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Enzymatic synthesis of polysaccharide-based copolymers†

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The design of enzymatic routes for the production of biosourced copolymers represents an attractive alternative to chemical synthesis from fossil carbon. In this paper, we explore the potential of glyco-synthesizing enzymes to produce novel block copolymers composed of various covalently-linked α -glucans with contrasting structures and physicochemical properties. To this end, various glucan-sucrases able to synthesize α -glucans with different types of α -osidic bonds from sucrose were tested for their ability to elongate oligosaccharide and polysaccharide acceptors with different structures from the native polymer synthesized by each enzyme. We showed that two enzymes – namely, the alternansucrase from *Leuconostoc mesenteroides* NRRL B-1355 (specific for $\alpha(1 \rightarrow 6)/\alpha(1 \rightarrow 3)$ -linked alternan synthesis) and the dextranucrase DSR-M Δ 1 from *Leuconostoc citreum* NRRL B-1299 (specific for $\alpha(1 \rightarrow 6)$ -linked dextran formation) – were able to elongate $\alpha(1 \rightarrow 4)$ -linked amylose and $\alpha(1 \rightarrow 6)/\alpha(1 \rightarrow 3)$ -linked alternan respectively. Carrying out stepwise acceptor reactions, and after optimization of the acceptor size and donor/acceptor ratio, two types of diblock copolymers were synthesized – a dextran-*b*-alternan and an alternan-*b*-amylose – as well as the triblock copolymer dextran-*b*-alternan-*b*-amylose. Their structural characterization, performed by combining chromatographic, NMR and permethylation analyses, showed that the copolymer polymerization degree ranged from 29 to 170, which is the highest degree of polymerization ever reported for an enzymatically synthesized polysaccharide-based copolymer. The addition of dextran and alternan blocks to amylose resulted in conformational modifications and related flexibility changes, as demonstrated by small angle X-ray scattering.

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1. Introduction

Due to their carbon neutrality and renewability, biosourced polymers are seen as an attractive alternative to synthetic polymers derived from fossil carbon when it comes to producing a wide range of materials. However, the intrinsic properties of natural polymers, for example in terms of rigidity and solubility, are often insufficient to meet functionality requirements for most non-food uses. In this context, the design of block copolymers is a promising strategy. Indeed, the physico-chemical properties of polymers directly derive from their

molecular structure and supramolecular organization, which vary considerably based on the nature of the covalently assembled building blocks that they are constructed from. Until now, the design of polysaccharide-based copolymers was mainly focused on the combination of cellulose, amylose, dextran, alginate or chitosan building blocks with synthetic blocks for obtaining hybrid materials used as surfactants, chromatography supports, or controlled release systems.^{1–5} Very few studies have looked at the synthesis of copolymers formed solely of blocks of different polysaccharides.^{6,7} Moreover, in most of these cases, the grafting of polysaccharides (or polysaccharide primers) onto synthetic or polysaccharidic blocks was achieved by chemical reaction⁸ or polymerization from chemically modified monomers.⁹ The *in vivo* or *in vitro* enzymatic synthesis of biosourced copolymers has also scarcely been investigated. Yadav *et al.*¹⁰ demonstrated the potential of using a glycosyltransferase for the microbial production of modified bacterial cellulose (MBC) with increased biodegradability properties. In this case, the MBC was a statistical copolymer rather than a block copolymer, since the glucosyl (Glc) and *N*-acetyl-glucosaminyl (GlcNAc) units were incorpor-

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ated on a random basis by cellulose synthase from *Gluconacetobacter xylinus*, which uses both UDP-Glc and UDP-GlcNAc as the glycosyl donor. Using the same principle, another type of glycan-synthesizing enzyme, $\alpha(1 \rightarrow 4)$ glucan phosphorylase (SP, EC 2.4.1.1) from *Aquifex aeolicus* VF5, belonging to the GT35 family of carbohydrate active enzymes (CAZy¹¹), was tested for the *in vitro* enzymatic synthesis of hybrid polysaccharides.¹²⁻¹⁵ The substrate promiscuity of this enzyme towards glycosyl donors (α -D-Glc-1-phosphate, α -D-GlcNAc-1-phosphate and α -D-Man-1-phosphate) was exploited to produce non-natural polysaccharides such as hetero-mannosides and aminopolysaccharides.¹³⁻¹⁵ The three sugar-phosphate donors were used in the presence of a maltotriose acceptor to obtain different copolymers with maltotriose at the reducing end, on which were grafted $\alpha(1 \rightarrow 4)$ -mannan, $\alpha(1 \rightarrow 4)$ -linked glucosaminoglycans or heteromannans (composed of either Glc and GlcNAc units, or Glc and Man units). Nevertheless, the degree of polymerization of the products was limited to 30 residues,¹⁴ with a very short block of only three glucosyl residues at the reducing end. Moreover, the impact of the Glc/GlcN or Glc/Man composition on the polymer chain conformation, and therefore its physicochemical properties, was not investigated.

Retaining transglucosylases, which catalyse polymerization reactions from non-nucleotide sugars, represent another category of glycan-synthesising enzymes that could be of interest in the production of block copolymers. Some α -transglucosylases, such as dextran dextrinases¹⁶⁻¹⁸ (DDase; EC2.4.1.2; CAZy family GH15), 4,3- α -glucanotransferase¹⁹ (4,3- α -GTase; E.C. 2.4.1.-; CAZy family GH70) and 4,6- α -glucanotransferase^{20,21} (4,6- α -GTase; E.C. 2.4.1.-; CAZy family GH70), have the ability to cleave the $\alpha(1 \rightarrow 4)$ -linkages of maltooligosaccharides (MOSs) and successively transfer the glucosyl units from one MOS chain to an acceptor molecule. If the acceptor molecule is another MOS chain, $\alpha(1 \rightarrow 6)$ (DDase and 4,6- α -glucanotransferase)¹⁶⁻¹⁹ or $\alpha(1 \rightarrow 3)$ (4,3- α -glucanotransferase) linkages can be formed.¹⁹ Besides, the 4,6- α -GTase from *Lactobacillus reuteri* 121 has been used to produce isomalto/malto-polysaccharides (IMMPs) with a DP of up to 30.²² These products contain one or more $\alpha(1 \rightarrow 4)$ -linked glucosyl units at the reducing end, and at least one $\alpha(1 \rightarrow 6)$ linkage at the non-reducing end. However, there are other α -transglucosylases, such as glucansucrases (GSs) from the GH13 and GH70 families, which also appear to be very interesting candidates for producing block copolymers. GSs use sucrose, a low cost agroresource, as the glucosyl donor, and catalyse the formation of homopolymers of α -D-glucosyl units. Depending on their characteristics, a wide variety of α -glucans with different molar masses, as well as with different distributions, numbers or types of glucosidic linkage (namely, $\alpha(1 \rightarrow 2)$, $\alpha(1 \rightarrow 3)$, $\alpha(1 \rightarrow 4)$ and/or $\alpha(1 \rightarrow 6)$), can be obtained.²³⁻²⁵

