Granule-bound starch synthase I in isolated starch granules elongates malto-oligosaccharides processively

Kay DENYER^{*1}, Darren WAITE^{*}, Saddik MOTAWIA[†], Birger Lindberg MØLLER[†] and Alison M. SMITH^{*}

^{*}John Innes Centre, Norwich Research Park, Norwich, Norfolk NR4 7UH, U.K., and [†]Plant Biochemistry Laboratory, Department of Plant Biology, The Royal Veterinary and Agricultural University, 40 Thorvaldsensvej, DK-1871 Frederiksberg C, Copenhagen, Denmark

Isoforms of starch synthase belonging to the granule-bound starch synthase I (GBSSI) class synthesize the amylose component of starch in plants. Other granule-bound isoforms of starch synthase, such as starch synthase II (SSII), are unable to synthesize amylose. The kinetic properties of GBSSI and SSII that are responsible for these functional differences have been investigated using starch granules from embryos of wild-type peas and *rug5* and *lam* mutant peas, which contain, respectively, both GBSSI and SSII, GBSSI but not SSII and SSII but not GBSSI. We show that GBSSI in isolated granules elongates

malto-oligosaccharides processively, adding more than one glucose molecule for each enzyme–glucan encounter. Granule-bound SSII can elongate malto-oligosaccharides, but has a lower affinity for these than GBSSI and does not elongate processively. As a result of these properties GBSSI synthesizes longer malto-oligosaccharides than SSII. The significance of these results with respect to the roles of GBSSI and SSII *in vivo* is discussed.

Key words: amylose, GBSSI, pea mutant.

INTRODUCTION

Amylose is an essentially linear polymer of glucose that constitutes 20–35% of the starch in plant-storage organs. The study of mutants of several species has shown that one particular class of isoform of starch synthase, granule-bound starch synthase I (GBSSI), is responsible for the synthesis of amylose [1]. Other soluble and granule-bound isoforms of starch synthase act together with starch-branching enzyme to synthesize amylopectin, the branched glucan that constitutes the remainder of storage starches. We have shown [2] that the ability of GBSSI to synthesize amylose is likely to be due to an interaction between this isoform and malto-oligosaccharides. Amylose-like material is synthesized by GBSSI in starch granules isolated from developing pea embryos when these are incubated with both ADPglucose and malto-oligosaccharides of 2–7 glucose units. In the absence of malto-oligosaccharides, GBSSI elongates amylopectin chains but does not synthesize amylose [3].

There is no evidence that isoforms of starch synthase other than those of the GBSSI class can synthesize amylose [1]. In starch granules of developing wild-type pea embryos, a second isoform of starch synthase, starch synthase II (SSII), contributes 10–20% of the granule-bound starch synthase activity [4]. SSII is present in starch granules of *lam* mutant pea embryos that lack GBSSI, and yet this starch contains no amylose. In isolated granules, SSII is unable to synthesize amylose-like material in the presence or absence of malto-oligosaccharides [2]. This suggests that SSII cannot synthesize amylose due to its inability to interact with malto-oligosaccharides in the same manner as GBSSI.

In this paper, we use intact, isolated starch granules to investigate the ways in which granule-bound SSII differs from GBSSI with respect to its interactions with malto-oligo-

saccharides. This is a semi-physiological experimental system that takes account of any influence of the granule matrix on the properties of the enzymes. Starch granules from pea embryos are particularly useful for this investigation for several reasons. First, unlike the starch granules of several other storage organs, essentially all of the granule-bound starch synthase activity of the granules of pea embryos is contributed by only two isoforms, GBSSI and SSII. Other isoforms are present in the soluble fraction of pea embryos but are not incorporated into the granules [4–10]. In wheat, barley and maize endosperms and in potato tubers, granule-bound starch synthase activity is contributed by at least three different isoforms [11–13]. Second, the contribution of SSII to granule-bound activity is high. At least 10–20% of the granule-bound activity of intact granules from wild-type embryos is estimated to be contributed by this isoform [4]. Third, two mutants of pea are available that each lack one of the granule-bound isoforms but retain normal amounts of the other (as judged by SDS/PAGE of granule-bound proteins and immunoblots using specific antibodies). Granules from *lam* mutant embryos lack GBSSI but retain SSII [4], and granules from *rug5* mutant embryos lack SSII but retain GBSSI [5]. Extensive studies of these starches have not revealed any further isoforms of starch synthase [4,5,9]. Thus the use of granules isolated from *lam* and *rug5* mutant embryos allows the properties of SSII and GBSSI to be investigated separately.

In our previous experiments with isolated granules [2], we examined the incorporation of glucose from ADPglucose into polymers that were insoluble and therefore assumed to be part of the structure of the granules. In this paper, we consider the possibility that the elongation of malto-oligosaccharides by granule-bound enzymes also leads to the synthesis of products that are soluble. We report differences between GBSSI and SSII in the size and solubility of their products and show that the

Abbreviations used: GBSSI, granule-bound starch synthase I; SSII, starch synthase II; DTT, dithiothreitol; DMF, *N,N*-dimethylformamide; HPAEC, high-performance anion-exchange chromatography.

