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The effect of *waxy* mutations on the granule-bound starch synthases of barley and maize endosperms

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Abstract. The effects of *waxy* mutations on starch-granule-bound starch synthases (EC 2.4.1.18) in the developing endosperm of barley (*Hordeum vulgare* L.) and maize (*Zea mays* L.) have been investigated. Three granule-bound starch synthases in barley endosperm were identified by use of antibodies to known starch synthases, by reconstitution and assay of individual proteins from sodium dodecyl sulphate-polyacrylamide gels of granule-bound proteins, and by partial purification of proteins released by enzymic digestion of starch. These are proteins of 60, 77 and 90 kDa. Use of antibodies to known starch synthases and partial purification of proteins released by enzymic digestion of starch indicated that there may be at least four granule-bound starch synthases in maize endosperm: proteins of 59, 74, 77 and 83 kDa. Mutations at the *waxy* loci of both species affected only the 60- (barley) and 59- (maize) kDa isoforms. No evidence was found that other putative isoforms are altered in abundance or activity by the mutations. The contribution of our results to understanding of the starch synthase activity of intact starch granules and the mechanism of amylose synthesis is discussed.

Key words: Endosperm – *Hordeum* – Starch granule – Starch synthase – *waxy* mutation – *Zea*

Introduction

The aim of this work was to improve our understanding of the effects of *waxy* mutations on starch synthases located on starch granules in cereal endosperms. There is good evidence that a class of granule-bound isoforms of starch synthase (which will be referred to as granule-bound starch synthase I: GBSSI) is exclusively responsible for the synthesis of the amylose component of storage starch (Preiss 1992; Smith and Martin 1993). Mutations in genes encoding GBSSI isoforms (at the *waxy* loci in cereals, e.g. Shure et al. 1983; Rohde et al. 1988) almost eliminate both the granule-bound starch synthase activity and the amylose component of the starch from storage organs. In the wild type, GBSSI is usually the major granule-bound protein of storage organs. It has a molecular mass of about 60 kDa, and is highly conserved in primary amino-acid sequence between species (Dry et al. 1992; Ainsworth et al. 1993).

Isoforms of starch synthase distinct from GBSSI are also located on starch granules in storage organs. Starch granules of pea embryo have an isoform of 77 kDa (pea GBSSII) in addition to GBSSI, which is quite distinct from GBSSI in predicted amino-acid sequence (Smith 1990; Dry et al. 1992). Starch granules of wheat endosperm and potato tuber also have an isoform similar to pea GBSSII (Denyer et al. 1995b; Edwards et al. 1995) and we have also detected antigenically-related proteins of 77 kDa on the storage starches of several other species. It seems likely there are also other granule-bound starch synthases, the nature of which may differ from one species to another. Starch granules of wheat endosperm have a 100- to 105-kDa isoform (Denyer et al. 1995b) and granules from wild-type maize endosperm are reported to have isoforms of 93 kDa (Macdonald and Preiss 1985) and 76 kDa (Mu et al. 1994).

The roles of granule-bound starch synthases other than GBSSI are not known. The phenotype of *waxy* mutants indicates that these isoforms are not important in amylose synthesis, and that they make only a minimal contribution to the activity of granule-bound starch synthase. However, these conclusions are based on the assumption that these isoforms are not affected by the *waxy* mutations. For maize, there is evidence that this assumption is incorrect. It has been reported that starch of wild-type maize endosperm has a single isoform of starch synthase other than GBSSI, a protein of 93 kDa, whereas the starch of waxy maize has two distinct activities, associated with proteins of 70 and 60 kDa (Macdonald and Preiss 1983, 1985). In pea embryo and potato tuber, however, mutations which eliminate GBSSI (at the *amf* locus of potato and the *lam* locus of pea: Visser et al. 1989; Denyer et al. 1995a) do not appear to affect the nature of other granule-bound starch synthases (Denyer et al.

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1995a; Edwards et al. 1995). Cereal endosperms may thus differ in this respect from other starch-storing organs.

Information about the effects of *waxy* mutations on all of the granule-bound isoforms of starch synthase in cereal endosperms is clearly required if the roles of isoforms other than GBSSI are to be understood. To provide this information, we have examined the effect of *waxy* mutations on the number, nature and activity of granule-bound isoforms in the developing endosperms of barley and maize. This work makes use of two antibodies which recognise starch synthases from a variety of starch-storing organs. The antibody to GBSSI from pea embryos recognises GBSSI from wheat endosperm, potato tuber and several other organs (Denyer et al. 1995b; Edwards et al. 1995). The antibody to GBSSII from pea embryos specifically recognises proteins identified as starch synthases in a variety of storage organs. In wheat endosperm it recognises exclusively a granule-bound and soluble protein of 77 kDa, shown to be a starch synthase by purification to homogeneity (Denyer et al. 1995b). In potato tuber it recognises exclusively a granule-bound and soluble protein with an apparent size of 92 kDa. The primary amino acid sequence of this protein deduced from a cDNA clone is very similar to that of pea GBSSII, and the protein displays starch synthase activity when expressed in *Escherichia coli* (Edwards et al. 1995).

Materials and methods

Plant material. Wild-type and mutant lines of barley (*Hordeum vulgare* L.) were cv. Hector and cv. Waxy Hector, respectively. Seed was the kind gift of Dr. Roger Ellis, SCRI, Dundee, UK. The wild-type line of maize (*Zea mays* L.) was the dent genotype B73. The mutant line was derived from it by mutagenesis of pollen with ethyl methyl sulphonate (Neuffer and Chang 1989). It was shown to carry a mutation at the *waxy* locus by an allelism test in which it was crossed with a known *waxy* mutant line, and its kernels have the appearance and iodine-staining characteristics typical of *waxy* mutants. Developing endosperm was used for all experiments. Barley plants were grown in a greenhouse maintained above 12 °C, with supplementary lighting in winter. Grains were removed from the ear and stored at − 80 °C before use. Maize plants were grown in field conditions in central Iowa, USA. Whole ears were harvested and stored at − 80 °C before use.