Of the GSs, dextransucrases (DS; E.C. 2.4.1.5; CAZy family GH70) catalyse the synthesis of dextrans that are α -glucans of molar masses ranging from 10^4 to 10^9 g mol⁻¹ with at least 50% $\alpha(1 \rightarrow 6)$ linkages and various branching points. Recently, a recombinant dextransucrase from *Leuconostoc citreum* NRRL

B-1299 (DSR-M) with a rather unusual specificity was characterized. Unlike other dextransucrases, which usually produce very high molar mass polymers, this enzyme produces only low molar mass linear $\alpha(1 \rightarrow 6)$ -linked dextran.^{26,27} Alternansucrase (ASR; E.C. 2.4.1.140; CAZy family GH70) specificity is also remarkable due to the mixture of $\alpha(1 \rightarrow 6)$ and $\alpha(1 \rightarrow 3)$ linkages found in the main polymer chain. The first ASR to be characterized, isolated from *L. mesenteroides* NRRL B-1355, produces a high molar mass alternan,²⁸ 4×10^7 g mol⁻¹ in size,²⁹ containing 58% $\alpha(1 \rightarrow 6)$ and 42% $\alpha(1 \rightarrow 3)$ linkages²⁸ mainly alternated. This alternan displays interesting properties such as a high solubility in water, low viscosity and resistance to dextranase degradation.³⁰ A third example is amylosucrases (AS; E.C. 2.4.1.4; CAZy family GH13), which catalyse the synthesis of an insoluble amylose-like polysaccharide containing exclusively $\alpha(1 \rightarrow 4)$ -linked glucosyl residues from sucrose,³¹ the size of which does not exceed 2×10^4 g mol⁻¹.³² The potential of amylosucrases has also been extensively investigated in terms of the synthesis of hyperbranched α -glucans and glycoclusters through elongation of native glycogen chains.^{33,34} In addition to polymer formation, GSs can also transfer the glucosyl unit of sucrose to a large variety of carbohydrate acceptors, including mono-, di- and trisaccharides such as galactose, xylose, maltose, isomaltose, mannose, maltotriose, panose, cellobiose, nigerose, lactose, raffinose and melibiose, as well as aglycon molecules such as polyols or flavonoids.^{25,35-39} However, there are very few publications describing the glucosylation using GSs of oligosaccharides with a DP higher than 3. The first example of this involved the strict elongation of maltooligosaccharides (MOSs) with a DP between 2 and 8 by the GTF-I and GTF-S enzymes from *S. mutans* 6715⁴⁰ and the dextransucrase from *L. mesenteroides* NRRL B-512FM.⁴¹ Only up to six glucosyl units could be added to the acceptor with a DP of 8. Although the physicochemical properties of these oligomers were not characterized, these findings reveal that GSs can be used for *gluco-co-oligomer* formation. In this study, we have gone one step further and explored various enzymatic routes for the production of *gluco-copolymers* composed of di- and tri- α -glucan blocks that differ in terms of their linkage types and physical properties. To this end, various combinations of glucansucrases and acceptor blocks were screened, and the synthesis conditions optimized. The new block copolymers that were produced were then characterized in detail in order to determine their structure and conformation in solution.

2. Experimental

Enzyme sources

The amylosucrase gene from *Deinococcus geothermalis* DSM11300 (*DgAS*) (accession number ABF44874) was amplified by means of PCR from a pGST-*DgAS* plasmid template⁴² using the two following primers: 5'-CACCAGCTGAAAGACGTGC TCACTTCTG-3' as the forward primer and 5'-TGCTGGAGC CTCCCCGGCGGTAGC-3' as the reverse primer. The addition



of a CACC sequence to the 5'-forward primer allowed the gene to be correctly inserted into the pENTR/D-TOPO® vector (Life Technologies). The recombinant gene was then transferred to the pET-53-DEST (Novagen) destination vector using the Gateway® LR Clonase® II enzyme mix (Life Technologies). Recombinant clones were selected on Luria–Bertani (LB) agar plates supplemented with 100 µg mL⁻¹ of ampicillin. Plasmids were extracted using the Sigma-Aldrich GenElute HP Plasmid Miniprep kit, and verified using restriction analyses and sequencing (GATC Biotech). *DgAS* was produced as a (His)₆-*DgAS* fusion protein by the recombinant *E. coli* BL21 star strain carrying the pET-53-DEST-*DgAS* plasmid, after 24 hours in a ZYM 5052 medium at 28 °C. The alternansucrase from *Leuconostoc mesenteroides* NRRL B-1355 (ASR) was produced as a (His)₆-ASR fusion protein by the recombinant *E. coli* BL21 strain carrying the pBad *asr C-APY-del Δthio* plasmid.²⁸ The dextranase from *Leuconostoc citreum* NRRL B-1299 (DSR-M Δ1) was produced as a (His)₆-DSR-M Δ1 fusion protein by the recombinant *E. coli* BL21 star strain carrying the pET-53-DEST-*dsrMΔ1* plasmid.²⁷ (His)₆-*DgAS*, (His)₆-ASR and (His)₆-DSR-M Δ1 were further purified by means of affinity chromatography using ProBond nickel-charged resin (Invitrogen), following the manufacturer's instructions, as described elsewhere.^{27,42} In order to make the reading of this study easier, (His)₆-*DgAS*, (His)₆-ASR and (His)₆-DSR-M Δ1 will be referred to hereafter as *DgAS*, ASR and DSR-M respectively.

Enzyme activity assays

One unit of glucansucrase corresponds to the amount of enzyme that catalyses the production of one µmol of fructose per minute under the assay conditions. Assays involving ASR and DSR-M were performed at 30 °C in a 50 mM sodium acetate buffer at pH 5.75 with 100 g L⁻¹ sucrose, while assays involving amylosucrase (*DgAS*) were performed at 50 °C in a 50 mM Tris-HCl buffer at pH 7.0 with 100 g L⁻¹ sucrose. The concentration of the fructose released during the reactions was determined using the dinitrosalicylic (DNS) acid method,⁴³ using fructose as the standard.

Production of acceptors

Maltooligosaccharides and the amylose block were synthesized *in vitro* from sucrose and maltose using the purified recombinant *DgAS*, under the same conditions as those described for *NpAS*.⁴⁴ The synthesis reaction was performed at 50 °C for a period of 24 hours in a 50 mM Tris-HCl buffer at pH 7.0, from 600 mM sucrose as the glucosyl donor and 100 mM maltose as the acceptor, using 1 U mL⁻¹ *DgAS*. Oligoalternans and the low molar mass (LMM) alternan block were synthesized *in vitro* using the purified ASR.²⁸ This synthesis reaction was performed at 30 °C for a period of 24 hours in a 50 mM sodium acetate buffer at pH 5.75, from 1 M sucrose as the sole substrate, using 1 U mL⁻¹ ASR. As described elsewhere,²⁸ under these conditions, ASR synthesizes three α-glucan populations: the HMM alternan (around 10⁷ g mol⁻¹), the LMM alternan, and oligoalternans. Isomaltooligosaccharide (dextran 1500 g mol⁻¹) was purchased from Sigma-Aldrich. In order to prepare

the blocks that would serve as acceptors for elongation, maltooligosaccharides, short amylose, isomaltooligosaccharides, oligoalternans, and LMM alternans were subsequently fractionated by means of preparative size-exclusion chromatography (SEC) using a 3L column of Biogel P6DG (Biorad). Sugars were eluted with water at 60 °C using a flow rate of 10 mL min⁻¹. High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) was used to determine the DP distribution of each fraction.

