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Note

Chemical synthesis of methyl 6′-α-maltosyl-α-maltotrioside and its use for investigation of the action of starch synthase II

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Abstract

The branched pentasaccharide methyl 6′-α-maltosyl-α-maltotrioside was chemically synthesised and investigated as a primer for particulate starch synthase II (SSII) using starch granules prepared from the low-amylose pea mutant *lam* as the enzyme source. For chemical synthesis, the trichloroacetimidate activation method was used to synthesise methyl *O*-(2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranosyl)-(1→4)-*O*-(2,3,6-tri-*O*-benzyl-α-D-glucopyranosyl)-(1→6)-*O*-[(2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranosyl-(1→4)]-*O*-(2,3-di-*O*-benzyl-α-D-glucopyranosyl)-(1→4)-2,3,6-tri-*O*-benzyl-α-D-glucopyranoside, which was then debenzylated to provide the desired branched pentasaccharide methyl 6′-α-maltosyl-α-maltotrioside as documented by ^{1}H and ^{13}C NMR spectroscopy. Using a large excess of the maltoside, the pentasaccharide was tested as a substrate for starch synthase II (SSII). Both of the non-reducing ends of methyl 6′-α-maltosyl-α-maltotrioside were extended equally resulting in two hexasaccharide products in nearly equal amounts. Thus, SSII catalyses an equimolar and non-processive elongation reaction of this substrate. Accordingly, the presence of the α-1,6 linkages does not dictate a specific structure of the pentasaccharide in which only one of the two non-reducing ends are available for extension.

Keywords: Carbohydrates; Enzymes; Glycosylation; Methyl 6′-α-maltosyl-α-maltotrioside; Starch synthase II

Abbreviations: ADPG, Adenosine 5′-diphospho-D-glucose; ADP[U-14C]glucose, Adenosine 5′-diphospho-(D-glucose-UL-14C); AGPase, ADPglucose pyrophosphorylase; Bicine, *N*,*N*-Bis(2-hydroxyethyl)glycine; BSA, Bovine serum albumine; DP, Degree of polymerisation; DPM, Disintegrations per minute; DTT, Dithiothreitol; EDTA, Ethylenediaminetetraacetate; GBSS, Granule-bound starch synthase; HPAEC, High-performance anion-exchange chromatography; LC–MS, Liquid Chromatography-Mass Spectrometry; MES, 2-[N-Morpholino]ethanesulfonic acid; Mops, 4-Morpholinepropanesulfonic acid; PAD, Pulse amperometric detection; SBE, Starch branching enzyme; SS, Starch synthase.

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Starch accumulates as granular structures in plastids in plants and is usually defined as a mixture of two distinct components, amylopectin and amylose. Amylose is a linear α-(1→4)-glucan molecule decorated with very few α-(1→6)-branches. Amylopectin is composed of intermediate size α-(1→4)-linked glucans that are clustered together and hooked to longer spacer glucans by α-(1→6)-linkages.[1,2] The amylopectin molecules in the isolated starch granules form alternative crystalline and amorphous lamellae with a constant combined length of 90 Å. This supports the existence of a highly ordered, precise and well-defined structure of amy-

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lopectin and a tightly regulated biosynthetic pathway.[1,3] Amylopectin is synthesised from Adenosine 5′-diphospho-D-glucose (ADPG) and starch primers by reactions catalysed by isozymes of starch synthase that are either bound to the granule or soluble and by isozymes of starch branching enzyme, that are responsible for introduction of α-(1→6) branch points.

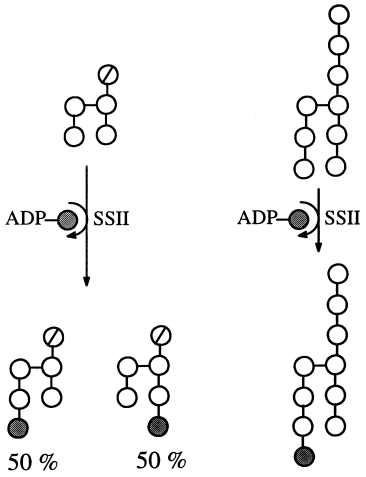
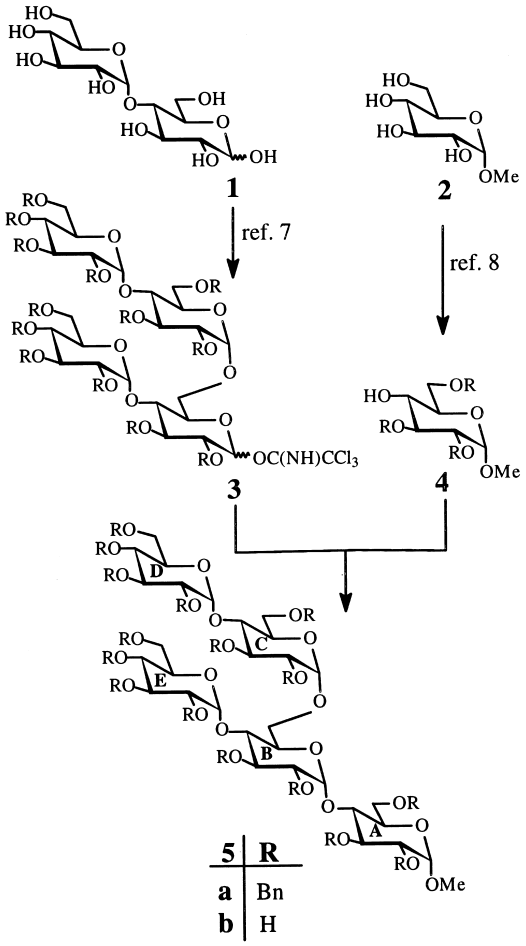