Preparation and analysis of starch. Approximately 200 g of barley grains of between 30 and 60 mg, or maize kernels of 150–180 mg, were homogenized in about 2 vol. extraction medium containing 50 mM Tris-acetate (pH 7.8), 1 mM dithiothreitol (DTT), 1 mM EDTA at 4 °C in a blender. The homogenate was filtered through four layers of cheesecloth and the residue re-extracted and filtered twice, each time with 1 vol. extraction medium. The combined filtrate was centrifuged at $10000 \cdot g$ for 10 min at 4 °C. The supernatant and the grey/green (barley) or grey-yellow (maize) material from the surface of the pellet were discarded, and the pellet resuspended in 100–200 ml extraction medium. This centrifugation and resuspension step was repeated four to five times until the starch pellet appeared completely white. The final pellet was suspended in 50 ml acetone at − 20 °C and allowed to settle under gravity. The acetone and the finer fraction of particulate material were removed. The pellet was washed twice more with acetone at − 20 °C, air-dried, and stored at − 20 °C for up to three months before use.

The size distribution of barley starch granules in a typical preparation was measured with a Malvern MasterSizer (Malvern Instruments, Malvern, UK). The average granule diameter was 12.1 μm, and the size distribution suggested that A-type granules made up at least 91% by volume of the starch. In these respects our starch samples are typical of barley starches generally (McDonald et al. 1991).

The amylose content of wild-type and mutant maize was analysed by gel permeation chromatography according to Denyer et al. (1995a).

Solubilization and fractionation of starch-granule-bound proteins. Starch (1–5 g) was ground in 0.5 g or 1 g batches in a mortar at room temperature for 2.5 min, suspended at $0.17\ g \cdot ml^{-1}$ in Medium A [50 mM Tris-acetate (pH 7.5), 10 mM KCl, 1 mM EDTA, 1 mM DTT, $50\ ml \cdot l^{-1}$ glycerol] that contained 30 unit $\cdot ml^{-1}$ α-amylase (porcine pancreas; Boehringer Mannheim, Lewes, Sussex, UK), incubated at 30 °C for 2.5 h (barley) or 1.5 h (maize), then centrifuged at $40000 \cdot g$ for 15 min. The supernatant is referred to as the solubilized, starch-granule-bound protein.

The solubilized, starch-granule-bound protein was passed through a 0.2-μm filter and applied to a Mono Q anion-exchange column (HR 5 × 5, FPLC system; Pharmacia-LKB, Milton Keynes, UK) equilibrated with Medium A. The column was washed with Medium A until all the unadsorbed protein had passed through, then eluted with a 45-ml linear gradient of 0 to 1 M KCl in Medium A at a flow rate of $1\ ml \cdot min^{-1}$. Fractions of 1 ml were collected.

Assay of starch synthase. Starch synthase was assayed as the incorporation of ^{14}C from ADP[^{14}C]glucose into starch in the presence of added amylopectin, either according to Smith (1990: methanol-KCl method, assay includes 0.5 M citrate) for all barley experiments, or according to Jenner et al. (1994: assay does not include citrate) for all maize experiments. For maize the resin method was used for soluble extracts and the methanol-KCl method was used where the assay contained starch granules.

Analysis by SDS-PAGE and immunoblotting. Samples were analysed by SDS-PAGE on 7.5% polyacrylamide gels (80 × 50 × 0.75 mm) according to Smith (1990). Immediately prior to analysis, starch granules were washed twice with $20\ g \cdot l^{-1}$ aqueous SDS then heated at 100 °C for 4 min at $50\ mg \cdot ml^{-1}$ in gel sample buffer (Laemmli 1970, but using 70 mM DTT instead of β-mercaptoethanol) and centrifuged at $10000 \cdot g$ for 10 min. The resulting suspension from waxy starch and the supernatant from wild-type starch were applied to the gel. Gels were stained with Brilliant Blue R.

Immunoblots were prepared and developed according to Denyer et al. (1993).

Reconstitution of enzyme activity from SDS-PAGE gels. Wild-type starch granules were prepared as above for SDS-PAGE. Reconstitution of enzyme activity was by an adaption of the method of Hager and Burgess (1980), according to Denyer et al. (1993). Enzyme activity was measured as above, for 3 h at 30 °C.

Results

Barley

Granule-bound proteins. Analysis by SDS-PAGE revealed that the protein compositions of starch from wild-type and *waxy* mutant barley are very similar except for proteins of about 60 kDa. The relative amount of protein of this molecular mass is very much less in waxy than in wild-type starch (Fig. 1).

An antibody to the GBSSI of pea embryo (Smith 1990) recognised the major band of protein of 60 kDa from wild-type starch (Fig. 1). This protein will be referred to as barley GBSSI. An antibody to pea GBSSII (Smith 1990) recognised GBSSI and bands of protein of 77 and 90 kDa

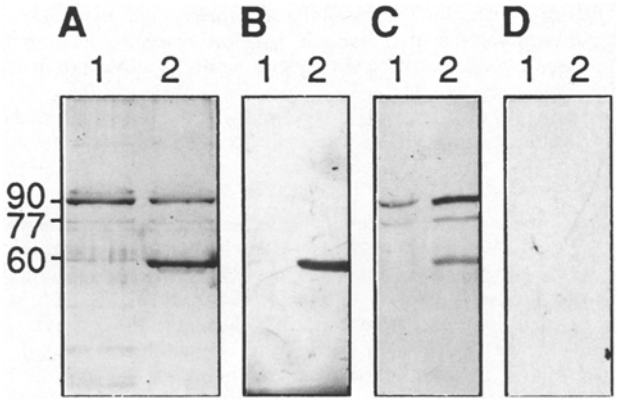


Fig. 1A–D. Granule-bound proteins of barley starch. Granule-bound proteins were extracted from purified waxy (*track 1*) and wild-type (*track 2*) starch by boiling in an SDS-containing solution, then subjected to SDS-PAGE (**A**). Sizes of proteins (estimated from several gels on which they were compared with molecular-weight standards) are given in kDa. Gels were blotted onto nitrocellulose, and blots were developed with antibodies to GBSSI of pea embryos (**B**: 1/1000 dilution of the Ig fraction of serum) or GBSSII of pea embryos (**C**: 1/1000 dilution of the Ig fraction of serum). Development of blots with pre-immune sera revealed no bands (**D**: 1/1000 dilution of the Ig fraction of preimmune serum for GBSSII)

on wild-type starch, and bands of protein of 77 and 90 kDa on waxy starch (Fig. 1).