Oligosaccharide elongation and copolymer synthesis

Reactions were performed at 30 °C, in a 50 mM sodium acetate buffer at pH 5.75 containing 1 U mL⁻¹ of purified ASR or DSR-M, with acceptor concentrations between 10 and 50 g L⁻¹ and sucrose concentrations between 50 and 150 g L⁻¹. The reaction media were stirred at 30 °C for 24 hours to allow total sucrose consumption. After 24 hours, the reaction media were heated for five minutes at 95 °C to stop the reaction. Two controls were included in the experiment. The first relates to the enzyme with sucrose and the second relates to the enzyme in the presence of the acceptor. The second control was used to determine whether the enzyme was able to modify the acceptor in the absence of sucrose. After a reaction period of 24 hours, the acceptor modification (*i.e.* elongation) was checked using HPAEC-PAD.

Copolymer purification

The reaction products obtained from sucrose and the amylose block acceptor using ASR were purified by means of preparative SEC, as described in the previous section. The reaction products obtained from sucrose and the alternan block acceptor using DSR-M were purified by means of preparative SEC using a HiPrep 26/60 column of Sephadryl S200 HR (GE Healthcare). Sugars were eluted at 25 °C in water using a flow rate of 1.5 mL min⁻¹. The reaction products obtained from sucrose and the alternan-*b*-amylose acceptor using DSR-M were purified using a semi-preparative 9 × 250 mm Dionex CarboPac PA100 column. A gradient of sodium acetate in 150 mM NaOH was applied at a flow rate of 2.5 mL min⁻¹ as follows: 0–10 min, 0–200 mM; 10–15 min, 200–200 mM; 15–30 min, 200–300 mM. Carbohydrate detection was performed in the same way as analytic HPAEC-PAD (see below). After HPAEC-PAD separation, the samples were desalting online using a carbohydrate membrane desalter (CMD; Dionex) according to the manufacturer's recommendations. The resulting purified polymer fractions were freeze-dried.

α-Glucan structure analysis

High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Aliquots of the reaction media were collected and diluted with water to a final concentration of 1 g L⁻¹. HPAEC-PAD analysis was performed on a 2 × 250 mm Dionex CarboPac PA100 column. A gradient of sodium acetate in 150 mM NaOH was applied at a flow rate of 0.25 mL min⁻¹ as follows: 0–10 min, 0–140 mM; 10–26 min, 140–400 mM; 26–29 min, 400–500 mM. Detection was per-



formed using a Dionex ED40 module with a gold working electrode and an Ag/AgCl pH reference.

¹H NMR. Freeze-dried polymer samples were re-suspended in deuterated water at a final concentration of 20 mg mL⁻¹. ¹H NMR spectra were recorded on a Bruker Avance 500 MHz spectrometer and the data were processed with TOPSPIN 3.0 software, as described elsewhere.⁴⁵ The percentage of $\alpha(1 \rightarrow 4)$, $\alpha(1 \rightarrow 6)$ and $\alpha(1 \rightarrow 3)$ linkages in the products was determined by integration of the respective peak areas of the anomeric proton signals. Of note is that the $\alpha(1 \rightarrow 3)$ linkage content may correspond to different $\alpha(1 \rightarrow 3)$ linked glucosyl units including terminal residue (α -D-Glcp-(1 \rightarrow 3)), monosubstituted residues ((\rightarrow 6)- α -D-Glcp-(1 \rightarrow 3)- or \rightarrow 3)- α -D-Glcp-(1 \rightarrow 3)-units) or disubstituted residues ((\rightarrow 3,6)- α -D-Glcp-(1 \rightarrow 3)-units) as their anomeric signals are too close to be distinguished.⁴⁶

The percentage of the alternan-*b*-amylose copolymer made up of the amylose block was determined as follows: % of the amylose block = % of $\alpha(1 \rightarrow 4)$ linkages in alternan-*b*-amylose. Similarly, the percentage of the dextran-*b*-alternan copolymer made up of the alternan block was determined using the following formula: % of the alternan block = (% of $\alpha(1 \rightarrow 3)$ linkages in alternan-*b*-dextran)/(% of $\alpha(1 \rightarrow 3)$ linkages in the alternan block) \times 100. With regard to the triblock dextran-*b*-alternan-*b*-amylose, the following formulae were used: % of amylose in the triblock = % of $\alpha(1 \rightarrow 4)$ linkages in the dextran-*b*-alternan-*b*-amylose; % of the alternan block in the triblock = [(% of $\alpha(1 \rightarrow 3)$ linkages in dextran-*b*-alternan-*b*-amylose)/(% of $\alpha(1 \rightarrow 3)$ in alternan-*b*-amylose)] \times [(% of $\alpha(1 \rightarrow 3)$ in alternan-*b*-amylose + % of $\alpha(1 \rightarrow 6)$ in alternan-*b*-amylose)]/100; % dextran block in the triblock = 100 - % alternan block - % amylose block.

Permethylation analyses. In preparation, the samples were lyophilized and dried under vacuum at 40 °C. One mg was dissolved in 1 mL of DMSO for 20 minutes at 80 °C. Methylation was performed by adding 1 mL of NaOH-DMSO reagent and 0.5 mL of methyl iodide.⁴⁷ To enhance the reaction, the samples were sonicated and vortexed for a period of two minutes. This step was repeated three times. The reaction was stopped by adding 2 mL of water, and the methylated products were extracted with 2 mL of chloroform. After vigorous vortex mixing, the samples were centrifuged for five minutes at 5000g. The aqueous supernatant phase was removed and the organic phase, after washing three times with 4 mL of water, was dried under an air ramp. An internal standard, inositol (50 µg), was added to each sample and the whole sample was then hydrolysed with 2 M trifluoroacetic acid solution and converted to alditol acetate. The partially methylated alditol acetates were analysed by means of gas chromatography, on a TraceGOLDTM TG-1MS GC non-polar column (30 m \times 0.25 mm \times 0.25 µm, Thermo ScientificTM, H₂ as the carrier gas at 1.5 mL min⁻¹), and mass spectrometry (TRACE-GC-ISQ, ThermoTM). The sample was injected at 240 °C. The column oven temperature was maintained at 60 °C for five minutes and gradually increased to 315 °C (3 °C min⁻¹), at which temperature it was maintained for two minutes. The ion source temperature of the electron impact

(EI) mass spectrometer was 230 °C. Masses were acquired with a scan range between *m/z* 100 and 500. Partially methylated alditol acetates were identified based on a combination of the analytes' relative retention times (relative to inositol) and their fragmentation spectrum, before being compared against a homemade library.

The proportion of each type of osidic linkage was calculated as follows:

$$\%_{\text{linkages}} i = \frac{\text{Area}_i}{f \times \sum \text{Area}} \times 100$$

where area_i is the area of the sugar *i* being examined, and *f* is its response factor.⁴⁸

Small angle X-ray scattering (SAXS). SAXS experiments were performed on the SWING beamline at Synchrotron SOLEIL, Gif-sur-Yvette, France. The wavelength was set to $\lambda = 1.033 \text{ \AA}$. A 17 \times 17 cm² low-noise Aviex CCD detector was positioned at 1800 mm from the sample, with the direct beam off-centre. The resulting exploitable *q*-range was 0.006–0.45 Å⁻¹, where *q* = $4\pi \sin \theta/2$, and the scattering angle was taken to be 2θ . The samples were circulated in a thermostated quartz capillary with a diameter of 1.5 mm and a wall thickness of 10 µm, which had been placed in a vacuum chamber.