Chart 1. Schematic draw of the different reactions catalysed by SSII using the branched pentasaccharide **5b** and the branched nonasaccharide as primers (the anomeric centre of **5b** is blocked at the α-position as indicated).



Scheme 1. Strategy for chemical synthesis of methyl 6′-α-maltosyl-α-maltotrioside.

The biological importance of the different isoforms in controlling and defining the chemical structure of starch during its synthesis is unresolved and the formation of the molecular basis for different types of structures also remains elusive. Elucidation of the functional properties of each of the different isoforms of these enzymes is therefore a prerequisite to obtain a better understanding of the different parts of the starch biosynthetic machinery and to enable predictive biotechnological tailoring of starch.

In pea embryos, the granule-bound starch synthase activity is exclusively provided by the two isoforms, granule bound starch synthase (GBSSI) and soluble starch synthase II (SSII). The low-amylose pea mutant *lam* completely lacks GBSSI.[4] Accordingly, granules from *lam* embryos constitute a pure SSII enzyme source that can be used to investigate the function of SSII in amylopectin synthesis. Hence, the use of the fraction of SSII that is granule bound mimics the *in vivo* situation where the enzyme is embedded in a carbohydrate matrix and stabilised. In previous studies, SSII was shown to act non-processively on well defined linear oligosaccharides and a branched nonasaccharide[5,6] based on the fact that only one glucose residue was attached using excess of primer substrate. This is in contrast to the processive action of GBSS demonstrated using the same experimental set up.[5] Also, it was demonstrated that for the branched nonasaccharide only the short trisaccharide branch was significantly elongated.[6]

In this study, we further investigate the substrate specificity of SSII by the same strategy using the chemically synthesised branched pentasaccharide substrate methyl 6′-α-maltosyl-α-maltotrioside (**5b**) to provide a better understanding of the mode of action of SSII and its precise role in the biosynthesis of α-glucan polymers. The methyl group fixes the conformation at the anomeric centre in the α-conformation. Further, it enables discrimination of the products resulting from the SSII treatment. We demonstrate that, in contrast to the branched nonasaccharide, the branched pentasaccharide **5b** is elongated at both of the non-reducing ends but still in a non-processive manner (Chart 1).

Chemical synthesis of methyl 6′-α-maltosyl-α-maltotrioside (**5b**): Synthesis of the substrate **5b** is shown in Scheme 1. The coupling reaction of glycosyl donor 3[7] with the glycosyl acceptor **4**[8] (Scheme 1) in diethyl ether was achieved using trimethylsilyl triflate as catalyst and provided the desired branched pentasaccharide methyl glycoside derivative **5a** in 65% yield after purification on silica gel column chromatography. Minor amounts (~ 5%) of the β-isomer were removed by chromatogra-

Table 1
^{1}H NMR (400 MHz) and ^{13}C NMR (101 MHz) data [a,b] for Pentasaccharide derivative **5a**

^{1}H NMR data	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
Residue	($J_{1,2}$)	($J_{2,3}$)	($J_{3,4}$)	($J_{4,5}$)	($J_{5,6a}$ & $J_{5,6b}$)	($J_{6a,6b}$)	
	C-1	C-2	C-3	C-4	C-5	C-6	
E[c]	5.70 d	3.45 dd	3.96 t	3.64 t	3.77–3.72 m	3.34 dd	3.47 dd
α-D-Glc *p*-(1→4)	(3.2)	(9.9)	(9.9)	(9.9)	(1.7 & 2.6)	(11.5)	
	97.0	79.9	81.0	70.9	69.8[d]	68.1	
D[c]	5.69 d	3.55 dd	3.98 t	3.74 t	3.96–3.94 m	3.72 dd	3.86 dd
α-D-Glc *p*-(1→4)	(3.0)	(9.6)	(9.6)	(9.6)	(1.8 & 2.7)	(12.0)	
	96.5	80.3	81.7	72.0	74.3[d]	68.8[e]	
B	5.52 d	3.18 dd	4.01 t	4.25 t	3.88–3.79 m	3.97–3.79 m	
α-D-Glc *p*-(1→4)	(3.5)	(9.7)	(9.0)	(9.1)	(--)	(-)	
	95.6	80.1	81.9	77.6	72.4[d]	68.7[e]	
C	5.34 d	3.59 dd	4.05 t	4.09 t	3.88–3.79 m	3.97–3.79 m	
α-D-Glc *p*-(1→6)	(3.7)	(9.1)	(9.1)	(9.2)	(--)	(-)	
	95.4	79.4	82.0	77.5	71.5[d]	64.3	
A	4.60 d	3.65 dd	4.07 t	4.07 t	3.88–3.79 m	3.62–3.55 m	
α-D-Glc *p* OMe	(3.2)	(9.1)	(9.1)	(9.1)	(--)	(-)	
	97.8	79.2	82.0	82.0	69.4[d]	68.9[e]	