Activity of granule-bound starch synthase. Purified, intact starch granules from wild-type barley endosperm have considerable starch synthase activity, whereas those from mutant endosperm have almost none (Table 1). Mechanical disruption of starch granules generally results in a considerable increase in their starch synthase activity (Frydman and Cardini 1967; Macdonald and Preiss 1983; Smith 1990). We therefore examined the effect of mechanical disruption on the activity of barley starch granules. After grinding in a mortar (see *Material and methods*), granules swelled considerably on contact with aqueous solutions and – although the magnitude of the effect varied from one preparation of starch to another – their starch synthase activity increased (Table 1). In general, the activity of wild-type granules increased about twofold and that of waxy granules increased at least tenfold. The amount of activity revealed by disruption of waxy granules was approximately 60% of that revealed by disruption of wild-type granules. The activity of damaged waxy granules was about 30% of that of damaged wild-type granules. Further treatments of mechanically-disrupted granules did not result in any further increase in starch synthase activity. These included sonication, prolonged grinding, and incubation of ground granules with urea (8 M), dimethyl sulphoxide (up to 800 ml $\cdot$ l^{-1}), α-amylase (from bacterial, mammalian and plant sources) or amyloglucosidase for up to 16 h at up to 30 °C.

Fractionation of granule-bound starch synthases. To discover which of the granule-bound proteins of barley starch have starch synthase activity, proteins were solubilised from mechanically-disrupted starch granules by incubation with α-amylase. About 15% of the activity

Table 1. Effect of mechanical disruption on the starch synthase activity of barley and maize starches. Suspensions of intact granules and granules which had been disrupted by grinding in a mortar were assayed for starch synthase activity. Values are either means ± SE of the number of measurements (each in an independent experiment) given in parentheses or, for intact, waxy barley granules, three measurements made in independent experiments. nd = not detected. Values for disrupted maize starch varied considerably between experiments, and are discussed in the text

	Starch synthase activity (μmol $\cdot$ min^{-1} $\cdot$ g^{-1} starch)	
Starch	Intact granules	Disrupted granules
Wild-type barley	0.219 ± 0.022 (3)	0.419 ± 0.026 (3)
Waxy barley	nd, 0.012, nd	0.116 ± 0.021 (3)
Wild-type maize	0.204 ± 0.023 (3)	
Waxy maize	0.031 ± 0.013 (3)	

originally present on the damaged granules was solubilised during the incubation. The sum of the activities in the soluble and insoluble material after incubation was routinely greater than 80% of that originally present on the damaged granules, indicating that little starch synthase activity was lost during the incubation. The SDS gels of granule-bound proteins and proteins solubilised by incubation with α-amylase appeared to be identical (not shown), indicating that the solubilised proteins were quantitatively and qualitatively representative of those on the granule.

Solubilised proteins were fractionated by anion-exchange chromatography on Mono Q. Although there was some variation between experiments in the precise elution profiles of proteins and starch synthase activity, the following features of the elution profiles were highly reproducible between experiments. Starch synthase activity eluted from the column routinely accounted for 90% or more of that applied to the column. Activity from both wild-type and waxy starch eluted as a sharp peak followed by a broad peak or shoulder (Fig. 2). The sharp initial peak from both wild-type and waxy starch contained the 77-kDa protein recognised by the antibody to pea GBSSII. This protein eluted from the column with the same profile as the peak of activity, and was always one of the major proteins visible on SDS gels of the peak fractions. The first peak did not contain the 90-kDa protein recognised by the pea GBSSII antibody (Fig. 2).

The second peak or shoulder of activity did not contain the 77-kDa protein. It contained several proteins including the 90-kDa protein recognised by the pea GBSSII antibody, and this protein eluted from the column with a profile similar to that of the peak of activity (Fig. 2).

The GBSSI of wild-type starch eluted in two broad regions of the salt gradient, the first roughly coinciding with the first peak of activity and the second coinciding with the tail of activity from the second peak or shoulder.

To provide further information about the starch synthase activity of granule-bound proteins, proteins were solubilised from wild-type granules by heating in an SDS-containing buffer, separated by SDS-PAGE, eluted from the gel and renatured. We have used this method to