¹ To whom correspondence should be addressed (e-mail denyerk@bbsrc.ac.uk).

malto-oligosaccharides required for amylose synthesis serve as substrates for GBSSI rather than acting as activators of this isoform. The significance of these results with respect to the roles of GBSSI and SSII *in vivo* and the involvement of malto-oligosaccharides in amylose synthesis is discussed.

EXPERIMENTAL

Materials

Peas were of a round-seeded, wild-type line (*Pisum sativum* L., line BC1/RR, John Innes Germplasm Collection; see [14] for the derivation of this line), a low-amylose (*lam*) mutant line (SIM 503) or a wrinkled-seeded (*rug5*) mutant line (SIM 51, *rug-5^a*) [5]. The *lam* and *rug5* mutant lines were derived from the round-seeded line [4,15]. Plants were grown as described in [4].

Preparation of starch

Approximately 1–3 g of embryos of fresh weight 200–400 mg was homogenized in a mortar with 5–15 ml of Medium A containing 100 mM Mops (pH 7.2), 5 mM $MgCl_2$, 0.68 M glycerol, 2 mM dithiothreitol (DTT) and $1\text{ g}\cdot\text{l}^{-1}$ BSA. The homogenate was filtered through two layers of Miracloth (Behring Diagnostics, La Jolla, CA, U.S.A.), and the residue was washed in the filter cloth with a further 5–15 ml of Medium A. After centrifugation at 2000 g for 10 min, the supernatant and the green material from the surface of the pellet were discarded. The pellet was then washed first in aqueous media and then in acetone at -20°C as described by [10], and dried at room temperature. The starch was either used immediately or stored at -20°C for up to a month.

Assay of starch synthase activity

Assays contained (in a final volume of 100 μl) 10 μl of a suspension of starch granules ($25\text{ mg}\cdot\text{ml}^{-1}$ in Medium A), 1 mM ADP-[U- ^{14}C]glucose (Amersham International, Little Chalfont, Bucks., U.K.) at $2.3\text{ GBq}\cdot\text{mol}^{-1}$, 100 mM Bicine (pH 8.5), 25 mM potassium acetate, 10 mM DTT, 5 mM EDTA and maltotriose (concentration as specified in the Figure legends). Assays were incubated at 25°C for 20 min. Controls were terminated immediately after the addition of starch granules to the assay. All assays were duplicated. Assays were terminated and processed by one of the following methods.

Methanol/KCl method

Assays were terminated by the addition of 3 ml of 18.4 M methanol/0.13 M KCl (methanol/KCl). After incubation at $0-4^\circ\text{C}$ for at least 5 min, starch was collected by centrifugation at 2000 g for 5 min and resuspended in 0.3 ml of water. This methanol/KCl wash, centrifugation and resuspension were repeated twice more. Radioactivity in the resuspended pellet was determined by liquid-scintillation counting.

Resin method

Assays were terminated by heating to $90-100^\circ\text{C}$ for 2 min. To ensure that the products passed freely through the resin, the assays were incubated at 37°C for 3 h with 100 μl of 50 mM sodium acetate (pH 5.2) containing 1 unit of amyloglucosidase and 20 units of α -amylase. After incubation, assays were heated to 90°C for 2 min and then subjected to centrifugation at 10000 g for 2 min. Columns of Dowex 1-X8 anion-exchange resin (200–400 mesh, Cl^- form; Sigma Chemical Co., Poole, Dorset, U.K.) were prepared in 1-ml pipette tips plugged with cotton wool by adding 1 ml of a suspension of $0.5\text{ g}\cdot\text{ml}^{-1}$ resin in

water. Excess water was removed by centrifugation of the columns at 50 g for 5 min immediately before use. A 175- μl sample of the assay supernatant was added to the resin column and it was subjected to centrifugation at 50 g for 5 min. To elute the column, three 50- μl aliquots of water were added in succession. After each addition, the column was subjected to centrifugation as above. Radioactivity in the column eluate was determined by liquid-scintillation counting.

To investigate whether amylose was synthesized as part of the amylopectin molecule and then cleaved off, wild-type starch granules were incubated for 15 min in the assay medium described above, collected by centrifugation at 14000 g for 2 min and resuspended in 200 μl of assay medium as above except that the ADPglucose was unlabelled. Granules were incubated at 25°C for a further 0, 45, 180 or 360 min and then processed by the methanol/KCl method. In the longer incubations, the rate of incorporation of glucose into starch decreases with time. The rate of starch synthesis is linear for 60 min after which the rate of incorporation slows (results not shown). To ensure that ADPglucose was available throughout the longest incubation of 360 min, the incubation medium was replaced after 180 min by centrifugation and resuspension of the granules in fresh medium.

Assays containing ^{14}C -labelled maltose

These were as above for the starch synthase assays except that maltotriose was replaced by 0.5 mM [^{14}C]maltose ($7.9\text{ GBq}\cdot\text{mmol}^{-1}$) and ADPglucose was unlabelled. A low concentration of maltose was used to achieve a high specific radioactivity. After incubation, assays were processed by the methanol/KCl method.