[a] $CDCl_3$; Chemical shifts in ppm, J in Hz.
[b] Chemical shifts for OMe and $PhCH_2O$ are given in Section 1.
[c,d,e] Assignment may be interchanged.

phy. The glucopyranosyl units of the branched pentasaccharide derivative **5a** are labelled **A** to **E** (Scheme 1). The complete ^{1}H and ^{13}C NMR chemical-shift assignments for **5a** listed in Table 1 were obtained using homonuclear (COSY) and ^{1}H-^{13}C-heteronuclear (HETCOR) chemical shift correlations. The ^{1}H NMR spectrum of **5a** showed a singlet at δ 3.35 ppm integrated to three protons and assignable to the methoxy group. The signal of the α-anomeric proton H^A-1 is resonating at δ 4.60 ppm ($J_{1,2} = 3.2$ Hz). The four doublets at δ 5.34 ($J_{1,2} = 3.7$ Hz), 5.52 ($J_{1,2} = 3.5$ Hz), 5.69 ($J_{1,2} = 3.0$ Hz) and 5.70 ($J_{1,2} = 3.4$ Hz) correspond to the four remaining-internal glycosidic protons. The chemical shifts and the characteristic small $J_{1,2}$-couplings for these anomeric protons, clearly indicate that the newly formed glycosidic linkage has α-configuration and confirms the stereochemistry of **5a**. Also, the ^{1}H-decoupled ^{13}C NMR spectrum of **5a** contains a diagnostic signal for the anomeric carbon of the glucose unit **A** at δ 97.8 due to the α-linkage of the methoxy group. The four additional anomeric carbon atoms resonate at δ 97.0, 96.5, 95.6 and 95.4 ppm in good agreement with an α-configuration.[9] Hydrogenolysis of **5a** for removal of the benzyl ether protecting groups was performed under hydrogen in the presence of 10% palladium on carbon in EtOAc/EtOH (1:1 v/v) and gave the desired branched pentasaccharide **5b** in 85% yield after chromatographic purification. The ^{1}H and ^{13}C NMR spectra of the anomeric region of compound **5b** are presented in Fig. 1a,b. In these figures the doublet at δ 4.99 ppm with $J_{1,2} = 3.6$ Hz (Fig. 1a) represent the anomeric proton at the branch point and the corresponding anomeric carbon at the branch point resonating at δ 99.4 ppm (Fig. 1b) are in good agreement with the values reported[10] (δ 4.96 with $J_{1,2} = 3.6$ Hz and δ 99.6 ppm, respectively) for the β-methyl glycoside analogue of **5b**.

Elongation of the branched primer methyl 6′-α-maltosyl-α-maltotrioside (**5b**): Starch granules isolated from *lam* pea embryos[5]containing only the SSII type starch synthase and insignificant hydrolytic and phosphorylytic activities[6] were incubated with adenosine 5′-diphospho-(D-glucose-UL-14C) (ADP[U-^{14}C]glucose) and **5b**. The structure of the saccharide primer allows for detection of the elongation site by high-performance anion-exchange chromatography (HPAEC), because the immediate products after debranching (maltotriose and/or methyl maltotetraoside) are readily separated from the substrate derived parts. The soluble fraction of the reaction mixture containing the extended primer oligosaccharides was separated from the starch granules and purified on a mixed-bed ion exchange resin and