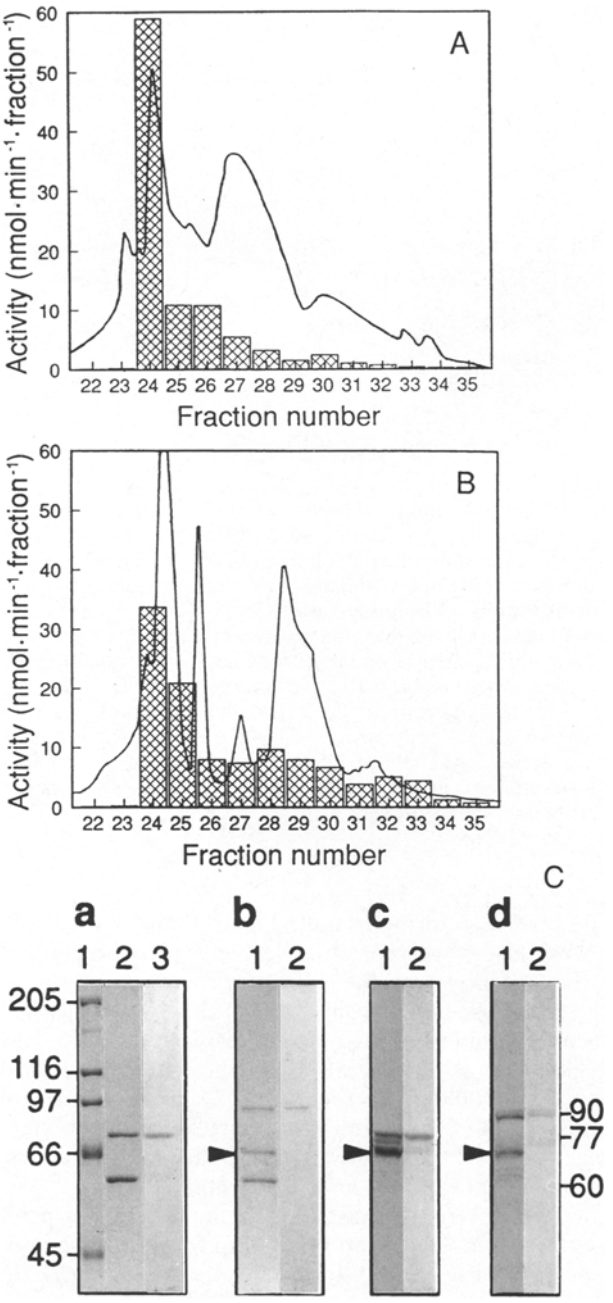


Fig. 2A, B. Fractionation of solubilised, granule-bound proteins from barley starch on a Mono Q column. Proteins were solubilised from disrupted starch granules (5 g) with α-amylase and subjected to chromatography on a Mono Q column. Fractions collected from the column were assayed for starch synthase activity and subjected to SDS-PAGE followed by immunoblotting with an antibody to GBSSII of pea embryos. **A** Solubilised protein from waxy starch. *Columns* represent starch synthase activity and the *solid line* is A_{280}, on a scale from 0 to 1.7 A. **B** Solubilised protein from wild-type starch. *Columns* represent starch synthase activity and the *solid line* is A_{280}, on a scale from 0 to 0.5 A. **C** SDS-polyacrylamide gels (7.5%) and immunoblots (1/5000 dilution of the Ig fraction of serum containing antibody to GBSSII) of fractions from **A** and **B**. The *arrowheads* indicate bands which are attributable to an inactive contaminant of the α-amylase used to digest the starch (experimental evidence for this not shown). Estimated sizes of bands are indicated in kDa on the right of the figure. *a*, gel (*2*) and blot (*3*) of fraction 24 from wild-type starch, Molecular-weight standards (kDa) are shown in *track 1*. *b*, gel (*1*) and blot (*2*) of fraction 29 from wild-type starch. *c*, gel (*1*) and blot (*2*) of fraction 24 from waxy starch. *d*, gel (*1*) and blot (*2*) of fraction 27 from waxy starch

identify granule-bound proteins with starch synthase activity in pea embryos (Denyer et al. 1993). The GBSSI and proteins of 77 and 90-kDa from wild-type barley starch yielded starch synthase activity when renatured (Fig. 3). The total activity associated with renatured proteins was only a few percent of that of the original sample of starch, hence no conclusions can be drawn about the specific or relative starch synthase activities of these proteins in vivo.

We were not able to examine the starch synthase activity of proteins from waxy starch by this renaturation method. In common with other waxy starches, waxy barley starch forms a paste on heating in aqueous solution. Granule-bound proteins can be resolved from the paste by direct loading onto an SDS gel (Fig. 1). However, extraction of proteins from the paste at sufficiently high concentrations for renaturation experiments proved impossible.

Maize

Granule-bound proteins. Experiments with maize were done on the dent genotype B73 and on a *waxy* mutant line derived from it by chemical mutagenesis. The starch of the *waxy* mutant stains red with iodine, and contains no detectable amylose when solubilised and subjected to gel-permeation chromatography (data not shown).

Analysis by SDS-PAGE revealed that the protein compositions of the wild-type and waxy starch are very similar (Fig. 4). Both starches contain a protein of 59 kDa which is recognised strongly by an antibody to pea GBSSI. This protein will be referred to as maize GBSSI. The retention of the GBSSI protein in the starch of maize *waxy* mutants has been observed previously: Echt and Schwartz (1981) reported that 4 out of 26 amylose-free mutants of maize retained a 60-kDa protein in their starch. Both starches also contain proteins of 74, 77 and 83 kDa which are recognised by an antibody to pea GBSSII. This antibody also weakly recognises the maize GBSSI (Fig. 4). The 74-kDa protein is strongly recognised by an antibody raised to a 76-kDa putative starch synthase of maize endosperm (Fig. 4; Mu et al. 1994). It is thus likely that the 74-kDa protein and the 76-kDa protein described by Mu et al. are one and the same.

Granule-bound starch synthase activity. The starch synthase activity of intact, wild-type maize granules was comparable to that of wild-type granules of barley (Table 1). The average activity of waxy maize granules was somewhat higher than that of waxy barley granules, but was still about sevenfold lower than that of wild-type maize or barley granules. The effect of mechanical disruption on the starch synthase activity of the granules varied considerably from one batch of starch to another, but activity was usually increased by this treatment. The largest increases we observed in four experiments were threefold for wild-type starch and fivefold for waxy starch.