Samples for gel-permeation chromatography

^{14}C -labelled soluble products of granule-bound starch synthases were obtained by incubating 10 μl of a suspension of starch granules ($200\text{ mg}\cdot\text{ml}^{-1}$ in Medium A), 1 mM ADP-[U- ^{14}C]glucose (Amersham International) at $18.5\text{ GBq}\cdot\text{mol}^{-1}$, 100 mM Bicine (pH 8.5), 25 mM potassium acetate, 10 mM DTT, 5 mM EDTA and 100 mM maltotriose in a final volume of 100 μl at 25°C for 60 min. After centrifugation at 10 000 g for 2 min, the supernatant was added to a resin column which was prepared, centrifuged and eluted as described above, except that the eluent was two 20- μl aliquots of water. The column eluate was concentrated to a volume of 50 μl by centrifugation under vacuum.

^{14}C -labelled insoluble products were prepared in the same manner except that incubations contained 5 μl of starch-granule suspension. Incubations were terminated and processed as described above (methanol/KCl method). After the final centrifugation, the pellet was dried in air at room temperature for at least 10 min, dissolved in 50 μl of 1 M NaOH and diluted with 50 μl of distilled water. Samples for chromatography on Sepharose CL2B were prepared as described in [2].

Gel-permeation chromatography

Chromatography on columns [0.5 cm (inner diameter) $\times$ 30 cm] of Sepharose CL6B or Sepharose CL2B was carried out as described in [2]. Glucans in fractions from unlabelled samples were detected by their reaction with iodine solution as described in [4]. Radioactivity was determined by liquid-scintillation counting. The recovery of radioactivity from each Sepharose column was monitored and was routinely greater than 80% of that applied.

Preparation of samples for high-performance anion-exchange chromatography (HPAEC) analysis

Samples of labelled soluble products of granule-bound starch synthases were prepared as described above for Sepharose CL6B chromatography except that the column eluate was not concentrated by centrifugation under vacuum.

Analysis by HPAEC

All samples were passed through a 0.2 μm filter before injection of an aliquot (25 μl) on to a CarboPac PA 100 column [4 mm (inner diameter) $\times$ 250 mm] fitted with a guard column [4 mm (inner diameter) $\times$ 50 mm] and attached to an HPLC system with a pulsed amperometric detector [Dionex (UK), Camberley, Surrey, U.K.]. The column was equilibrated with 107.5 mM NaOH/75 mM sodium acetate and eluted with a 45-ml linear gradient of 107.5–130 mM NaOH/75–300 mM sodium acetate at 1 ml $\cdot$ min $^{-1}$ (soluble products). The position of elution of the labelled products was compared with the position of elution of a mixture of authentic glucose, maltose and malto-oligosaccharides ranging from maltotriose to maltoheptaose.

Chemical synthesis of maltose analogues with a modified non-reducing end

The strategy for synthesis of the target maltose analogue (**4**) from maltose is outlined in Scheme 1(A). Benzylidenation of maltose monohydrate (**1**) with α,α -dimethoxytoluene in *N,N*-dimethylformamide (DMF) in the presence of *p*-toluenesulphonic acid according to [16] afforded 4',6'-*O*-benzylidenemaltose. Benzyl-ation of the product using benzyl bromide in DMF-NaH and subsequent removal of the benzylidene group using 80 % acetic acid gave compound **2**. The 6'-OH group of **2** was selectively

silylated with *t*-butylchlorodiphenylsilane in DMF-imidazole and the 4'-OH group of **2** was then acetylated with acetic anhydride in pyridine to give compound **3**. The silyl group at the 6'-OH group serves to protect acetyl migration during the dibenzylation. Removal of benzyl protecting groups by catalytic hydrogenation over 10 % palladium on carbon in ethanol/ethyl acetate and subsequent desilylation with tetrabutylammonium fluoride in tetrahydrofuran gave the target compound (**4**).

The strategy for the synthesis of the other maltose analogue (**10**) is shown in Scheme 1(B), and involves the conversion of D-glucose into a suitable glycosyl acceptor (**6**) as well as the conversion of D-galactose (**7**) into a glycosyl donor (**8**).

Previously, we reported the synthesis of the glycosyl acceptor phenyl 2,3,6-tri-*O*-benzyl-1-thio- β -D-glucopyranoside (**6**) from D-glucose (**5**) in six steps [17]. In a similar manner, D-galactose (**7**) was modified and converted into the glycosyl donor, *O*-(α,β -D-galactopyranosyl)trichloroacetimidate (**8**) in five steps. The glycosidation reaction between **6** and **8** in diethyl ether using trimethylsilyl triflate as a catalyst resulted in the formation of the desired thioglycoside product (**9**). Replacement of the phenylthio group of **9** by a free OH group [18] and the removal of the benzyl protecting groups yielded the target molecule (**10**).

All intermediates were purified to homogeneity and their structures ascertained by ^1H - and ^{13}C -NMR spectroscopy. Experimental details and proof of the identity of the synthesized products will be published elsewhere.

RESULTS

In the presence of malto-oligosaccharides, starch synthases in isolated granules synthesize soluble as well as insoluble glucans

We showed previously that, in the presence of malto-oligosaccharides and ADP-[^{14}C]glucose, GBSSI within the granule synthesized ^{14}C -labelled material that was insoluble in methanol/

Scheme 1 Strategy for chemical synthesis of two analogues of maltose

The pathway of synthesis of two maltose analogues (**4** and **10**) from maltose (**1**) is shown in (A) and (B), respectively. The 4'-hydroxyl group was modified by acetylation (A) or shifted to the epimeric position (B) to prevent elongation of the molecule by starch synthase.