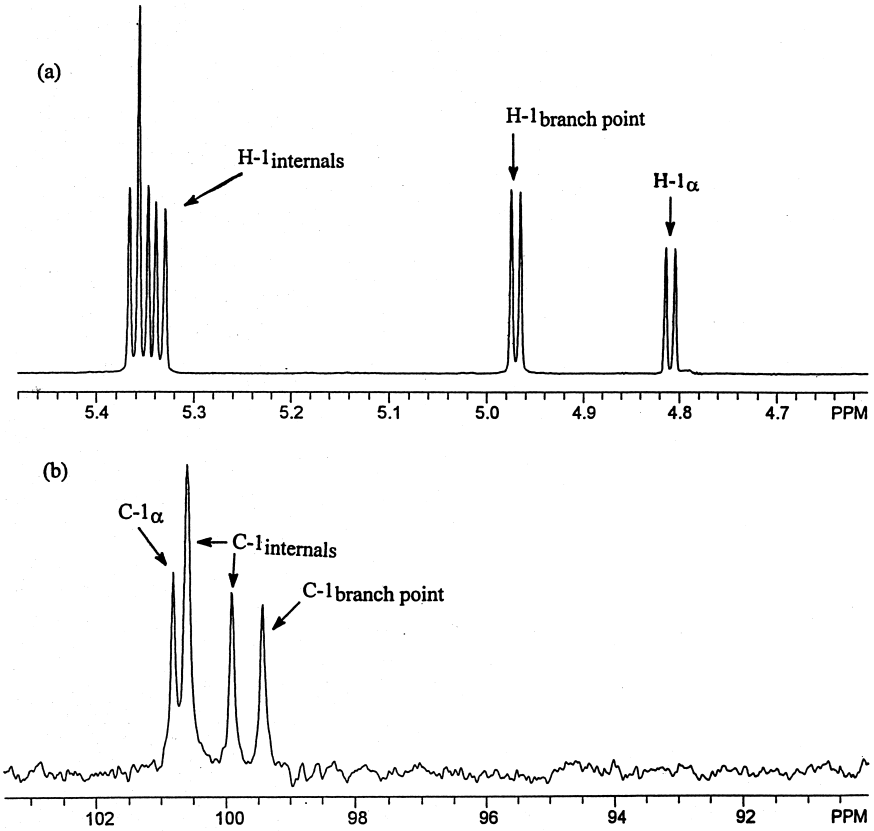


Fig. 1. (a) 400 MHz ^{1}H NMR spectrum of **5b** in D_2O; (b) 101 MHz ^{13}C NMR spectrum of **5b** in D_2O.

analysed by HPAEC (solid trace) and liquid scintillation counting (dotted trace) (Fig. 2A). The primer **5b** elutes at 7.5 min. The radioactivity profile monitors extension of the primer and reveals a single peak with a retention time of 10.5 min. To discriminate between elongation of the two different branch chains, the soluble fraction of the reaction mixture was treated with pullulanase to cleave α-1,6-linkages and the linear chains obtained were analysed by HPAEC (Fig. 2B). The two oligosaccharides eluting at 3.5 min and 6.5 min (solid line) are derived from the substrate and represent methyl α-maltotrioside and maltose, respectively. Two radioactively labelled oligosaccharides eluting at 6.0 min and at 9.5 min (dotted line) were detected. This indicated that both of the non-reducing ends had been elongated. The radiolabelled oligosaccharide eluting at 9.5 min was identified as maltotriose based on its retention time compared with an authentic standard (Fig. 2C). In the mass spectrum of oligosaccharides present in the soluble fraction after incubation with SSII, the substrate is recognised by the mass of 841 (Fig. 3, lower). Only one additional component was detected at 1003.5 and corresponds to elongation of **5b** with one glucose unit. Therefore, the products produced by the enzymatic reaction consisted of two hexasaccharides and the radiolabelled oligosaccharide eluting at 6.0 min is methyl α-maltotetraoside.

The observed non-processive action of SSII is in agreement with previous results.[5,6] However, the result differs from similar studies using the branched nonasaccharide 6‴-α-maltotriosyl-maltohexaose as primer,[6] where SSII showed selectivity and elongated only one of the non-reducing ends, namely the maltotriose part. In combination, the results led us to deduce that SSII

Fig. 2. (A) Soluble products formed by SSII using methyl 6′-α-maltosyl-α-maltotrioside as primer. Starch granules from pea *lam* mutant were incubated with ADP[^{14}C]glucose and 1.0 mM of the branched pentasaccharide methyl 6′-α-maltosyl-maltotrioside. The solid line represents the HPAEC chromatogram of methyl 6′-α-maltosyl-α-maltotrioside (retention time: 7.5 min). The dotted line shows the extension of methyl 6′-α-maltosyl-α-maltotrioside with radiolabelled D-glucose (retention time: 10.5 min); (B) Soluble products formed by SSII using methyl 6′-α-maltosyl-α-maltotrioside as primer after debranching using pullulanase. The two components eluting at 3.5 min and 6.5 min (solid line) represent methyl maltotrioside and maltose, respectively. The two radiolabelled oligosaccharides eluting at 6.0 min and 9.5 min (dotted line) represent methyl maltotetraoside and maltotriose, respectively. Panels (A) and (B): The graphs shown are typical several two repeated experiments. Due to instrumental artefact a peak at 1–2 min are seen; (C) HPAEC elution profile of linear α-glucans (D-glucose to maltoheptaose).

