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Carbohydrate Research 338 (2003) 1951–1960
CARBOHYDRATE RESEARCH
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Solid-supported enzymatic synthesis of pectic oligogalacturonides and their analysis by MALDI-TOF mass spectrometry

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Received 27 March 2003; received in revised form 28 June 2003; accepted 5 July 2003

Abstract

Solid-phase biosynthetic reactions, followed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis (MALDI-TOF), was used to gain insight into the biosynthesis of pectin oligomers. Sepharose supports bearing long pectic oligogalacturonides (OGAs) anchored through a disulfide-containing cleavable linker, were prepared. The OGAs (degrees of polymerization of 13 and 14) were efficiently immobilized through the reducing end via formation of an oxime linkage. These OGA-derivatized matrices were subsequently employed in novel solid-phase enzymatic reactions, with the pectin biosynthetic enzyme, α-1,4-galacturonosyltransferase, GalAT (solubilized from *Arabidopsis thaliana*) and the glycosyl donor, uridine diphosphate-galacturonic acid (UDP-GalA). Solid-supported biosynthesis was followed by cleavage of the immobilized OGAs and direct analysis of the products released into the liquid phases by MALDI-TOF mass spectrometry. In time course studies conducted with an immobilized $(\alpha\text{-D-GalA})_{14}$ and limiting amounts of the glycosyl donor, the predominant product was an OGA extended by one GalA residue at the non-reducing end (i.e., $(GalA)_{15}$). When UDP-GalA was added in $\approx$ excess compared to immobilized $(GalA)_{13}$, OGAs up to the 16-mer were synthesized, confirming the non-processivity of the GalAT in vitro.

Keywords: Biosynthesis; Enzymatic glycosylation; Galacturonosyltransferases; Glycosyltransferases; Pectin; Solid-phase

1. Introduction

Pectin is a complex polysaccharide that is located in all plant primary cell walls and that exhibits a multitude of functions in plants.[1–3] An important feature of pectin is its ability to form gels under defined conditions and this property has been widely exploited within the food industry for the preparation of jams and jellies.[4] Pectin has also been employed as a stabilizer in beverages and dairy products. Pharmaceutical effects of pectin are also known and include the lowering of serum glucose levels in diabetics and the reduction of blood cholesterol.[5] This

Abbreviations: Aoa, Aminooxy acetyl; Boc, *tert*-Butyloxycarbonyl; BSA, Bovine serum albumin; DMF, Dimethylformamide; DP, Degree of polymerization; DTT, Dithiothreitol; α-D-GalA, Galacturonic acid; GalAT, α-1,4-Galacturonosyltransferase; HBTU, *N*-[(1H-Benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; *m*-HDP, *meta*-Hydroxybiphenyl; HEPES, [4-(2-Hydroxyethyl)-1-piperazineethane sulfonic acid]; HG, Homogalacturonan; HPAEC, High performance anion exchange chromatography; MALDI-TOF MS, Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; NC, Nitrocellulose; OGA, Oligogalacturonide; PEGA, Polyethylene glycol polyacrylamide; Pyr, Pyridine; RG, Rhamnogalacturonan; rt, Room temperature; SEC, Size exclusion chromatography; THAP, 2,4,6-Trihydroxyacetophenone; TLC, Thin-layer chromatography; Tris, Tris(hydroxymethyl)aminomethane; UDP-GalA, Uridine diphosphate-galacturonic acid.

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doi:10.1016/S0008-6215(03)00321-5

polysaccharide and some of its derivatives have also been used in the treatment of diarrhea and in the regulation and protection of the gastrointestinal tract.[4] Additionally, there are indications that pectin may inhibit cancer metastasis.[6,7] Three polysaccharides have been identified and characterized in all pectins, namely homogalacturonan (HG), rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II).[8] HG, which accounts for 60% of pectin, consists of a linear chain of α-(1→4)-linked galacturonic acid (GalA) residues that can be partially methyl esterified at the C-6 carboxylic acid position.[1] Depending on the plant source, a proportion of the secondary alcohol groups may also be *O*-acetylated at O-2 and O-3. Other pectic polysaccharides, such as xylogalacturonans and apiogalacturonans, have also been structurally characterized from a limited number of plants.[2] The sheer complexity of pectin is highlighted by the fact that at least 58 enzyme activities are predicted to be involved in its biosynthesis.[9] These enzymes include glycosyl-, methyl-, and acetyltransferases. The identification of the biosynthetic enzymes and the elucidation of their modes of action are two of the most important challenges for biochemical studies of pectin.