In order to obtain the oligosaccharide form factor, concentrated solutions of amylose, alternan-*b*-amylose and dextran-*b*-alternan-*b*-amylose (50 µL at 50 mg mL⁻¹) were injected into a size exclusion column (BIOSEC, Agilent) using an Agilent high-performance liquid chromatography (HPLC) system, and eluted directly into the SAXS flow-through capillary cell at a flow rate of 200 µL min⁻¹.

SAXS data were collected online for the duration of the elution time, with a time frame of 1 s, and a dead time between frames of 0.5 s. For each sample, the frames corresponding to the main elution peak were checked for the stability of the associated radius of gyration and global curve shape, and the resulting selection of curves was averaged, as described elsewhere.⁴⁹ Selected frames corresponding to the main elution peak were averaged using FOXTROT, a custom homemade application. A large number of frames were collected during the first minutes of elution, and these were averaged to account for buffer scattering, which was subsequently subtracted from the signal during the elution of the polymer. Data reduction to absolute units, frame averaging, and subtraction were carried out using FOXTROT.

High-performance SEC coupled with multi-angle laser light scattering (HPSEC-MALLS). The equipment, method, data acquisition and analysis used here were the same as those described elsewhere,⁵⁰ except for the columns: three Suprema columns (8 mm \times 300 mm, PSS, Germany) and a guard column (8 mm \times 50 mm, PSS, Germany) were used, connected in series and maintained at 30 °C. The eluent was a 0.2 g L⁻¹ sodium azide solution, at 0.5 mL min⁻¹. Mono- and diblocks were solubilized in water at 0.5 g L⁻¹, and filtered through 0.45 µm filters before a sample (100 µL) was injected into the HPSEC-MALLS system.



3. Results and discussion

Three different GSs, specific for the synthesis of α -glucans of various structures and properties, were tested for copolymer synthesis: the DSR-M dextranase from *Leuconostoc citreum* NRRL B-1299, the ASR alternansucrase from *L. mesenteroides* NRRL B-1355 and the *DgAS* amylosucrase from *Deinococcus geothermalis* DSM11300. The approach consisted of using one GS to elongate the α -glucans produced by the other two. In order to select the best GS/ α -glucan combination, we first tested the ability of each enzyme to glucosylate short oligosaccharides, whose structure differed from that of their natural acceptors: $\alpha(1 \rightarrow 6)$ -linked (IMOS), $\alpha(1 \rightarrow 6)/\alpha(1 \rightarrow 3)$ -linked (OAL) and $\alpha(1 \rightarrow 4)$ -linked (MOS) oligosaccharides for DSR-M, ASR and *DgAS* respectively. Six acceptor reactions were carried out and the reaction products were analysed by means of HPAEC-PAD. A given enzyme was considered to be able to glucosylate a given acceptor if acceptor conversion into new products was observed, which was determined by the presence of new peaks in the HPAEC chromatogram. As can be seen from Table 1, only the three couples DSR-M/OAL, ASR/MOS and ASR/IMOS produced positive results. The acceptor reaction conditions were then optimized by varying the acceptor concentration from 10 to 50 g L⁻¹, and the sucrose concentration from 50 to 150 g L⁻¹. The best acceptor glucosylation was always obtained using an acceptor/donor mass ratio of 0.15. These conditions were selected for the next experiments. Almost total acceptor consumption, alongside the appearance of new peaks in the HPAEC-PAD chromatograms (Fig. 1), proved that covalent grafting had occurred. Similar results were obtained for the three couples being tested, providing the first piece of evidence to suggest that some GSs are able to glucosylate α -linked glucooligosaccharide acceptors with DPs > 8 and structures different from those of the enzyme's natural acceptor.

Only MOSSs of up to DP 8 were indeed shown to be glucosylated with GTF-I and GTF-S from *S. mutans* 6715,⁴⁰ and the dextranase DSR-S from *L. mesenteroides* NRRL-B-512FM.⁴¹ Subsequently, with a view to synthesizing copolymers, and not

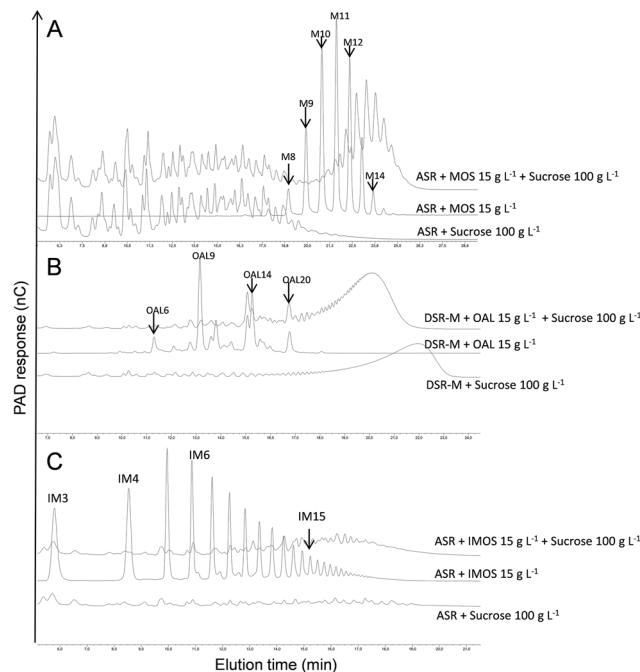


Fig. 1 HPAEC-PAD chromatograms of the acceptor reaction media involving the following couples: ASR/maltooligosaccharides (A), DSR-M/oligo-alternan (B), and ASR/isomaltoligosaccharides (C). Two controls are also presented below the acceptor reaction products, these being (i) the enzyme with sucrose alone, and (ii) the enzyme with the acceptor alone. Only chromatograms corresponding to the final reaction times are reported here. For the control 'enzyme + acceptor', it was verified that the chromatograms obtained at the initial and final reaction times were identical.

just oligosaccharides with new structures, the potential of DSR-M and ASR to act on α -glucans with higher DPs was investigated. Accordingly, α -glucan acceptors of intermediate molar mass were prepared that could be easily separated from the natural polymers synthesized by the glucansucrases selected to perform the elongation. At this stage, and in order to limit the number of combinations, we selected the following couples: DSR-M/OAL and ASR/MOS.