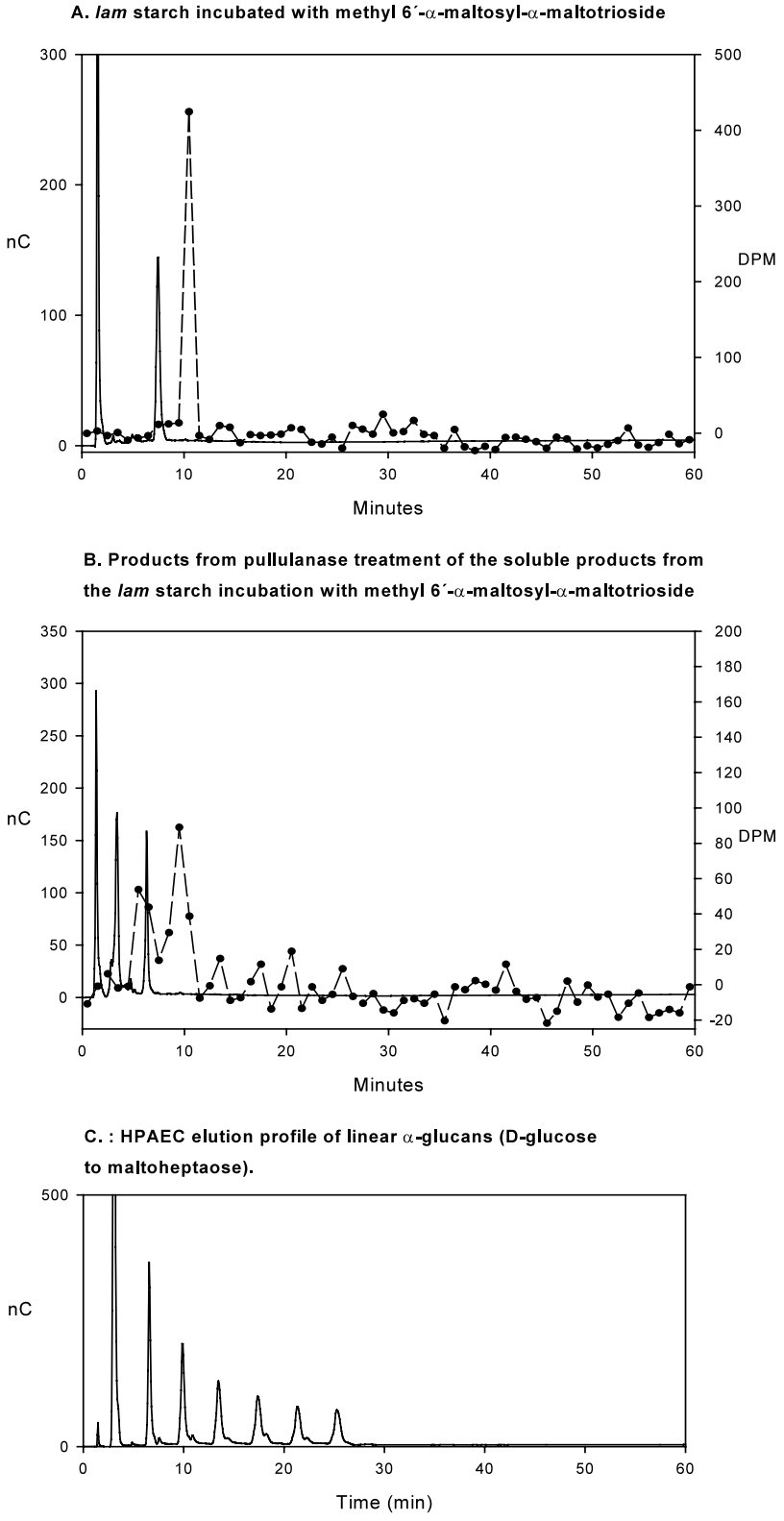


Fig. 2.

exerts higher activity towards shorter glucan chains than towards longer chains. We also deduced that SSII may catalyse the extension of a non-reducing end although the neighbouring glucose residue is involved in the formation of an α-1,6 branch point as part of an A or B chain. When the branched nonasaccharide was used as substrate, SSII preferentially elongated the shorter chain. While no incorporation in the malto-

