9a). With 0.03 U/mL, the molecular weight distribution became similar to that of the solubilised first 1% using this concentration (Fig. 8), and, with 0.3 U/mL, extensive

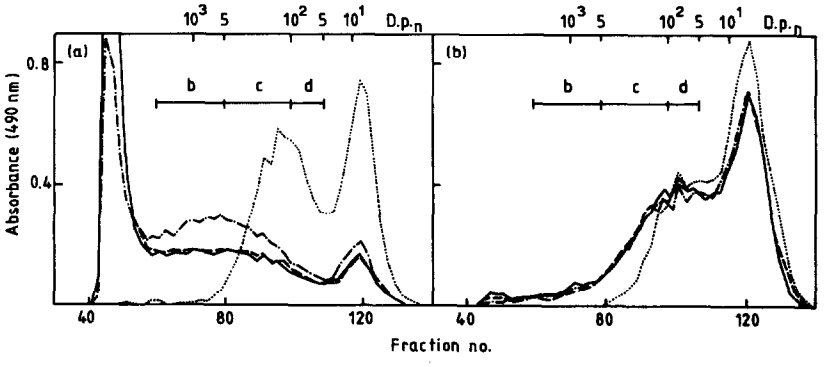

Fig. 9. Fractionation on Sepharose CL 6B of the dextrins solubilised from WMS with 0.003 U of alpha-amylase per mL (——), and after a further treatment with enzyme concentrations of 0.003 (-----), 0.03 (----), and 0.3 U/mL (·····) for 6 min: (a) the first 1% of solubilised material (flow rate, 1.0 mL/min) and (b) the fourth 1% (0.33 mL/min).

hydrolysis ocurred, resulting in large amounts of fractions **c** and **d** together with the dextrins of d.p. ~6. The smaller dextrins obtained as the fourth 1% of solubilised material (with 0.003 U/mL and 0.33 mL/min), which had d.p. <200 (Fig. 8), were resistant to hydrolysis with the more dilute enzyme solutions. Only with 0.3 U/mL did further hydrolysis occur (Fig. 9b).

When gelatinised NMS was hydrolysed with the alpha-amylase (0.03 U/mL), the molecular weight distribution of the hydrolysate at different stages of the reaction (Fig. 10) was similar to that of WMS hydrolysates[47,48]. Initially, the reaction was fast and the material of high molecular weight (fraction **a**) was hydrolysed to dextrins of fraction **b** that were further hydrolysed to dextrins of fractions **c** (Fig. 10a). The rate of reaction then decreased, so that dextrins of fractions **c** and **d** with d.p. 50–200 accumulated (Fig. 10b). Simultaneously with the production of these intermediate dextrins, a distinct peak corresponding to dextrins with d.p. ~6 was formed.

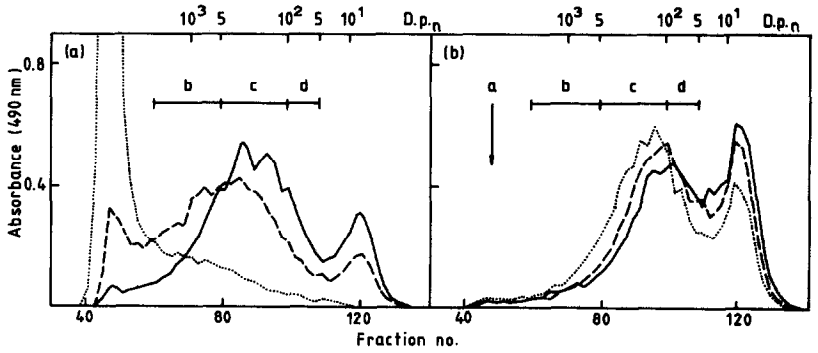

Fig. 10. Fractionation on Sepharose CL 6B of the hydrolysates of gelatinised NMS obtained after the action of alpha-amylase (0.03 U/mL). (a) NMS before (·····) and after hydrolysis for 20 (-----) and 40 min (——); (b) the hydrolysate after 60 (·····), 90 (-----), and 120 min (——): a–d show areas of fractions with different molecular weights.