Fractionation of granule-bound starch synthases. Proteins were solubilised from starch by incubation with α-amylase and fractionated by anion-exchange chromatography. Accurate estimation of the percentage of starch synthase activity solubilised and its recovery from the Mono Q

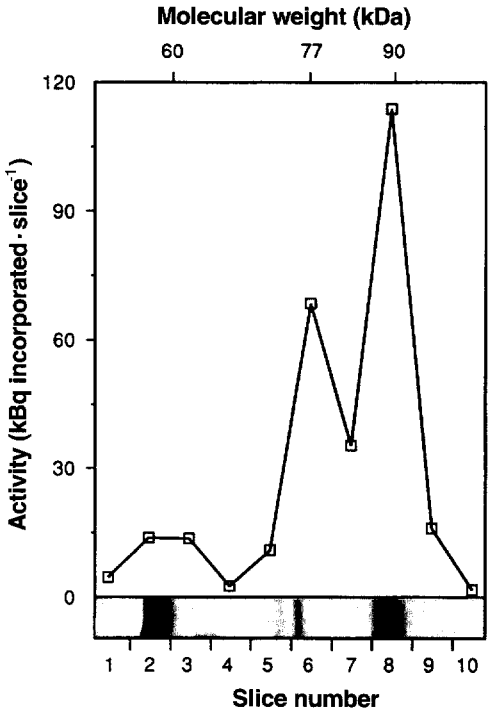


Fig. 3. Starch synthase activity of granule-bound proteins reconstituted from an SDS-polyacrylamide gel. Approximately 0.5 ml of supernatant from boiled starch prepared as in Fig. 1 was applied to a 7.5% SDS-polyacrylamide gel (10 cm wide, 0.75 mm thick). Protein bands were visualised by staining with KCl. Sequential slices 2 mm wide were excised from top to bottom of the gel, and protein in them was extracted and precipitated with acetone to remove SDS. Precipitated protein was dissolved in a small volume of 6 M guanidine-HCl, then diluted rapidly with a large volume of renaturation medium (Hager and Burgess 1980), incubated for 16 h, and assayed for starch synthase activity. A typical experiment is shown. The upper panel shows the activity of starch synthase (as kBq incorporated from ADP[U^{14}-C]glucose into glucan polymer, mean of two replicate assays) in each slice. The lower panel is a part of the same gel, stained with Brilliant Blue R, showing the positions of the protein bands relative to the slices. The molecular weights of the main bands of protein are indicated

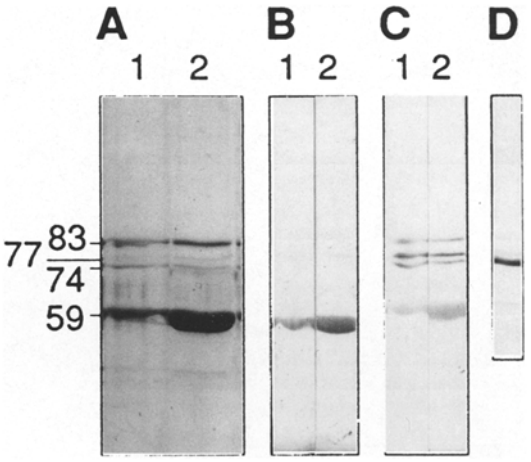


Fig. 4A–D. Granule-bound proteins of maize starch. Granule-bound proteins were extracted from purified waxy (*track 1*) and wild-type (*track 2*) starch by boiling in an SDS-containing solution, then subjected to SDS-polyacrylamide gel electrophoresis (**A**). Sizes of proteins (estimated from several gels on which they were compared with molecular-weight standards) are given in kDa. Gels were blotted onto nitrocellulose, and blots were developed with antibodies to GBSSI of pea embryos (**B**: 1/1000 dilution of the Ig fraction of serum), GBSSII of pea embryos (**C**: 1/1000 dilution of the Ig fraction of serum), or an antibody to a 76-kDa putative starch synthase described by Mu et al. (1994) (**D**: wild-type starch only, 1/10 000 dilution of crude serum). Development of blots with pre-immune sera revealed no bands (not shown)

column was not possible because activity recovered after chromatography was always severalfold greater than that measured in the solubilised, unfractionated material. This is a problem specific to maize starch: it did not occur in essentially identical solubilisation experiments with barley starch (see above), and pea and wheat starch (Smith 1990; Denyer et al. 1995b).

Both the amount and the distribution of starch synthase activity eluted from the Mono Q column were reproducible between experiments. For both wild-type and waxy starch, starch synthase activity eluted as two peaks, the first peak to elute containing most of the activity. In two experiments with wild-type starch, activities of 0.027 and 0.028 $\mu mol \cdot min^{-1} \cdot g^{-1}$ starch were recovered from the column, and 86 and 80% of these, respectively, were in the first peaks. In three experiments with waxy starch, somewhat lower activities of 0.013, 0.021 and 0.022 $\mu mol \cdot min^{-1} \cdot g^{-1}$ starch were recovered from the column, and 85, 80 and 76% of these were in the first peaks.