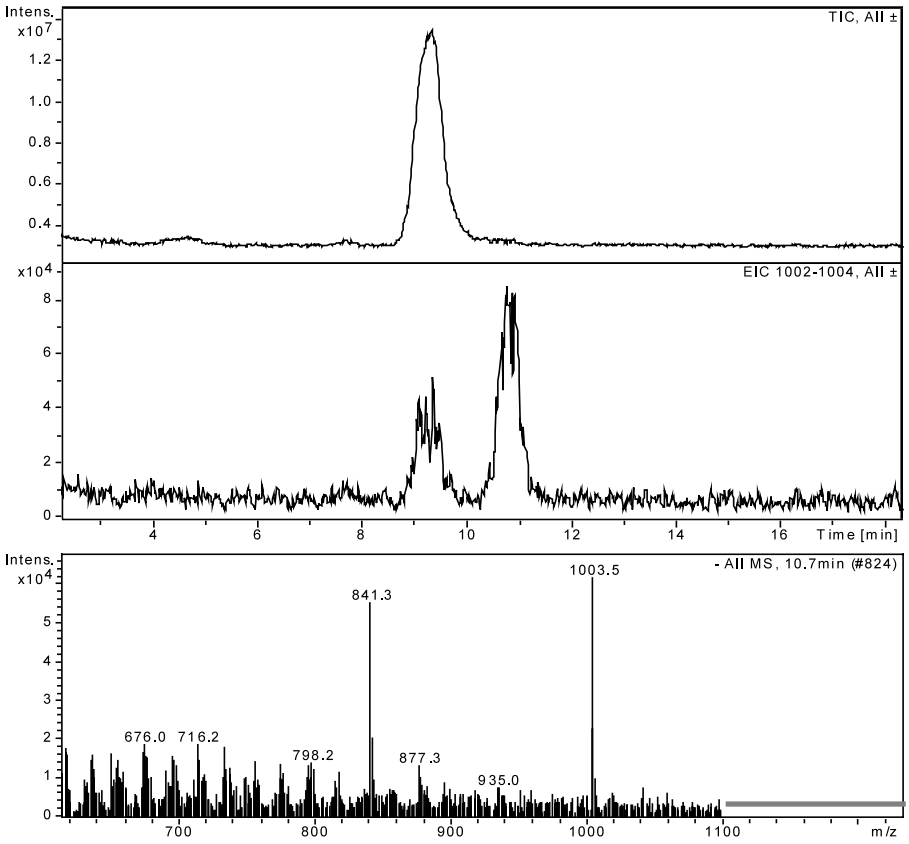


Fig. 3. Oligosaccharides present in the soluble fraction after elongation of methyl 6′-α-maltosyl-α-maltotrioside as detected by LC–MS (ESI (negative mode)). Upper: Total ion current. Middle: Extracted ion chromatogram (m/z 1002–1004). The peak at 10.7 min is the chain elongated product and the peak at 9.2 min is instrumental artefact from the huge amount of starting material. Lower: Mass spectrum at RT 10.7 min showing the chain elongated compound ($[M-H]^-$ at 1003.5) and trace of starting material due to tailing of the huge peak at 9.2 min.

hexaose part of the branched nonasaccharide was detected, incorporation of radiolabelled glucose in the similar linear molecule, maltohexaose, was observed.[6] Because incorporation is observed at both non-reducing ends in the branched pentasaccharide, the lack of extension of the maltohexaose chain of the branched nonasaccharide must reflect that this non-reducing end is inaccessible in the active site of SSII possibly due to steric hindrance as a result of the folding of the branched nonasaccharide.

Distribution of incorporation between starch granules and soluble primers: Starch synthase II present within starch granules is capable of catalysing elongation of the substrate **5b** as demonstrated above. However, the presence of endogenous primers within the starch granules was demonstrated by significant label in the starch granule fraction (Table 2). The incorporation in the starch granules was independent of the type of soluble primer used since the slight difference in incorporation is not significant (the results presented are averages of two independent assays showing a maximal variation of $\pm 14\%$). Partitioning of the activity of SSII between the soluble and particulate α-glucan primers can be considered physiologically relevant since linear soluble oligosaccharides can be formed *de novo* or as a result of amylopectin trimming reactions proposed as an integrated step in amylopectin biosynthesis.[11] Alternatively scavenging proposed to take place in the stroma would involve highly branched soluble molecules of various sizes.[12] The distribution of the incorporation of ^{14}C-glucose units between starch granules and soluble primers thus reflects the availability and efficiency of the two kinds of primers representing different metabolic pathways. The site of action of SSII within the granule is not known. However, the starch granule is growing from the surface and hence the main incorporation is assumed to take place at the outermost layer of the granule.

1. Experimental

1.1. General procedures and abbreviations

Optical rotations were measured at 29 °C with an Optical Activity Ltd AA-10 Polarimeter. All reactions were

Table 2
Incorporation of radiolabelled d-glucose moieties into soluble primers and starch granule bound primers

	Radioactivity (DPM):		Incorporation in soluble glucans (%) of the total incorporation	Total incorporation of ADPG (pmol) [a]:	
	Soluble glucans	Starch granule bound glucans		Soluble glucans	Starch granule bound glucans
Maltotriose [b]	750	2670	22.0	18.6	66.1
Maltohexaose [b]	300	2140	12.3	7.4	53.0
Methyl 6′-α-maltosyl-α-maltotrioside	425	2390	15.1	10.5	59.2
6‴-α-Maltotriosyl-maltohexaose [b]	400	2340	14.6	9.9	58.0