KCl and of a similar molecular mass to amylose [2]. In earlier experiments, Leloir et al. [19] showed that enzymes in starch granules isolated from *Phaseolus vulgaris* catalysed the transfer of glucose from UDPglucose to malto-oligosaccharides. As well as insoluble products that were not removed from the granules when these were washed with ethanol, Leloir et al. [19] observed small, ethanol-soluble malto-oligosaccharide products. Such soluble products are too small to precipitate in methanol/KCl and would not have been revealed in our previous experiments. To compare the synthesis of soluble and insoluble products by different granule-bound isoforms of starch synthase, we used an assay method designed to measure all of the products of starch synthase, regardless of their size or solubility in methanol/KCl, as well as the usual methanol/KCl assay method [20]. With the former method, labelled products were separated from the substrate, ADP[14 C]glucose, using columns of anion-exchange resin (see the Experimental section). All incubations contained 1 mM ADPglucose, a concentration likely to be very close to that in the plastids of wild-type pea embryos *in vivo* (B. R. Clarke, personal communication).

The starch synthase activities of starch granules isolated from wild-type, *lam* and *rug5* pea embryos were estimated using both the resin and methanol/KCl methods (Figure 1A). In incubations without malto-oligosaccharides, the two methods gave very similar estimates of the amount of product formed for all three types of granule. This showed that small soluble products were not formed and that degradation of starch during the incubations (which can interfere with estimates of starch synthase activity made by the methanol/KCl method) [14] was insignificant. In the presence of malto-oligosaccharide (maltotriose), the resin-assay method gave a considerably higher estimate of the amount of product formed than the methanol/KCl method for all three

A. Assays lacking maltotriose

B. Assays containing 100 mM maltotriose

Figure 1 Estimates of granule-bound starch synthase activity using the resin and methanol/KCl assay methods

Granules isolated from wild-type, *lam* or *rug5* embryos (containing GBSSI and SSII, only SSII or only GBSSI, respectively) were incubated with ADP-[14 C]glucose with or without maltotriose. After incubation, the assays were processed as described in the Experimental section by either the methanol/KCl method (hatched bars) or the resin method (black bars). Data are means $\pm$ S.D. of measurements on three independent preparations of starch. All assays were done in duplicate.

Figure 2 Estimation of $S_{0.5}$ values of granule-bound starch synthases for maltotriose

Isolated granules were incubated with ADP-[14 C]glucose and various concentrations of maltotriose. After incubation, the incorporation of label into fractions containing amylose (A) and amylopectin (results not shown) and into soluble products (B) was determined. The results of typical experiments are shown. (A) The effect of maltotriose concentration on the incorporation of label into amylose (●) in incubations containing granules isolated from wild-type pea embryos. Values are for individual incubations, each processed separately on a column of Sepharose CL2B. The line joins mean values of duplicate assays. (B) The effect of maltotriose concentration on the incorporation of label into soluble products in incubations of granules isolated from wild-type embryos (GBSSI and SSII, ●), *lam* embryos (SSII, ○) and *rug5* embryos (inset panel, GBSSI, ▲). Values are for individual incubations. The lines join mean values of duplicate assays.

types of granule (Figure 1B). This suggests that, in the presence of malto-oligosaccharide, products that are soluble in methanol/KCl are made by both GBSSI and SSII.

We investigated the nature of the methanol/KCl-insoluble products of GBSSI and SSII to determine the extent to which they were part of the structure of the granules. These products could include not only glucans within the granules but also malto-oligosaccharides that were soluble in the aqueous incubation medium but were of a sufficient size to be precipitated by methanol/KCl. After incubations of the granules from wild-type embryos with ADP[14 C]glucose and maltose, the granules were washed three times with either water or methanol/KCl solution. In two separate experiments, the amounts of label recovered in the insoluble fractions after washing with water were 103% and 113% of those recovered after washing with methanol/KCl. This showed that the methanol/KCl-insoluble product was also water insoluble and therefore likely to be part of the structure of the granules.

Figure 3 Elution of glucan polymers and the soluble and insoluble products of granule-bound starch synthases from columns of Sepharose CL6B

Granules isolated from wild-type, *rug5* or *lam* embryos were incubated with ADP[^{14}C]glucose and 100 mM maltotriose. After incubation, the assays were either processed by the methanol/KCl method or the soluble material was separated from the granules by centrifugation and processed by the resin method. The insoluble ($\circ$) and soluble products (Δ) were subjected to chromatography on columns of Sepharose CL6B and the elution of labelled glucans was monitored by liquid-scintillation counting. The elution of samples of unlabelled starch (prepared as described in the Experimental section) was determined by mixing fractions eluted from the columns with iodine/KI solution and measuring the absorbance of the starch-iodine complex at 595 nm (dotted lines).