DISCUSSION

NMS and WMS appeared to be similar in light microscopy. Each contained angular and rounded granules with diameters in the range 12–15 μm, typical for normal and waxy-maize starches[31,49,50]. Therefore, the total surface areas to which the alpha-amylase could adsorb were approximately the same. However, the adsorption characteristics of the starches were different (Fig. 1). The enzyme was adsorbed onto the WMS granules with high efficiency, whereas, with the NMS granules, it was dependent on the time and the concentration of the enzyme. This finding could indicate that either the surface of the granules changed so that the adsorption became weaker, or that there was a shift in the equilibrium between the binding of the enzyme to the granular starch and the starch solubilised from the granules.

The adsorption of alpha-amylases onto starch granules is dependent on pH and

temperature[33,51]. The temperature (25°) and the pH (6.5) were chosen in order to obtain a reasonable rate of solubilisation and to make the enzyme negatively charged so that it could bind to the ion-exchanger.

Despite the differences in the adsorption, only a limited difference in the rate of solubilisation between the starches was detected (Fig. 2). Initially, the waxy-starch granules were solubilised somewhat faster and similar results have been reported[31,32]. However, the amount of enzyme used affected the relative rate of solubilisation so that, when the granules were saturated with enzyme, the NMS granules were attacked with higher efficiency. When the granules were hydrolysed in the column, they behaved similarly (Fig. 6). When the rate at which the enzyme was pumped through the starch bed was low, the effect on the NMS granules was more pronounced, which depended, possibly, on the weaker adsorbtion to this starch. The rate of solubilisation was not proportional to the concentration of the enzyme added or to the enzyme that was adsorbed onto the granules (Figs. 1, 2, and 6). A ten-fold increase in the concentration of the enzyme only increased the rate of solubilisation by a factor of 2–3. Thus, a substantial part of the adsorbed enzyme did not participate in the solubilisation of the starch components.

The dextrins that could be analysed by gel-permeation chromatography after the batch-wise solubilisation experiments represented only small fragments of the material released initially (Figs. 3 and 5), because it was necessary to use either a high concentration of enzyme or a long time of incubation in order to obtain sufficient amounts of solubilised material. When the starch granules were packed into the column, a more detailed study of the solubilisation was possible. In order to obtain intact released material, low concentrations of alpha-amylase had to be used and the enzyme had to be removed as soon as possible. Therefore, the ion-exchanger should have a high enzyme-binding capacity. The capacity of the DEAE Sepharose was indicated when $<1\%$ of 120 U of alpha-amylase (3 U/mL, 40 mL, and 1 mL/min) added to the column was leached out. When the same amount of enzyme was applied, but the enzyme concentration was reduced to 0.3 U/mL, no trace of the enzyme in the eluate was detected.

The time during which the enzyme was in contact with the released products depended on the volume (~ 3.5 mL) between the starch and ion-exchange columns and the flow rate. With the flow rates used, this time varied in the range 3.5–10.6 min. The dextrins solubilised from WMS granules were resistant to hydrolysis when treated with the most dilute enzyme solution (0.003 U/mL) for 6 min (Fig. 9), which indicated that the samples collected during hydrolysis of the granule with this concentration of enzyme represented the true products (Figs. 7 and 8). With higher concentrations of enzyme, either limited or extensive further degradation occurred, which explained the great differences in the molecular weight distributions of the samples collected from the starch column at identical stages of solubilisation (Figs. 7 and 8).

Gelatinised starch is easily hydrolysed by alpha-amylases. The molecular weight distribution curves of the hydrolysates of the gelatinised NMS showed several peaks, which indicated that defined intermediate products were formed (Fig. 10). This pattern of hydrolysis was similar to that of gelatinised WMS in which branched dextrins with

d.p. 50–200 were resistant to hydrolysis and therefore accumulated[47,48]. As amylopectin comprised ~ 75% of the NMS, the gel-permeation chromatograms probably indicated mainly products therefrom.