[a] Radioactivity in each assay: 109000 DPM; Assay volume: 25 μL; ADP[^{14}C]G: 0.108 mM.
[b] Damager *et al.* (ref. 6).

monitored by TLC on aluminium sheets coated with silica gel 60F_{254} (0.2 mm thickness, E. Merck, Darmstadt, Germany) and the components present were detected by charring with 10% H_2SO_4 in MeOH. Column chromatography was carried out using silica gel 60 (particle size 0.040–0.063 mm, 230–400 mesh ASTM, E. Merck). Solvent extracts were dried with anhydrous $MgSO_4$ unless otherwise specified. The ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance 400 spectrometer at 400 and 101 MHz, respectively. In D_2O, dioxane was used as internal reference [δ_H (dioxane) = 3.75; δ_C (dioxane) = 67.4]. In $CDCl_3$, the δ_H-value is relative to internal Me_4Si and the δ_C-value is referenced to the solvent [δ_C ($CDCl_3$) = 77.0]. MALDI spectrum was recorded on a Tofspec E spectrometer (Micromass). LC/MS (ESI) spectrum was recorded on a Bruker Esquire-LC.

1.2. Methyl *O*-(2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranosyl)-(1→4)-*O*-(2,3,6-tri-*O*-benzyl-α-D-glucopyranosyl)-(1→6)-*O*-[(2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranosyl)-(1→4)]-*O*-2,3-di-*O*-benzyl-α-D-glucopyranosyl)-(1→4)-2,3,6-tri-*O*-benzyl-α-D-glucopyranoside (5a)

A solution of **3**[7] (0.88 g, 0.44 mmol) and **4**[8] (0.25 g, 0.53 mmol) in dry diethyl ether (50 mL) was stirred for 45 min at room temperature in the presence of 4 Å molecular sieves (0.5 g, activated powder) under Ar. The stirred mixture was then cooled to −50 °C and a solution of trimethylsilyl trifluoromethanesulfonate (14 μL, 0.077 mmol) in dry diethyl ether (5 mL) was added dropwise. Stirring was continued and the temperature was raised to room temperature over a period of 2.5 h. After dilution with diethyl ether (50 mL), solid $NaHCO_3$ (0.5 g) was added and stirring was continued for 10 min. The mixture was filtered through Celite. The filtrate was washed thoroughly with satd aq $NaHCO_3$ (2 × 25 mL), water (3 × 25 mL), and brine (25 mL), dried and evaporated to dryness. The residue was chromatographed on silica gel (120 g) with 0.5% EtOAc in CH_2Cl_2 and repeated chromatography on silica gel (65 g) with diethyl ether/*n*-pentane 2:3 as eluent provided pure **5a** (0.6 g, gum, 59%) as amorphous white material. An analytical sample was crystallised from EtOH as a white powder. $[\alpha]_D$ +63.8° (c = 0.28, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ = 7.30–6.95 (m, 80 H; ArH), 5.05–4.18 (m, 32 H; 16 × OCH_2Ph), 3.35 (s, 3 H, OCH_3); ^{13}C NMR ($CDCl_3$): δ = 139–126.5 (ArC), 75.4, 75.4, 75.1, 74.9, 74.3, 74.2, 74.1, 73.6, 73.4, 73.4, 73.2, 73.2, 73.1, 72.8, 72.8, 71.7 (16 × OCH_2Ph), 55.2 (OCH_3).

1.3. Methyl 6′-α-maltosyl-α-maltotrioside (5b)

To a solution of **5a** (0.4 g, 0.175 mmol) in EtOAc–EtOH (20 mL, 1:1 v/v) was added 10% Pd on carbon (400 mg) and the mixture was stirred under hydrogen for 48 h at room temperature. The catalyst was removed by filtration through a Celite pad and a silica gel layer. The filtrate was evaporated and the residue was applied to a column of silica gel (28 g) eluted with methanol/water 4:1 to give a pure compound **5b** (124.0 mg, 84%) as a white powder; $[\alpha]_D$ +177.8° (c = 0.14, H_2O); ^{1}H NMR data (D_2O): δ 5.36 (d, $J_{1,2}$ = 3.9 Hz, 1 H; H^E-1), 5.35 (d, $J_{1,2}$ = 3.8 Hz, 1 H; H^D-1), 5.33 (d, $J_{1,2}$ = 3.7 Hz, 1 H; H^B-1), 4.97 (d, $J_{1,2}$ = 3.7 Hz, 1 H; H^C-1), 4.81 (d, $J_{1,2}$ = 3.7 Hz, 1 H; H^A-1), 3.41 (s, 3 H, OCH_3); ^{13}C NMR data (D_2O): δ = 100.8 (C^A-1), 100.6 (C^E-1), 100.6 (C^D-1), 99.9 (C^B-1), 99.4 (C^C-1), 78.8, 78.6, 78.4, 74.3, 74.1, 73.9, 73.8, 73.7, 73.6, 73.6, 72.7, 72.7, 72.3, 72.0, 71.8, 71.2, 71.0, 71.0, 70.2, 70.2 (C^{A-E}-2, C^{A-E}-3, C^{A-E}-4, and C^{A-E}-5), 68.1 (C^C-6), 61.6, 61.5, 61.3, 61.3 ($C^{A,B,D,E}$-6), 55.9 (OCH_3).