Table 1 Results of acceptor reactions performed using DSR-M, ASR and *DgAS*, and various exogenous oligosaccharide acceptors

Enzyme	Product from sucrose only	Acceptors ^a		
		MOS ^b DP 8–14	OAL ^c DP 6–20	IMOS ^d DP 1–25
ASR	Alternan (58% of $\alpha(1 \rightarrow 6)$ and 42% of $\alpha(1 \rightarrow 3)$)	+	n.d.	+
DSR-M	Dextran (100% of $\alpha(1 \rightarrow 6)$)	–	+	n.d.
<i>DgAS</i>	Amylose (100% of $\alpha(1 \rightarrow 4)$)	n.d.	–	–

^aThe reactions resulting in the elongation of the acceptors are indicated by the symbol “+”. ^bMaltooligosaccharides (MOS) with 100% $\alpha(1 \rightarrow 4)$ linkages. ^cOligo-alternan (OAL) with 63% $\alpha(1 \rightarrow 6)$ and 37% $\alpha(1 \rightarrow 3)$ linkages. ^dIsomaltoligosaccharides (IMOS) with 95% $\alpha(1 \rightarrow 6)$ and 5% $\alpha(1 \rightarrow 3)$ linkages. Linkage percentages determined by ¹H proton NMR.

Amylose block synthesis and elongation using ASR

DgAS was used to produce $\alpha(1 \rightarrow 4)$ -glucan fractions with suitable DPs, which would be tested as primers for copolymer synthesis with ASR. The synthetic reaction was performed using a sucrose donor/maltose acceptor ratio of 6. The resulting amylose-like products with DPs between 14 and 23 were fractionated by means of SEC. This fraction (DP 14–23) was further incubated with ASR and 100 g L⁻¹ sucrose.

The HPAEC-PAD analysis of the reaction products showed that the initial $\alpha(1 \rightarrow 4)$ -glucan chains were used as acceptors, leading to the formation of new compounds with higher retention times, as revealed by the clear displacement of the peaks corresponding to the initial acceptor (Fig. 2A). This population is, in theory, composed of an $\alpha(1 \rightarrow 3)/\alpha(1 \rightarrow 6)$ -glucan block at the non-reducing end, grafted to the non-reducing end of the



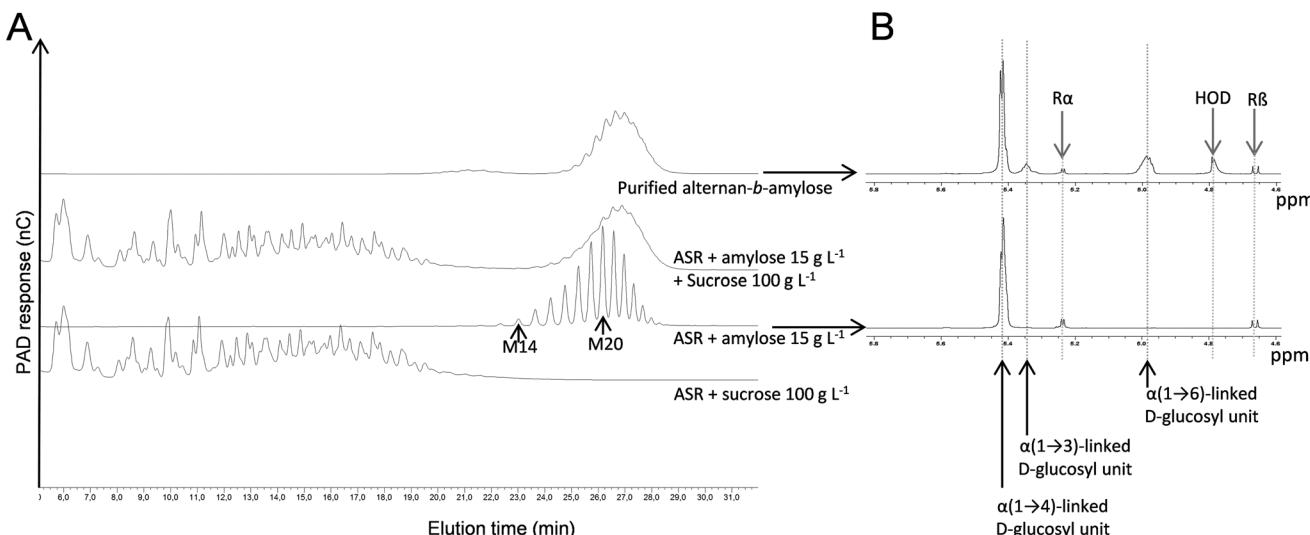


Fig. 2 HPAEC-PAD chromatograms of the copolymer synthesis reaction medium involving ASR and amylose (A), and ^1H NMR spectra of alternan-*b*-amylose and amylose (B). The chemical shifts at 5.45 ppm, 5.35 and 5 ppm were assigned to $\alpha(1 \rightarrow 4)$, $\alpha(1 \rightarrow 3)$ and $\alpha(1 \rightarrow 6)$ linkages respectively. $\text{R}\alpha$ and $\text{R}\beta$ correspond to the anomeric signals of the reducing ($\rightarrow 4$)-D-GlcP units.

$\alpha(1 \rightarrow 4)$ -glucan block acceptor. It was thus considered to be an alternan-*b*-amylose copolymer. To further characterize the polymer, the diblock was separated by means of SEC from the fructose side-product and the natural α -glucans produced by ASR (OAL). The purity of the fraction was evaluated by means of HPAEC-PAD, which revealed the absence of contamination from the natural ASR products (Fig. 2A). The various α -glucosidic linkages comprising the pure diblock were determined by means of ^1H NMR in order to evaluate the relative proportion of each glucan block (Fig. 2B and Table 2). In the alternan-*b*-amylose copolymer, $\alpha(1 \rightarrow 4)$ linkages made up

69%, indicating that the alternan block made up 31%. As the average DP of the initial amylose fraction was 20 (determined by HPAEC-PAD analysis), it was estimated that the amylose chains were grafted with oligoalternans with a DP of 9, assuming that all the chains were homogeneously elongated.

The structure of the copolymer was also analysed by means of permethylation. The relative proportions of each type of permethylated glucosyl units are reported in Table 3. The presence of 3 substituted, 6 substituted and 3,6 di-substituted glucosyl units confirms that the amylose chains were grafted with glucosyl units linked in $\alpha(1 \rightarrow 6)$, $\alpha(1 \rightarrow 3)$ and branching

Table 2 α -Glucosidic linkages in the purified acceptor blocks and copolymers, identified using ^1H NMR. Average DPs were determined either by HPAEC-PAD (amylose), HPSEC (alternan), and HPSEC-MALLS (dextran-*b*-alternan), or calculated from the relative proportion of each block determined by ^1H NMR (alternan-*b*-amylose and dextran-*b*-alternan-*b*-amylose). The details of the calculation of the synthesis yields and final production yields (including both the synthesis and purification steps) are provided in the ESI

Samples	Enzymes	Synthesis conditions		% of α -glucosidic linkages			Estimated synthesis yields (%)	Production yields (%)
		Donor	Acceptor	$\alpha(1 \rightarrow 6)$	$\alpha(1 \rightarrow 3)$	$\alpha(1 \rightarrow 4)$		
Alternan	ASR	Sucrose	—	62.9	37.1	0	33	
Dextran- <i>b</i> -alternan	DSR-M	Sucrose	Alternan	91.0	9.0	0	171	44.3
Amylose	DgAS	Sucrose	Maltose	0	0	100	20	
Alternan- <i>b</i> -amylose	ASR	Sucrose	Amylose	21.3	9.9	68.8	29	35.8
Dextran- <i>b</i> -alternan- <i>b</i> -amylose	DSR-M	Sucrose	Alternan- <i>b</i> -amylose	61.3	4.5	34.2	59	45.2