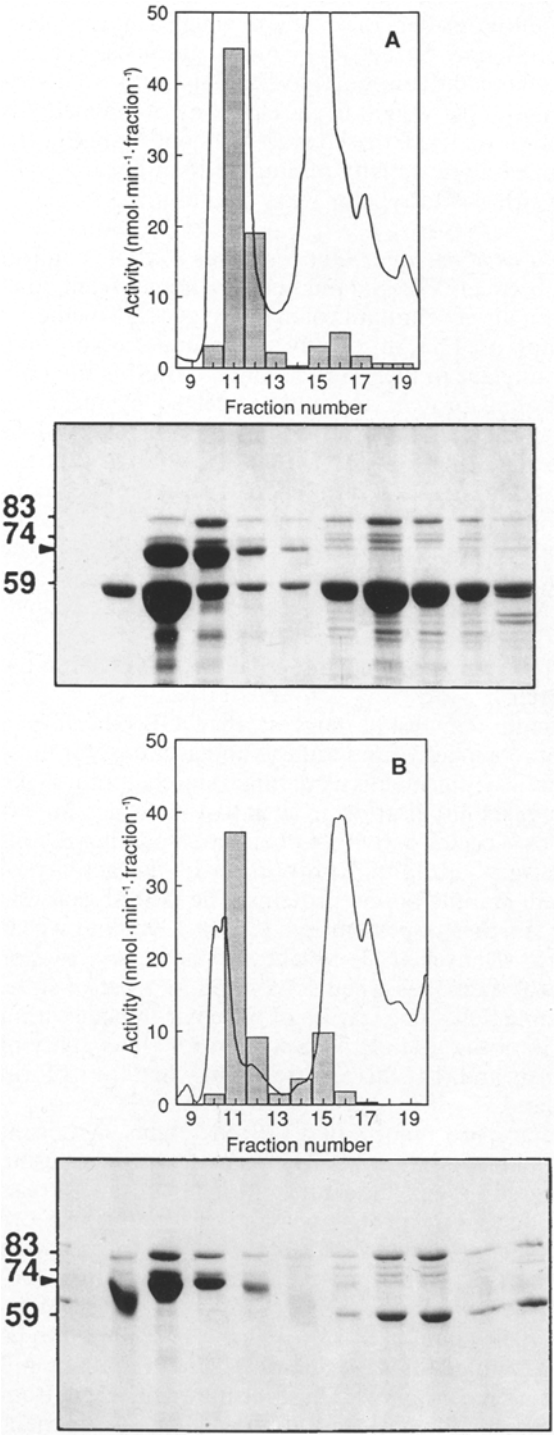
The profiles of proteins eluted from the Mono Q column were also very reproducible between experiments, with the exception of the GBSSI protein from waxy starch. The degree of solubilisation of this protein varied between experiments, and in some cases very little was solubilised. However, variation in the amount of this protein that eluted from the Mono Q column had no discernible effect on the amount or profile of starch synthase activity or other proteins, hence this variation does not affect the interpretation of the results.

There were strong similarities in the profiles of proteins eluted from the Mono Q column for wild-type and waxy starches (Fig. 5A–C). In both cases the first peak of starch synthase activity to elute from the column contained the 74- and 83-kDa proteins antigenically related to pea GBSSII, and the second peak contained the 74-, 77- and 83-kDa proteins (Fig. 5C). The relative amount and elution profiles of these proteins from the two sorts of starch were virtually indistinguishable.

There were differences between wild-type and waxy starches in the degree of coincidence between the first peak of starch synthase activity and the elution profiles of particular proteins (Fig. 5). For waxy starch, the elution profiles of the 74- and 83-kDa proteins closely matched that of the activity. For wild-type starch, however, there was no obvious coincidence between the elution profile of starch synthase activity and that of any particular protein. The peak of 74- and 83-kDa proteins was later than that of starch synthase activity, and the peak of GBSSI protein was sharper than that of starch synthase activity. These results indicated that the 74- and 83-kDa proteins might

be responsible for the starch synthase activity of the first peak from waxy starch, and these proteins together with GBSSI might contribute to the first peak from wild-type starch.

To investigate this further, samples containing equal starch synthase activities from the first peak for the two starches were subjected to SDS-PAGE (Fig. 5D). Staining with Brilliant Blue revealed that the waxy sample contained no GBSSI, and that the wild-type sample contained less of the 74- and 83-kDa proteins than the waxy sample.



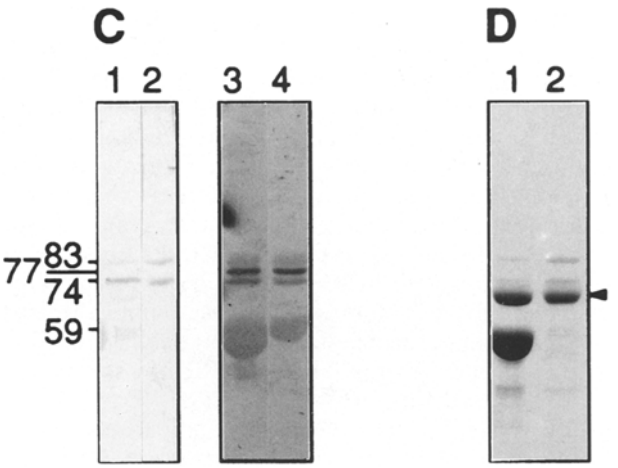


Fig. 5A, B. Fractionation of solubilised, granule-bound proteins from maize wild-type (**A**) and waxy (**B**) starch on a Mono Q column. Proteins were solubilised from disrupted starch granules (3 g) with α-amylase and subjected to chromatography on a Mono Q column. Fractions collected from the column were assayed for starch synthase activity and subjected to SDS-PAGE followed by immunoblotting with an antibody to GBSSII of pea embryos. Sizes of proteins are given in kDa. In the *upper panels*, *columns* represent starch synthase activity and the *solid line* is A_{280}, on a scale from 0 to 0.11 A. Fractions were between 0.15 M and 0.4 M on a linear salt gradient. *Lower panels* are SDS-polyacyrylamide gels (7.5%) of fractions 9–19 in the upper panels. Tracks contain 1/4 of the protein from the fractions. The *arrowheads* indicate bands which are attributable to an inactive contaminant of the α-amylase used to digest the starch (experimental evidence for this not shown). **C** Immunoblots (1/5000 dilution of the Ig fraction of serum containing antibody to GBSSII) of fractions from the Mono Q column. *Track 1*, fraction 12 from wild-type starch. *Track 2*, fraction 12 from waxy starch. *Track 3*, fraction of the highest activity from the second peak, from wild-type starch. *Track 4*, fraction of the highest activity from the second peak, from waxy starch. This waxy fraction was taken from an experiment in which more GBSSI eluted from the column than in the experiment in **B**. **D** SDS-polyacrylamide gel (7.5%) of fractions of highest activity from the first peak of wild-type (*track 1*) and waxy (*track 2*) starch, loaded so that both tracks contain protein equivalent to about 10 $nmol \cdot min^{-1}$ starch synthase activity