1.4. Plant material

The pea low-amylose (*lam*) mutant line (SIM 503)[13] was derived from round-seeded wild-type pea *Pisum*

sativum L. (line BCl/RR) as described by Denyer *et al.*[4] Plants were grown as previously described.[4]

1.5. Preparation of starch

Embryos (totally 1–3 g) with individual fresh weights between 200 and 400 mg were homogenised using a mortar and pestle with 5–15 mL of Medium A (100 mM Mops (pH 7.2), 5 mM $MgCl_2$, 0.68 M glycerol, 2 mM DTT and 1 g/L BSA). The homogenate was filtered through two layers of Miracloth (Behring Diagnostics, La Jolla, CA, U.S.A), and the residue was washed with an additional 5–15 mL of Medium A. After centrifugation (2.000 g, 10 min), the supernatant and the green material observed at the top of the pellet were discarded. The white pellet was washed in Medium A and subsequently in acetone at -20 °C as described previously[14] and dried at room temperature. The starch grains were either used immediately or stored at -80 °C for up to 6 months.

1.6. Starch synthase activity

Assays (final volume: 25 μL) contained 100 mM Bicine (pH 8.5), 25 mM KCl, 5 mM EDTA, 0.108 mM ADP[U-^{14}C]glucose (1.0×10^5 DPM, Amersham International, Little Chalfont, Buckinghamshire, U.K), 1.0 mM malto-oligosaccharide and a suspension (2.5 μL) of starch granules (25 mg/mL) in 10 mM Mops (pH 7.2), 0.5 mM $MgCl_2$, 0.068 mM glycerol, 0.5 mM DTT, BSA (1 mg/mL). This suspension was incubated with gentle continuous rotation at 22 °C for 4 h. The reaction was terminated by addition of 1 μL 2 M HCl followed by centrifugation for 1 min. The supernatant (containing the oligosaccharides) was collected and transferred to a new tube. The starch pellet was washed twice with 100 μL aliquots of H_2O, which were added to the supernatant. The combined fractions were added to a column containing a mixed-bed ion exchanger (1 mL, 1:1 v/v of Dowex 50W-X8 (450–100 mesh, H^+-form; Fluka AG, Buchs SG) and AG2-X8 (200–400 mesh, Cl^--form, BioRad; exchanged to $^-$OH-form by treatment of 2 M NaOH for 1 h). The starch pellet was resuspended in H_2O (100 μL) and centrifuged twice for 1 min (washing). The oligosaccharides were eluted in a total volume of 1.5 mL H_2O and the water was removed by freeze drying. The residue was dissolved in H_2O (100 μL) and the radioactivity was determined by liquid-scintillation counting.

1.7. Debranching of elongated methyl 6′-α-maltosyl-α-maltotrioside

6′-α-Maltosyl-maltotrioside elongated by SSII (50 μL aliquot) was debranched by the addition of 1 μL pullulanase (45 U/mL, Sigma). After incubation (2.5 h, 38 °C), the reaction was terminated by heating (100 °C, 1 min). The sample was centrifuged and the supernatant was desalted, freeze dried and the oligosaccharides were dissolved as described earlier for starch synthase activity.

1.8. High-performance anion-exchange chromatography/pulsed amperometric detection (HPAEC/PAD)

Separation of oligosaccharides was performed using a Dionex DX 500 system equipped with a GP40 pump and an ED40 PAD system fitted with a CarboPac PA-100 column (4×250 mm). Aliquots (50 μL) were injected using an S-3500 auto-sampler and the oligosaccharides were separated (flow-rate: 1 mL/min) using isocratic 150 mM NaOH and a linear gradient profile of NaOAc: 0–200 mM (0–60 min). Fractions (1 mL) were collected and radioactivity in these was measured after neutralisation with 2 M HCl using Ecoscint A scintillation liquid (National Diagnostics, Manville, NJ, USA) in a Wallac WinSpectral 1414 liquid scintillation counter (Wallac, Helsinki, Finland) using WinSpectral version 1.0 software.

1.9. Liquid chromatography/mass spectrometry (LC–MS)

LC–MS was done on a HP1100 LC coupled to a Bruker Esquire-LC ion trap mass spectrometer. Normal phase conditions were used with a 'CC 125/3 Nucleosil 100-3 NH2' column (Macherey-Nagel). The flow rate was 0.3 ml/min and a linear gradient of 65–50% acetonitrile in water was used. The mass spectrometer was run in negative ion mode.

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