Table 3 Summary of the different glucosyl units detected after sample permethylation

	Glct %	($\rightarrow 3$)-GlcP-1 \rightarrow %	($\rightarrow 4$)-GlcP-1 \rightarrow %	($\rightarrow 6$)-GlcP-1 \rightarrow %	($\rightarrow 3,6$)-GlcP-1 \rightarrow %
Alternan	8.8	21.6	0	62.3	6.9
Dextran- <i>b</i> -alternan	2.5	5.1	0	88.9	3.1
amylose	6.6	0	93.4	0	0
Alternan- <i>b</i> -amylose	5.4	5.2	58	28.4	1.9
Dextran- <i>b</i> -alternan- <i>b</i> -amylose	2.7	1.8	33.5	60.1	1.1

points. This is in accordance with the structure proposed for alternan⁴⁶ and what has been elsewhere deduced from the analysis of the ASR products obtained from sucrose and maltose.⁵¹

Alternan block synthesis and elongation using DSR-M

At the same time, ASR was also used to synthesize LMM alternans directly from sucrose. After synthesis and fractionation by means of preparative SEC, a fraction with an average molar mass of 5388 g mol⁻¹ (estimated from the HPSEC data with dextran calibration) was selected for extension by DSR-M (Fig. 3A). An HPAEC-PAD analysis of the reaction products revealed the presence of two α -glucan populations, one corresponding to the polymer synthesized by DSR-M from sucrose only, and a second that had been eluted later than the initial alternan acceptor (Fig. 3A).

These results indicate that the alternan block was elongated by DSR-M, resulting in a diblock referred to here as a dextran-*b*-alternan copolymer, since DSR-M is specific for linear $\alpha(1 \rightarrow 6)$ glucan formation. The diblock was isolated by means of preparative SEC (Fig. 3A) and analysed by means of ¹H NMR and permethylation (Tables 2 and 3).

According to the ¹H NMR analysis, the number of $\alpha(1 \rightarrow 3)$ linkages (including the $\alpha(1 \rightarrow 3,6)$ branching points) decreased from 37% in the alternan acceptor to 9% in the diblock, indicating that the alternan and dextran blocks represented 24% and 76% of the diblock respectively. Methylation data confirmed the increased proportion of $\alpha(1 \rightarrow 6)$ linkages in the diblock and the decrease of $\alpha(1 \rightarrow 3)$ linkages and branching points. Finally, from the HPSEC-MALLS analysis of the diblock, a weight-average molar mass of 27 700 g mol⁻¹ (DP171) was determined. Since the molar mass of the alternan block was 5388 g mol⁻¹ (DP of 33), it was calculated that the

alternan and dextran blocks made up 19% and 81% of the diblock respectively. These values do not significantly differ from those obtained from the ¹H NMR analysis (24% and 76% respectively). Based on these results, it would appear that the DP of the alternan block in the diblock ranged from 31 to 36 and that of the dextran block from 135 to 140.

Production, purification and analysis of a triblock copolymer

As it had been proven that DSR-M was able to glucosylate LMM alternan, an attempt was made to glucosylate the alternan-*b*-amylose diblock in order to synthesize a triblock. The purified alternan-*b*-amylose copolymer was incubated with DSR-M and sucrose.

After the reaction, it was observed by means of HPAEC-PAD that the concentration of the alternan-*b*-amylose copolymer had decreased, indicating a covalent modification of the diblock copolymer structure (Fig. 4). Surprisingly, the elution time of the modified copolymer in the HPAEC chromatogram was lower than that of the acceptor. We can exclude that DSR-M caused hydrolysis of the diblock, since no modification of the diblock was observed when the enzyme was incubated with the diblock alone. A more plausible explanation is that the decrease in the retention time was due to the higher number of $\alpha(1 \rightarrow 6)$ -linked glucosyl units in the triblock, introduced by DSR-M.

As shown in Fig. 5, HPAEC-PAD elution times indeed depend on a product's linkage composition, $\alpha(1 \rightarrow 4)$ -linked oligosaccharides being eluted much later than those that are $\alpha(1 \rightarrow 6)$ -linked. In order to investigate the osidic linkage composition of the triblock copolymer, the product was isolated by means of preparative HPAEC-PAD. The product purity was then verified by means of analytical HPAEC-PAD, and the linkage pattern was analysed using ¹H NMR (Fig. 4B and

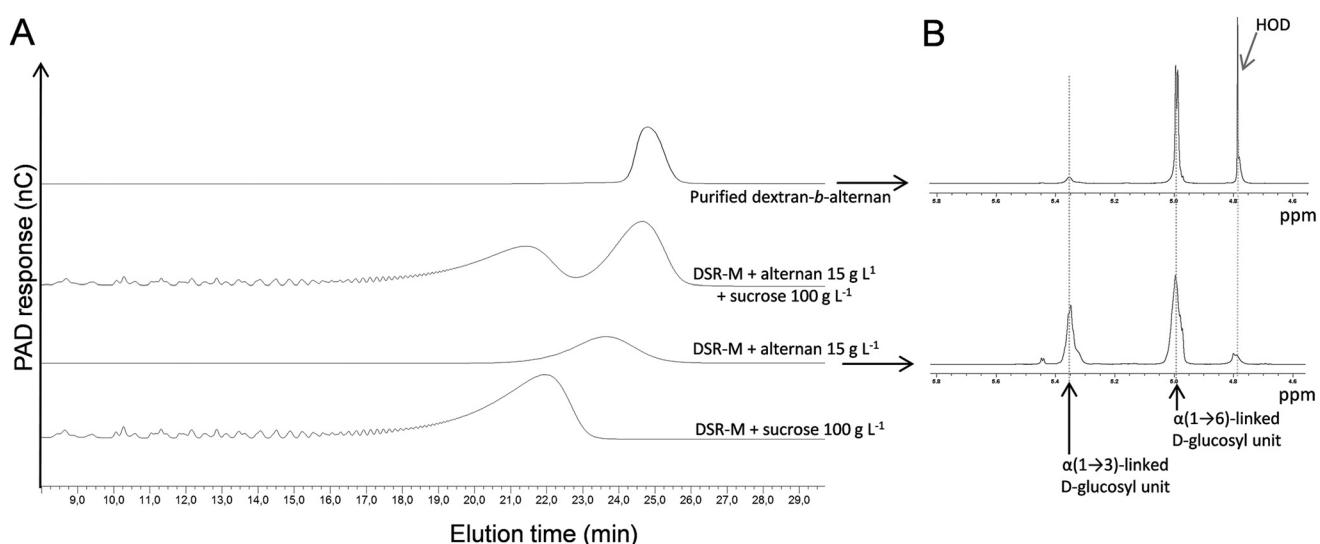


Fig. 3 HPAEC-PAD and ¹H NMR analysis of the copolymer synthesis reaction media involving DSR-M and alternan: (A) HPAEC-PAD chromatograms and (B) ¹H NMR spectra of dextran-*b*-alternan and alternan. The chemical shifts at 5.35 and 5 ppm were assigned to the $\alpha(1 \rightarrow 3)$ and $\alpha(1 \rightarrow 6)$ linkages respectively.