GBSSI has a greater affinity for malto-oligosaccharides than SSII

To determine the concentration of malto-oligosaccharides required for amylose synthesis by GBSSI, we measured the rate of amylose synthesis in incubations of granules with a range of concentrations of maltotriose (Figure 2A). A plot of the incorporation of [^{14}C]glucose into amylose/maltotriose concentration against maltotriose concentration (Hanes plot) was used to calculate $S_{0.5}$, the concentration of maltotriose at which the rate of amylose synthesis was half of its limiting rate (V_{max}). In two independent experiments, the $S_{0.5}$ was 0.10 mM and 0.06 mM. Thus GBSSI within the granule is saturated substantially at maltotriose concentrations of less than 1 mM.

To compare the affinities of GBSSI and SSII for malto-oligosaccharides, we measured the incorporation of label from ADP[^{14}C]glucose into soluble products by granules isolated from wild-type, *rug5* and *lam* embryos in incubations containing

a range of concentrations of maltotriose (Figure 2B). In independent experiments, the values for $S_{0.5}$ for the synthesis of soluble products were 2.0, 3.3 and 6.0 mM for wild-type granules, 0.16 and 0.5 mM for *rug5* granules and 11.25 and 14.3 mM for *lam* granules. Thus GBSSI has a high affinity for malto-oligosaccharides for the synthesis of both amylose and soluble products and its affinity for malto-oligosaccharides is at least 20-fold greater than that of SSII.

The insoluble products of GBSSI are longer on average than the soluble products of GBSSI and SSII

It seemed likely that the insoluble products of GBSSI were long glucans, trapped inside the granule, whereas the soluble products of GBSSI and SSII were shorter glucans able to diffuse out of the granule. To investigate this, we compared the positions of elution of insoluble and soluble products from a column of Sepharose CL6B (Figure 3). Most of the soluble products from wild-type, *lam* and *rug5* granules eluted from the column later than both insoluble products and the unlabelled amylose and amylopectin. However, there was some overlap in the positions of elution of the insoluble and soluble products from wild-type and *rug5* granules (Figures 3A and 3B). These data suggest that the average size of the insoluble amylose synthesized by GBSSI during the incubation was greater than that of the soluble products but that there may have been some glucans of similar sizes in the two fractions.

Comparison of the profiles of elution of the unlabelled starch and the labelled insoluble products for wild-type and *rug5* granules (Figures 3A and 3B), showed that much of the label was incorporated into glucans that eluted later than the bulk of the unlabelled amylose and amylopectin. This suggests that the amylose-like material synthesized by GBSSI during the 1-h incubations was smaller, on average, than the unlabelled amylose.

HPAEC analysis of the soluble products of GBSSI and SSII

To determine whether the soluble glucans synthesized by GBSSI and SSII were made by elongation of the supplied malto-oligosaccharide, their sizes were examined in detail by HPAEC. In incubations of granules from wild-type and *rug5* embryos with ADP[^{14}C]glucose and maltose, the major soluble product was [^{14}C]maltotriose (Figures 4A and 4D). When granules were incubated with ADP[^{14}C]glucose and maltopentaose, the major soluble product was [^{14}C]maltohexaose (Figure 4B and results not shown). Smaller amounts of longer malto-oligosaccharides were also produced in both cases. This suggests strongly that malto-oligosaccharide acts as a primer to which GBSSI adds one or more [^{14}C]glucose units from ADP[^{14}C] glucose. With *lam* granules, containing SSII but no GBSSI, all of the product was only one glucose unit longer than the supplied malto-oligosaccharide (maltotriose, Figure 4C; maltohexaose, results not shown). No longer products were detected. These results suggest that GBSSI and SSII elongate malto-oligosaccharides by different mechanisms.

Do malto-oligosaccharides act as primers for amylose synthesis?

The experiments described above show that the small soluble products of GBSSI are short-chain malto-oligosaccharides synthesized by elongation of the malto-oligosaccharides supplied in the incubation medium. The larger insoluble products of GBSSI, which are synthesized only in the presence of malto-oligosaccharides [2], could be: (i) products of the same process of elongation of the supplied malto-oligosaccharide; (ii) pre-existing

A. Wild-type starch incubated with maltose

B. Wild-type starch incubated with maltopentaose

C. *lam* starch incubated with maltotriose D. *rug5* starch incubated with maltotriose

Figure 4 HPAEC analysis of the soluble products of GBSSI and SSII

Granules isolated from wild-type, *rug5* or *lam* embryos were incubated with ADP[^{14}C]glucose and 100 mM maltose, maltotriose or maltopentaose. After incubation, the soluble material was separated from the granules by centrifugation, processed by the resin method to remove ADP[^{14}C]glucose and analysed by HPAEC on a CarboPac PA100 column as described in the Experimental section. The elution of labelled glucans was monitored by liquid-scintillation counting (■) and the elution of standards (glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose) was monitored with a pulsed amperometric detector (plain black line). The recovery of label from the column in these experiments was in the range 67–120% of that applied.

amylose molecules elongated by GBSSI when this enzyme is activated in some way in the presence of malto-oligosaccharides; or (iii) chains of amylopectin synthesized by GBSSI, which are cleaved off in the presence of malto-oligosaccharides. We have used three different approaches to determine whether malto-oligosaccharides act as substrates or as effectors of GBSSI in the synthesis of insoluble products.