The material solubilised from the starch granules also contained specific products. Dextrins solubilised from the granular WMS had d.p. similar to those[47] produced from gelatinised amylopectin and are indicated in some of the chromatograms in Fig. 8. Dextrins dIV and dV (within fraction **d**) and dextrin cI contained 5–8 short chains and were resistant to further hydrolysis because they probably possess clusters of branches[47]. These dextrins were suggested[6] to represent the building blocks of the larger dextrins cII, cIII, and cIV, which, in turn, represent the building units of the still-larger dextrins within fractions **c** and **b**. The products solubilised from the NMS granules had similar (if not identical) d.p. (Fig. 7) and it seems probable that they originated from the amylopectin component.

The first 1% of the solubilised products contained large products (fraction **a**, Figs. 7 and 8), the relative proportion of which was overestimated because the ion-exchanger also possessed some gel-permeation character. This conclusion was reflected by the carbohydrate content of the fractions collected initially which contained more solubilised material when WMS granules were hydrolysed (Fig. 6). Much more of the large products were solubilised from these granules than from the NMS granules (Figs. 7 and 8). This finding indicated that the amylopectin at the surface of the WMS granules was packed more loosely and therefore more easily accessible to the alpha-amylase. It would also explain the better adsorption of the enzyme onto these granules and, at least in part, the higher initial rate of solubilisation that was obtained with the lower concentrations of enzyme in the batch-wise experiments (Fig. 2). If larger fragments were released from the granules, fewer linkages would be broken. Lineback[50] suggested that the surface of starch granules has the appearance of a "hairy billiard ball".

As the solubilisation proceeded, less of the large fragments were formed and the smaller dextrins (dIV–cIII) of fractions **c** and **d** became the main products with each starch (Figs. 7 and 8). At this stage, the rate of solubilisation was similar for the starches and probably only densely packed amylopectin, largely found in the crystalline state, remained accessible to the enzyme. At this stage also, large quantities of the dextrins with d.p. ~ 6 were formed. Maltohexaose is produced from the external chains of gelatinised waxy-maize amylopectin simultaneously with the branched dextrins[52], and probably the small dextrins solubilised from the granules also originated from the external chains.

If the solubilised material originated solely from the amylopectin component, the amylose content of the residual granules should increase. However, the apparent amylose (c.l. >200) content of the NMS remained almost constant after the granules were hydrolysed in the column (Table I). In a control experiment, the blue values[53] of these granules were found to increase to 0.38–0.43 (*cf.* 0.34 for the untreated NMS). This finding indicated that the amylose content of the granules had increased. Possibly some of the amylose chains were shortened by the alpha-amylase to c.l. <200, but were still long enough to react with iodine.

In the batch-wise experiments, both the lengths and proportions of the amylose-like chains were reduced when high concentrations of enzyme were used (Table I, Fig. 4). The short chains that had been produced within the granules seemed to be associated largely with branched amyloses because the overall molecular weight distribution of the residual NMS granules did not show any increase of low molecular weight material before debranching (Fig. 3b). The reduction of the granular long-chain material seemed to be correlated with the concentration of enzyme (Table I) and probably a part of the enzyme (that not participating in the solubilisation) was not only passively adsorbed onto the granules, but also attacked the amylose component, although it was not, or only partly, solubilised. Although little is known about the organisation of starch granules, the amylose and amylopectin fractions may be distributed uniformly[32] throughout those of normal maize starch. If this is true, then the adsorbed enzyme penetrated throughout the granules in order to attack the amylose. However, it has been suggested that starch granules of wheat[54] and barley[55] contain an amylopectin-rich core and a gradient with increasing amounts of amylose towards the outer zones.

Thus, the column method enables the solubilisation of starch granules to be monitored and the products to be collected at any stage (until the granules eventually fragment and penetrate the filter). The method should be applicable to any combination of starch and an appropriate enzyme.

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