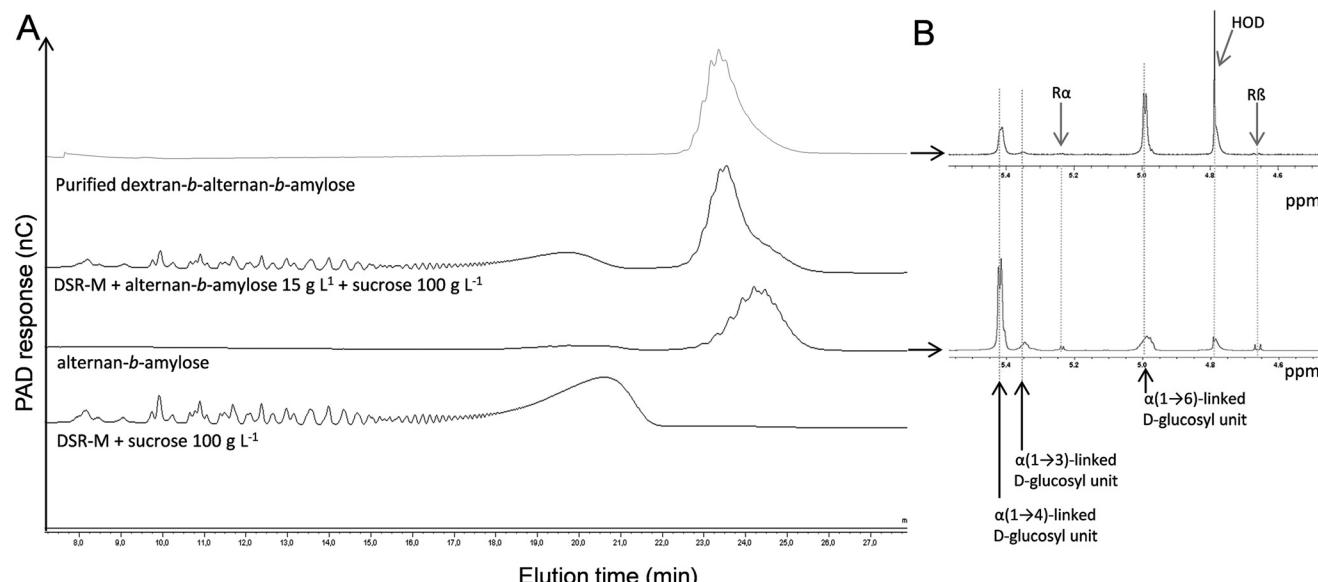


Fig. 4 HPAEC-PAD chromatograms of the purified copolymer obtained from the DSR-M/alternan-*b*-amylose pair (A), and ^1H NMR spectra of dextran-*b*-alternan-*b*-amylose and alternan-*b*-amylose (B). The chemical shifts at 5.45 ppm, 5.35 and 5 ppm were assigned to $\alpha(1 \rightarrow 4)$, $\alpha(1 \rightarrow 3)$ and $\alpha(1 \rightarrow 6)$ linkages respectively. $\text{R}\alpha$ and $\text{R}\beta$ correspond to the anomeric signals of the reducing $-(1 \rightarrow 4)\text{-D-Glc}\beta$ units.

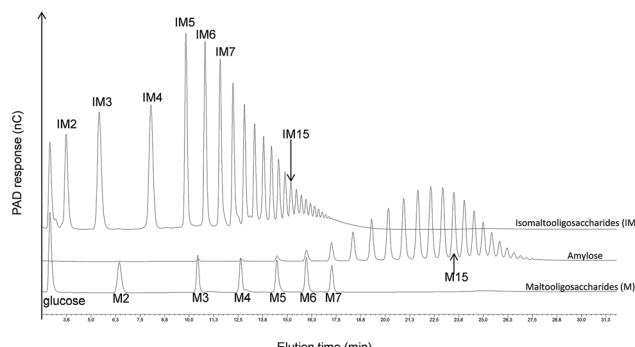


Fig. 5 HPAEC-PAD chromatograms showing the difference in retention times between $\alpha(1 \rightarrow 4)$ -glucans (maltooligosaccharides and amylose) and $\alpha(1 \rightarrow 6)$ -glucans (isomaltooligosaccharides).

Table 2) and permethylation (Table 3). The results show that the proportion of $\alpha(1 \rightarrow 6)$ linkages increased in this purified fraction, whereas $\alpha(1 \rightarrow 3)$ and $\alpha(1 \rightarrow 4)$ linkages decreased, while maintaining an almost constant $\alpha(1 \rightarrow 3)/\alpha(1 \rightarrow 4)$ linkage ratio. This is again consistent with the elongation of the diblock by DSR-M, which is specific for the formation of $\alpha(1 \rightarrow 6)$ linkages. Low numbers of $\alpha(1 \rightarrow 3,6)$ -linked glucosyl units in the triblock were also detected, indicating the presence of branched molecules (Table 3).

NMR analysis indicated that the proportion of $\alpha(1 \rightarrow 4)$ linkages in the triblock was 34%. It was thus deduced that the amylose block represented 34% of the triblock. Since the alternan block comprised 31% of the alternan-*b*-amylose copolymer, the proportions of the alternan and dextran blocks in the triblock were 14% and 52% respectively.

As the average DP of the initial alternan-*b*-amylose copolymer was 29, we can deduce that approximately 30 $\alpha(1 \rightarrow 6)$ -linked glucosyl units were grafted to the alternan-*b*-amylose. Thus, the dextran-*b*-alternan-*b*-amylose copolymer should contain 59 glucosyl residues (30 in the dextran block, 9 in the alternan and 20 in the amylose block) with a calculated molar mass of 9558 g mol⁻¹. These values are average values, as it was assumed that all the chains were elongated to the same extent. Altogether, these results indicate that a triblock copolymer could be obtained by following a stepwise process using the *DgAS*, *ASR* and *DSR-M* enzymes. Examples of structural arrangements of this triblock copolymer are presented in Fig. 6.

The specific locations of the osidic linkages, especially in the alternan block, are putative as several structures could correspond to our data.

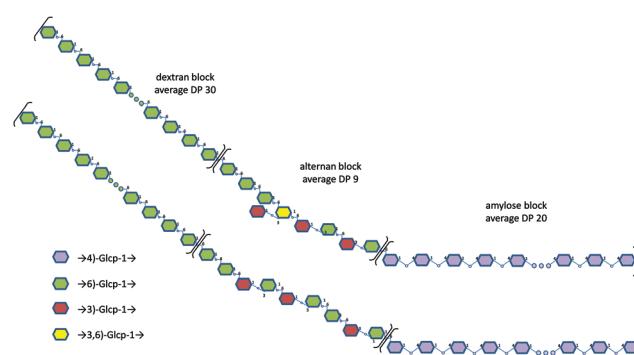


Fig. 6 Examples of structural models proposed for the triblock copolymer dextran-*b*-alternan-*b*-amylose.