Thus GBSSI is likely to contribute to the activity of this peak in the wild type. However, its specific activity must be much lower than that of other starch synthases contributing to activity in the first peak. The specific activity of starch synthase in fraction 10 on Fig. 5A, a fraction from wild-type starch in which GBSSI is by far the major protein, was 0.095 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein. In contrast, the specific activity of the peak fraction from waxy starch (fraction 11 on Fig. 5B) was 0.5 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein. Putative starch synthases form only a very minor component of the protein in this fraction, most of which is an inactive contaminant of the α-amylase used to solubilise the starch. The individual specific activities of the starch synthases must therefore be much higher than 0.5 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein. The fact that the second peak of activity from wild-type starch contributes only about 15% of the total activity and yet contains several times more GBSSI protein than the first peak (Fig. 5) also suggests that the specific activity of GBSSI in these experiments is very low.

Discussion

Occurrence of starch synthases on wild-type starches. Our data provide good evidence that wild-type barley and maize endosperms have granule-bound starch synthases in addition to GBSSI. In barley starch, proteins of 77 and 90 kDa exhibit starch synthase activity when reconstituted after SDS-extraction from starch granules, and are antigenically related to pea GBSSII. Starch synthase activity co-elutes with these proteins upon ion-exchange chromatography of solubilised, granule-bound proteins. Our examination of maize starch is less detailed, but it is clear that protein(s) other than GBSSI are starch synthases. Three proteins, of 74-, 77- and 83-kDa, are antigenically related to pea GBSSII. Comparison of the elution profiles of starch synthase activity and proteins from wild-type starch on a Mono Q column suggests that at least one of these proteins makes a substantial contribution to the activity that can be solubilised from the granule.

Our results are in general agreement with those from previous studies of the starches of cereal endosperms. Wheat starch has starch synthases of 77- and 100- to 105-kDa in addition to GBSSI (Denyer et al. 1995b). Maize starch is reported to have an isoform of 93 kDa (Macdonald and Preiss 1985), and there is good circumstantial evidence that it has an isoform of 76 kDa (Mu et al. 1994). Although the relationship between the former and the proteins identified in our study is not clear, the latter is highly likely to be the 74-kDa protein antigenically related to pea GBSSII.

Effects of waxy mutations on granule-bound starch synthases. The *waxy* mutations of maize and barley have no apparent effect on putative granule-bound starch synthases other than GBSSI. For barley, the amounts and starch synthase activities of the 77- and 90-kDa proteins identified as starch synthases appear to be unaffected by the mutation. For the lines of maize used in our experiments, amounts of all of the proteins strongly antigenically related to pea GBSSII are not affected by the mutation. Evidence that the activities of starch synthases other than GBSSI are not affected by the mutation is provided by the similarity between wild-type and waxy starches in the elution profiles from a Mono Q column of starch synthase activity and proteins related to pea GBSSII. Our results are not consistent in this respect with previous reports that waxy maize starch has two isoforms of starch synthase which differ in molecular mass and kinetic properties from the isoforms of wild-type starch (Macdonald and Preiss 1985).

Activity of granule-bound starch synthases. The effects of the *waxy* mutations on the starch synthase activity of starch granules provide evidence that the activity of intact, wild-type starch granules is due primarily to GBSSI. Intact wild-type granules have high activity whereas intact waxy granules, which lack GBSSI but retain other isoforms of starch synthase, have almost none. Although isoforms other than GBSSI contribute little to the activity of intact granules, they probably account for a substantial proportion of the activity revealed by mechanical disruption of wild-type granules. In barley, the amount of activity revealed by disruption is of the same order in waxy as in wild-type starch. The situation is less clear in maize because of variation between experiments, but here too disruption of waxy starch reveals considerable starch synthase activity. We suggest that GBSSI is in a location or conformation within the intact granule which allows it to be active, whereas other isoforms only display significant activity when granules are disrupted.

This interpretation must, however, be treated with caution. First, *waxy* mutations change the structure of the granule, and hence may alter the environment of granule-bound starch synthases. It is thus conceivable that the mutation alters the extent to which isoforms other than GBSSI may be active in intact granules. Second, while mechanical disruption gave the highest starch synthase activities per weight of starch of any of the methods of disruption we tried, these values may not represent the maximum catalytic activity of granule-bound starch synthase for either wild-type or waxy granules.

The synthesis of amylose. Our results as a whole confirm the link between GBSSI and the synthesis of amylose. Conventional *waxy* mutants of maize in general, including the mutant used in this study, have undetectably low levels of amylose in their starch. Since GBSSI is the only granule-bound protein apparently affected by the *waxy* mutation, it must play the dominant role in amylose synthesis. The situation for barley is similar in that the *waxy* mutation causes a large reduction in amylose content and affects only GBSSI. However, the Waxy Hector cultivar used here is reported to retain about 30% of the amylose content of Hector, the cultivar from which it was derived (Morrison et al. 1986). This amylose may be synthesised by starch synthases other than GBSSI, or it may be the product of a residual amount of active GBSSI in the waxy starch.

Although our results suggest that GBSSI plays a dominant role in amylose synthesis and accounts for most of the starch synthase activity of intact starch granules, the protein after solubilisation in a native state has an extremely low specific activity. Other starch synthases contribute a very substantial proportion of the activity of solubilised, granule-bound proteins. The GBSSI isoforms from the starches of pea embryo (Smith 1990) and wheat endosperm (Denyer et al. 1995b) also have very low or undetectable activities when solubilised in a native state. It is possible that some feature of the environment within the matrix of the granule is important for the activity of this isoform, and that this feature is lost when the isoform is solubilised.