First, we synthesized two analogues of maltose modified at the non-reducing end, namely 4-*O*-acetyl- α -D-glucopyranosyl-(1-4)-D-glucopyranose (**4**, Scheme 1A) and α -D-galactopyranosyl-(1-4)-D-glucopyranose (**10**, Scheme 1B). The modifications had minimal effects on the shapes and charges of the molecules and were therefore unlikely to prevent the modified maltose acting as an activator of starch synthase. However, the modifications at the non-reducing end specifically prevented elongation of the maltose analogue by starch synthase. Incubation of wild-type granules with maltose or the modified maltoses showed that, as expected, the modified maltoses could not be elongated to form soluble products (Figure 5A). Analysis of the insoluble products on columns of Sepharose CL2B showed that there was no incorporation of [^{14}C]glucose into amylose in incubations containing modified maltoses (Figure 5B). These results are consistent with the idea that amylose is synthesized by GBSSI by elongation of malto-oligosaccharides. However, we cannot rule out the possibility that chemical modification of the maltose also prevented it from acting as an effector of GBSSI.

Second, to determine whether malto-oligosaccharides were incorporated into the insoluble products, we incubated starch granules with [^{14}C]maltose and unlabelled ADPglucose and analysed the insoluble products by chromatography on

Sepharose CL6B (Figure 6). On this column, smaller amylose molecules were well separated from maltose and the largest amylose molecules eluted with the amylopectin. There was considerable labelling of insoluble products from [^{14}C]maltose. Most of the products were relatively small, but some were comparable in size with unlabelled amylose. These results suggest that the amylose synthesized by GBSSI in our incubations was generated by the elongation of the added malto-oligosaccharides.

Third, we considered the possibility that amylose could be synthesized as part of the amylopectin molecule and then cleaved off. The promotion of amylose synthesis by malto-oligosaccharides in this case could reflect either stimulation by these compounds of synthesis of long chains destined to become amylose, or stimulation of the rate of cleavage of amylose from its precursor by an unknown mechanism, or both of these. The synthesis of amylose by cleavage of chains from amylopectin has been demonstrated to occur during prolonged incubations of granules isolated from the monocellular alga *Chlamydomonas reinhardtii* [21]. To examine this in granules from pea embryos, we incubated the granules for a short period with ADP[^{14}C]glucose (the pulse, 15 min) and then with unlabelled ADPglucose (the chase, 45 min, 3 h or 6 h) and looked for transfer of label from the amylopectin to the amylose fraction. There were no detectable differences between the pulse and chase samples in the ratios or amounts of label in the amylopectin and amylose fractions, whether malto-oligosaccharide was present in the incubations or not. This was true both for granules prepared and dried before use in the usual manner, and freshly prepared granules that had not been dried before use (Figure 7 and results not shown). Thus [^{14}C]glucose incorporated into

A. Granule-bound starch synthase activity assayed using the methanol/KCl and resin methods.

B. The incorporation of [14 C]glucose into methanol/KCl insoluble products.

Figure 5 Effect of the chemical modification of maltose on granule-bound starch synthase activity

Granules isolated from wild-type embryos were incubated with ADP[14 C]glucose alone or with ADP[14 C]glucose and 25 mM of either maltose or analogues of maltose. The synthesis of the maltose analogues is described in Scheme 1. After incubation, the assays were either processed by the methanol/KCl method (A; hatched bars) or the resin method (A; black bars). Samples of the insoluble material were analysed by monitoring the elution of label from a column of Sepharose CL2B as described in the Experimental section (B: line with no symbol, no malto-oligosaccharide; $\circ$, maltose; $\triangle$, maltose analogue synthesized according to Scheme 1A; $\square$, maltose analogue synthesized according to Scheme 1B).

Figure 6 Incorporation of [14 C]maltose into amylose-like material

Granules isolated from wild-type embryos were incubated with 1 mM ADPglucose/0.5 mM [14 C]maltose or with 1 mM ADP[14 C]glucose/0.5 mM maltose. After incubation, the assays were processed by the methanol/KCl method and samples of the insoluble material were analysed by monitoring the elution of labelled glucans from a column of Sepharose CL6B as described in the Experimental section. To estimate the sizes of the labelled products, their positions of elution were compared with those of labelled maltose and unlabelled starch polymers prepared as described in the Experimental section. Unbroken line, unlabelled starch polymers; $\square$, products of incubations including ADP[14 C]glucose; $\triangle$, products of incubations including [14 C]maltose; and $\blacktriangle$, [14 C]maltose.

A. Incubations lacking malto-oligosaccharides

B. Incubations containing 100 mM maltotriose.

Figure 7 Incubation of granules with unlabelled ADPglucose following incubation with ADP[14 C]glucose

Granules isolated from wild-type embryos were incubated with ADP[14 C]glucose with or without 100 mM maltotriose. After incubation for 15 min, the granules were isolated by centrifugation and either processed immediately by the methanol/KCl method (pulse; $\circ$) or incubated for a further 45 min in unlabelled ADPglucose without (A) or with (B) 100 mM maltotriose before processing (pulse + chase; $\triangle$). The insoluble material was subjected to chromatography on a column of Sepharose CL2B as described in the Experimental section. Data are means of values from two individual incubations. Incubation of starch granules with ADP[14 C]glucose for 15 min followed by incubation with unlabelled ADPglucose for 3 or 6 h gave the same result as that shown.

amylopectin during the pulse was not transferred into amylose in the subsequent chase, providing good evidence that amylose is not derived from amylopectin in granules isolated from developing pea embryos.