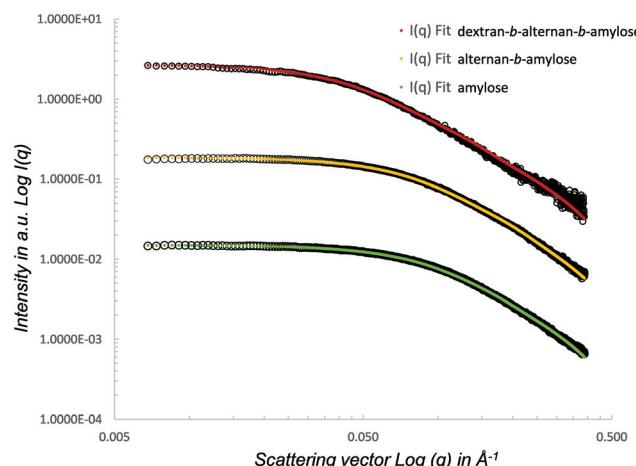


Fig. 7 SAXS curves of the purified triblock copolymer and its diblock and monoblock components.

Copolymer conformation in solution

SAXS was used to analyse the conformation of the initial amylose block, as well as of the alternan-*b*-amylose and dextran-*b*-alternan-*b*-amylose copolymers. The SAXS curves obtained for each polymer are shown in Fig. 7. $\log I(q)$ versus $\log(q)$ was used in order to highlight the region of small q angles where the Guinier approximation and different power law regimes can be determined. The radius of gyration (R_g) values obtained for the amylose block, alternan-*b*-amylose diblock and dextran-*b*-alternan-*b*-amylose triblock were 15.1, 17.1 and 30.0 Å, respectively. It can therefore be deduced that the addition of each block caused an increase in the copolymer R_g . The SAXS curves with a form factor corresponding to a worm-like chain were then fitted using SASfit.⁵² The worm-like model was adjusted, taking the contour length, persistence length and polymer radius as the fitting parameters.

A worm-like conformation is usually used to describe the low DP polysaccharide conformation in solution, and has been previously used to investigate the conformational tracking of amylose synthesized by amylosucrase from sucrose as the sole substrate.⁵³ Here again, it was observed that the worm-like model fits very well with the data collected from the single, di- and triblocks. The contour length (R_L) of the fitted worm-like chain ranged from 85.5 to 271.0 Å (Table 4). This model was also used to estimate the polymers' DPs using a contour

length (R_L)/monomer size (radius) ratio. The calculated DPs were 20, 30 and 59 for amylose, alternan-*b*-amylose and dextran-*b*-alternan-*b*-amylose, respectively. These values are very close to those deduced from the HPAEC-PAD and ¹H NMR analyses. The flexibility of the products in solution was assessed based on the persistence length (R_{LL}) factor values. The addition of the alternan block to the amylose block increased the flexibility, as revealed by the R_{RL} shift from 20.7 to 15.4 Å. With regard to dextran-*b*-alternan-*b*-amylose, the fitted values obtained from the worm-like model differed slightly from the experimental data for q values higher than 0.1 Å. In this specific case, the R_{LL} value must therefore be taken with caution. Nevertheless, the $\log I(q)$ versus $\log(q)$ slope is lower for the experimental values than for the worm-like model, indicating that the addition of the dextran block increased the flexibility of the diblock copolymer. It has been previously demonstrated by means of theoretical and experimental analyses that polysaccharides (amylose, dextran, mutan and cellulose) with the same monosaccharide composition but different linkage types present different behaviours in solution.^{54–56} Using ¹³C NMR relaxation experiments, Tylianakis and co-workers also showed that dextran flexibility was higher than that of amylose.⁵⁷ The higher flexibility of $\alpha(1 \rightarrow 6)$ -linked chains is due to the presence of an extra bond in the inter-residue linkages, which grants a higher degree of freedom.^{58,59} This is very consistent with our SAXS analysis results, as each copolymer block differs from the others in terms of its linkage type, which in turn affects its conformation and flexibility. The contrasting properties of each block would be expected to positively impact the applicative potential of the copolymers synthesized in this study. Due to its helical conformation, amylose could certainly prove to be of great interest in drug and flavour complexation. However, as a result of the weak solubility of amylose preparations in water, these kinds of applications would require amylose functionalization, such as hydroxypropylation, in order to increase the water solubility of the inclusion complexes.^{60,61} In this study, we demonstrated that the covalent grafting of alternan and dextran blocks to amylose increases the flexibility. Such copolymer structures could offer advantages with respect to the encapsulation of bioactive molecules, since the amylose block could serve as the complexing agent, while the alternan and dextran blocks could be used to increase the water solubility, due to their greater flexibility.

4. Conclusions

To our knowledge, this study represents the first successful enzymatic synthesis of biosourced block copolymers from sucrose by means of a stepwise approach. Previous studies involved a glycosyltransferase,^{10,40} a glycoside-phosphorylase,^{12–15} or transglycosidases,^{22,41} and produced either statistical copolymers or co-oligomers (of a total size of less than 30 glycosyl units) rather than block copolymers. Here, we demonstrated that the selected glucansucrases

Table 4 Structural parameters of amylose, alternan-*b*-amylose and dextran-*b*-alternan-*b*-amylose, obtained by SAXS analysis

	Amylose	Alternan- <i>b</i> -amylose	Dextran- <i>b</i> -alternan- <i>b</i> -amylose
Contour length R_L in Å	85.5	131.3	271.0
Persistence length (R_{LL}) in Å	20.7	15.4	21.5
Radius (R) in Å	4.7	4.3	4.8
Gyration radius (R_g) in Å	15.1	17.1	30.0
Maximal distance (D_{max}) in Å	70.0	80.0	120.0



(DSR-M and ASR) transfer α -glucosyl residues to acceptor polymers (i) with a conformation and linkage composition different from that of their natural acceptors, and (ii) a size of up to 30 glucosyl residues, producing copolymers comprising up to 171 residues. The original synthesis method designed for this study successfully resulted, for the first time, in several diblock polysaccharides – dextran-*b*-alternan with a molar mass of 27 700 g mol⁻¹ and alternan-*b*-amylose with a molar mass of 4700 g mol⁻¹ – and one triblock of dextran-*b*-alternan-*b*-amylose with a molar mass of approximately 9500 g mol⁻¹. Whether all GH70 enzymes are able to perform such reactions remains to be established. It was noted that ASR elongated amylose, whereas DSR-M did not, revealing that both the enzyme's specificity and its acceptor's site topology are important. With respect to the objective of producing HMM copolymers on a large scale, the problem appears to be more complex, as the purification techniques currently available do not allow HMM polymer acceptors to be easily separated from either the HMM polymers produced by acceptor elongation or those naturally produced by many glucansucrases. Fractionation based on polymer solubility differences could be a solution, but this remains tricky. Moreover, enzyme engineering may be required in some cases to improve the accommodation of long polymer chains differing in terms of their structure from those of the natural polymer. Nevertheless, the conformational analysis of the new copolymers synthesized here showed that covalent grafting of alternan and dextran to amylose increases the polymer flexibility. While such properties are likely to generate interest in terms of how these copolymers might be used to encapsulate various molecules of interest, such as aromas or drugs, their potential for such uses will have to be assessed. Scaling up this fully green production process to the dozen gram level will allow us to obtain more information on the structure/property relationship of these copolymers, as well as their potential applications.

Conflicts of interest

There are no conflicts to declare.

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