There are two reports that GBSSI retains significant activity after solubilisation from starch granules using starch-degrading enzymes. First, Macdonald and Preiss (1985) found that the protein responsible for 80% or more of the activity solubilised from starch of wild-type maize endosperm had a native molecular mass on sucrose-density gradients of 60 kDa. Second, Sivak et al. (1993) reported that a protein of 60 kDa, which they believed to be GBSSI, accounted for all of the solubilised granule-bound activity from pea embryos. These results are different from our own, and may suggest that subtle differences in experi-

mental protocol affect the degree to which GBSSI retains activity upon solubilisation.

Our results indicate strongly that granule-bound starch synthases other than GBSSI make little contribution to the synthesis of amylose. The possibility that they contribute to the synthesis of amylopectin cannot be ruled out. We have suggested previously that they may be soluble isoforms active in amylopectin synthesis at the surface of the granule, which become trapped within the growing matrix and then play no further role in starch synthesis (Denyer et al. 1993, 1995b). Consistent with this, a 76-kDa granule-bound protein of maize endosperm (likely to be the 74-kDa protein identified in this paper) appears also to account for a large proportion of the soluble starch synthase activity (Mu et al. 1994). However, a 100- to 105-kDa granule-bound starch synthase of wheat is not detectable in the soluble fraction of the endosperm (Denyer et al. 1995b). The roles of these isoforms remain to be elucidated.

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References

Ainsworth C, Clark J, Balsdon J (1993) Expression, organisation and structure of the genes encoding the *waxy* protein (granule-bound starch synthase) in wheat. Plant Mol Biol 22: 67–82

Denyer K, Barber LM, Burton R, Hedley CL, Hylton CM, Johnson S, Jones DA, Marshall J, Smith AM, Tatge H, Tomlinson K, Wang TL (1995a) The isolation and characterisation of novel low-amylose mutants of *Pisum sativum* L. Plant Cell Environ 18: 1019–1026

Denyer K, Hylton CM, Jenner CF, Smith AM (1995b) Identification of multiple isoforms of soluble and granule-bound starch synthase in developing wheat endosperm. Planta 196: 256–265

Denyer K, Sidebottom C, Hylton CM, Smith AM (1993) Soluble isoforms of starch synthase and starch-branching enzyme also occur within starch granules in developing pea embryos. Plant J 4: 191–198

Dry I, Smith AM, Edwards EA, Bhattacharyya M, Dunn P, Martin C (1992) Characterisation of cDNAs encoding two isoforms of granule-bound starch synthase which show differential expression in developing storage organs. Plant J 2: 193–202

Echt CS, Schwart D (1981) Evidence for the inclusion of controlling elements within the structural gene at the waxy locus in maize. Genetics 99: 275–284

Edwards A, Marshall J, Sidebottom C, Visser RGF, Smith AM, Martin C (1995) Biochemical and molecular characterisation of a starch synthase from potato tubers which is both soluble and granule-bound. Plant J 8: 283–294

Frydman RB, Cardini CE (1967) Studies on the biosynthesis of starch. II. Some properties of the adenosine diphosphate glucose: starch glucosyltransferase bound to the starch granule. J Biol Chem 242: 312–317

Hager DA, Burgess RR (1980) Elution of proteins from SDS-polyacrylamide gels, removal of SDS, and renaturation of enzymatic activity: results with sigma subunit of *E. coli* RNA polymerase, wheat germ DNA topoisomerase, and other enzymes. Anal Biochem 109: 76–86

Jenner CF, Denyer K, Hawker JS (1994) Caution on the use of the generally accepted methanol precipitation technique for the assay of soluble starch synthase in crude extracts of plant tissues. Aust J Plant Physiol 21: 17–22

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685

Macdonald FD, Preiss J (1983) Solubilization of starch-granule-bound starch synthase of normal maize kernels. Plant Physiol 73: 175–178

Macdonald FD, Preiss J (1985) Partial purification and characterisation of granule-bound starch synthases from normal and *waxy* maize. Plant Physiol 78: 849–852

McDonald ALM, Stark JR, Morrison WR, Ellis RP (1991) The composition of starch granules from developing barley genotypes. J Cereal Sci 13: 93–112

Morrison WR, Scott DC, Karklas J (1986) Variation in the composition and physical properties of barley starches. Stärke 38: 374–379

Mu C, Harn C, Ko Y-T, Singletary GW, Keeling BP, Wasserman BP (1994) Association of a 76 kDa polypeptide with soluble starch synthase I activity in maize (cv B73) endosperm. Plant J 6: 151–159

Neuffer MG, Chang M-T (1989) Induced mutations in biological and agronomic research. Vortr Pflanzenzüchtg. 16: 165–178

Preiss J (1992) Biology and molecular biology of starch synthesis and its regulation. In: Miflin BJ (ed) Oxford surveys of plant molecular and cell biology (Vol. 7). Oxford University Press, pp 59–144

Rohde W, Becker D, Salamini F (1988) Structural analysis of the *waxy* locus from *Hordeum vulgare*. Nucleic Acids Res 16: 7185–7186

Shure M, Wessler S, Fedoroff N (1983) Molecular identification and isolation of the *waxy* locus in maize. Cell 35: 225–233

Sivak MN, Wagner M, Preiss J (1993) Biochemical evidence for the role of the waxy protein from pea (*Pisum sativum* L.) as a granule-bound starch synthase. Plant Physiol. 103: 1355–1359

Smith AM (1990) Evidence that the waxy protein of pea (*Pisum sativum* L.) is not the major starch-granule-bound starch synthase. Planta 182: 599–604

Smith AM, Martin C (1993) Starch biosynthesis and the potential for its manipulation. In: Grierson D (ed) Biosynthesis and manipulation of plant products. Blackie and Son, Glasgow, pp 1–54

Visser RGF, Hergersberg M, van der Leij FR, Jacobsen E, Witholt B, Feenstra WJ (1989) Molecular cloning and partial characterisation of the gene for granule-bound starch synthase from a wildtype and an amylose-free potato (*Solanum tuberosum* L.). Plant Sci 64: 185–192