DISCUSSION

In previous experiments, we showed that GBSSI in isolated granules synthesized amylose-like material only when malto-oligosaccharides were included in the incubations [2]. We have now shown that this amylose is a result of the elongation of the supplied malto-oligosaccharide by GBSSI. First, label from [14 C]maltose was incorporated into amylose-like material. Second, amylose-like material was not synthesized in incubations containing chemically synthesized maltose analogues which could not be elongated by starch synthase because they had modified non-reducing ends.

We found that GBSSI synthesized short soluble glucan chains as well as the longer glucan chains that constituted the amylose-like material within the granule. A similar result was reported by Leloir et al. [19] using starch granules isolated from developing beans, although they used UDPglucose rather than ADPglucose as the glucose donor. The fate of the elongated malto-oligosaccharide products of granule-bound starch synthases may depend upon their size. Solutes larger than 1 kDa (maltoheptaose

has a molecular mass of approx. 1 kDa) do not readily penetrate hydrated starch granules [22]. Therefore, the larger products of GBSSI will probably not diffuse out of the granules into the surrounding medium whereas the shorter ones will do so. In support of this idea, we found that the amylose-like material was not removed from the granules by extensive washing with water, suggesting that these products were trapped within the granule matrix.

Like GBSSI, SSII is also able to elongate malto-oligosaccharides. However, there are fundamental differences between these two isoforms of starch synthase in their interactions with malto-oligosaccharides and the nature of the products that they synthesize. First, the isoforms differ with respect to their affinities for malto-oligosaccharides. The $S_{0.5}$ value of GBSSI was estimated to be 0.05–0.5 mM with respect to maltotriose, whereas that for SSII was greater than 10 mM. Thus GBSSI has a much higher affinity for maltotriose than SSII. In most of our assays, we used concentrations of malto-oligosaccharides (100 mM) that were saturating with respect to both GBSSI and SSII. To our knowledge, there are no estimates of the concentrations *in vivo* of malto-oligosaccharides in the plastids of any plant organ. Therefore it is not possible to determine whether malto-oligosaccharide elongation by either enzyme is likely to occur to any significant extent *in vivo*. However, we suggest that, due to the very high affinity of GBSSI for malto-oligosaccharides, elongation by this isoform is highly probable.

Second, the sizes of the soluble products synthesized by SSII and GBSSI differed in a manner which indicates that the two isoforms differ in their mechanism of malto-oligosaccharide elongation. When incubated in the presence of high concentrations of malto-oligosaccharides, GBSSI synthesized a range of sizes of glucan chains whereas SSII added only a single glucose unit to the supplied malto-oligosaccharide. The concentration of malto-oligosaccharide substrate (100 mM) in these experiments was much higher than the concentration of the malto-oligosaccharide product (≤ 1 mM). Thus it is highly improbable that a second glucose unit would be added to the initial malto-oligosaccharide product if the enzyme and product dissociated after each addition. The most likely explanation for our results is that GBSSI elongates malto-oligosaccharides processively, adding more than one glucose unit at each enzyme–glucan encounter, whereas SSII acts in a distributive manner, dissociating from the glucan after adding a single glucose unit.

A number of other enzymes involved in the synthesis, degradation or modification of polymers have been shown to act processively (e.g. DNA polymerase I [23] and bacterial cellulose synthase [24]). In some cases, the three-dimensional structure of the enzyme is known and provides an explanation for the processive nature of the reaction. For example, DNA polymerase I from *Escherichia coli* has a cleft that can accommodate a double-stranded DNA helix. Enclosure of the substrate, DNA, within the binding cleft is predicted to decrease the rate of dissociation of the enzyme–DNA complex while allowing the polymerase to move along the template DNA strand. Thus several nucleotides (typically 20–25) are added to the primer strand before the enzyme–DNA complex dissociates [23]. Comparison of the three-dimensional structures of GBSSI and SSII may help to explain the kinetic differences between these isoforms.

We stress that our results demonstrate differences between the mechanisms of action of GBSSI and SSII within isolated starch granules. We cannot determine from this work alone whether these mechanisms also operate *in vivo* or whether other amylopectin-synthesizing isoforms of starch synthase behave like SSII in elongating malto-oligosaccharides distributively. However, as a working hypothesis, we propose the following ex-

planation of the unique ability of GBSSI to synthesize amylose *in vivo*. We propose that small malto-oligosaccharides generated in the plastid (by the actions of enzymes such as amylases, starch-debranching enzymes, disproportionating enzyme and starch phosphorylase) diffuse into the starch granule where they are elongated processively by GBSSI to a size that is too great to allow them to diffuse readily out of the granule matrix. They thus become the amylose component of the granule. Other granule-bound isoforms of starch synthase such as SSII, at least in part because of their low affinity for malto-oligosaccharides and their distributive mode of action, are unable to elongate malto-oligosaccharides sufficiently to immobilize them within the granule. Further work is required to establish the validity of this model. In an as-yet-unpublished study (Denyer, K., Waite, D., Edwards, E. A., Martin, C. and Smith, A. M., unpublished work), we investigate whether processivity is an intrinsic property of GBSSI and not SSII, or whether this difference is conferred by features of the granule matrix, by studying the properties of these enzymes in soluble form.

We have found no evidence for amylose synthesis by cleavage of chains from amylopectin. There was no transfer of [14 C]glucose from amylopectin to amylose in incubations of up to 6 h. Such transfer has been shown to occur during prolonged incubations with ADPGlucose of starch granules isolated from the green alga *Chlamydomonas*, in addition to the synthesis of amylose by elongation of maltotriose [21]. The authors proposed that the main route of synthesis of amylose *in vivo* is by elongation by granule-bound starch synthase of amylopectin chains and the subsequent cleavage of these chains, rather than by elongation of malto-oligosaccharides. It is not clear whether these differences between results from pea starch and *Chlamydomonas* starch represent differences between higher plants and algae in the mechanism of amylose synthesis, or technical differences between the experiments.

This work was supported by a competitive grant from the Biotechnology and Biological Sciences Research Council, by the EU FAIR programme and by the Danish Food Technology programme. We thank Dr. Cliff Hedley and Dr. Trevor Wang, John Innes Centre, Norwich, U.K. for the gift of the peas, and Dr. Kim Tomlinson, Dr. Cathie Martin and Dr. Noel Ellis for their constructive criticism of the manuscript.

REFERENCES

- Smith, A. M., Denyer, K. and Martin, C. (1997) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **48**, 67–87
- Denyer, K., Clarke, B., Hylton, C., Tatge, H. and Smith, A. M. (1996) *Plant J.* **10**, 1135–1143
- Baba, T., Yoshii, M. and Kainuma, K. (1987) *Starch* **39**, 52–56
- Denyer, K., Barber, L. M., Burton, R., Hedley, C. L., Hylton, C. M., Johnson, S., Jones, D. A., Marshall, J., Smith, A. M., Tatge, H. et al. (1995) *Plant Cell Environ.* **18**, 1019–1026
- Craig, J., Lloyd, J., Tomlinson, K., Barber, L., Edwards, A., Wang, T., Martin, C., Hedley, C. and Smith, A. M. (1998) *Plant Cell* **10**, 413–426
- Craig, J. (1996) Ph.D. Thesis, University of East Anglia, U.K.
- Edwards, A., Marshall, J., Denyer, K., Sidebottom, C., Visser, V. G. F., Martin, C. and Smith, A. M. (1996) *Plant Physiol.* **112**, 89–97
- Denyer, K., Sidebottom, C., Hylton, C. M. and Smith, A. M. (1993) *Plant J.* **4**, 191–198
- Denyer, K., Barber, L., Edwards, E. A., Smith, A. M. and Wang, T. (1997) *Plant Cell Environ.* **20**, 1566–1572
- Smith, A. M. (1990) *Planta* **182**, 599–604
- Denyer, K., Hylton, C. M., Jenner, C. F. and Smith, A. M. (1995) *Planta* **196**, 256–265
- Hylton, C. M., Denyer, K., Keeling, P. L., Chang, M.-T. and Smith, A. M. (1996) *Planta* **198**, 230–237
- Marshall, J., Sidebottom, C., Debet, M., Martin, C., Smith, A. M. and Edwards, A. (1996) *Plant Cell* **8**, 1121–1135
- Hedley, C. L., Smith, C. M., Ambrose, M. J., Cook, S. and Wang, T. L. (1986) *Ann. Bot.* **58**, 371–379

-
- 15 Wang, T. L. and Hedley, C. L. (1993) *Pisum Genetics* **25**, 64–70
- 16 Takeo, K. and Shinmitsu, K. (1984) *Carbohydr. Res.* **133**, 135–145
- 17 Motawia, M. S., Olsen, C. E., Enevoldsen, K., Marcussen, J. and Møller, B. L. (1995) *Carbohydr. Res.* **277**, 109–123
- 18 Motawia, M. S., Marcussen, J. and Møller, B. L. (1995b) *J. Carbohydr. Chem.* **14**, 1279–1294
- 19 Leloir, L. F., de Fekete, M. A. and Cardini, C. E. (1961) *J. Biol. Chem.* **236**, 636–641
- 20 Jenner, C. F., Denyer, K. and Hawker, J. S. (1994) *Aust. J. Plant Physiol.* **21**, 17–22
- 21 van de Wal, M., D'Hulst, C., Vincken, J.-P., Buléon, A., Visser, R. and Ball, S. (1998) *J. Biol. Chem.* **273**, 22232–22240
- 22 French, D. (1984) in *Starch: Chemistry and Technology*, 2nd edition (Whistler, R. L., BeMiller, J. N. and Paschall, E. F., eds.), pp. 183–248, Academic Press, San Diego
- 23 Joyce, C. M. and Steitz, T. A. (1987) *Trends Biochem. Sci.* **12**, 288–292
- 24 Koyama, M., Helbert, W., Imai, T., Sugiyama, J. and Henrissat, B. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94**, 9091–9095
-

Received 16 November 1998/5 February 1999; accepted 26 February 1999