We previously identified an α-1,4-galacturonosyltransferase (GalAT) in microsomal membranes isolated from tobacco and radish, and in pea (*Pisum sativum* L. var. Alaska) Golgi.[10,11] The latter study showed that GalAT is a Golgi-localized enzyme with its catalytic site facing the Golgi lumen. Using detergents, we also solubilized membrane-bound GalAT from radish, tobacco, and *Arabidopsis thaliana* (var. Columbia) (Sterling, J.D., Mohnen, D., unpublished) and employed in vitro studies to show that the solubilized enzyme catalyzes the transfer of individual GalA residues from UDP-GalA onto HG oligosaccharides (i.e., oligogalacturonides; OGAs) with a preference for chains of degree of polymerization (DP) >9.[10,12,13] Since processive glycosyltransferases catalyze multiple glycosyl transfers before dissociating from the acceptor, the finding that GalAT synthesizes primarily an OGA extended by one GalA residue when reactions are carried out at equimolar or low UDP-GalA:OGA ratios,[12,13] suggests that, at least under in vitro conditions, the enzyme does not act processively. GalAT can however accept and elongate OGA acceptors synthesized in a prior catalytic cycle. Indeed, in our laboratories (Mohnen, D., unpublished) and elsewhere,[14] it has been demonstrated that at high (1 mM) concentrations of UDP-GalA and 10–1000-fold lower concentrations of uniformly sized OGAs (i.e., 1–100 μM), OGAs extended by up to twelve GalAs can be recovered. That said, the kinetics of product formation in all these studies strongly suggest a distributive (i.e., non-processive) mode of synthesis.

Scheller and co-workers showed that the elongation of the HG chain takes place at the non-reducing end.[13] The products resulting from reactions between GalAT, UDP-GalA and exogenous OGA acceptors have so far been identified using *comparative* analytical methods, such as high performance anion exchange chromatography (HPAEC) and size exclusion chromatography (SEC).[11–13] However, despite these efforts, the detailed mechanism of HG biosynthesis remains unresolved, and moreover, direct analytical evidence that the elongation of an HG (i.e., OGA) chain proceeds via the transfer of single GalA residues from UDP-GalA in the presence of GalAT (i.e., in a non-processive manner), is still required.

Solid-phase chemistry has played an increasingly important role in the synthesis of oligosaccharides over the past few years, given the multiple advantages it offers for product isolation and automation.[15] The recent use of enzymes to perform solid-supported oligosaccharide synthesis has revolutionized this growing field.[16–21] For instance, this technique has proved efficient for the synthesis of tetrasaccharides such as lacto-*N*-neotetraose and sialyl Lewis X.[20,22] In general, excellent selectivities, good efficiencies and high product recovery can be obtained without the need for protecting groups. We therefore envisioned that the combined use of solid-phase techniques and glycosyltransferases might provide novel information regarding HG biosynthesis, and further anticipated that OGAs of defined sizes and with a DP ≥ 10 could, in an immobilized state, serve as exogenous acceptors for GalAT. Employment of such OGAs would also constitute a new approach to the solid-phase synthesis of large oligosaccharides, and thus, be of general interest.

Recently, we developed a fast preparative procedure for the purification of large quantities of pectin OGAs of defined sizes, and with degrees of polymerization up to 20,[23] and additionally reported several immobilization strategies for anchoring OGAs to solid-supports through their reducing ends.[24] In the latter study, a disulfide bridge was installed, in some cases, between the linker unit connecting the OGA to the polyethylene glycol polyacrylamide (PEGA) support. This made it possible to release the OGAs into solution upon treating with dithiothreitol (DTT).

Here, we describe chemoselective 'reversible' immobilization of pure OGAs (DP = 13 or 14) onto solid-supports through their reducing ends. The linker contained a disulfide bond and an aminooxy-terminal group. Following solid-supported biosynthetic reactions with UDP-GalA and a solubilized GalAT isolated from *Arabidopsis thaliana* suspension cultured cells, the OGAs were cleaved from the support under mild reducing conditions, and analyzed as their thiol-derivatives by MALDI-TOF MS. In this study, Sepharose supports were chosen in preference to the PEGA resin, that we have employed in much of our previous work.[21,23,24] This was primarily because the Sepharose

6B matrix possesses a much higher size exclusion limit (4000 kDa)[25] for globular proteins than the PEGA material (66 kDa)[26]. Given that the size of the glycosyltransferase used in this study was not known, it was therefore anticipated that the Sepharose supports might be more appropriate. An additional advantage of the Sepharose matrix is that it is ideally suited for preparative affinity chromatography.[27–30] Although not a part of this work, we envisage that the OGA-functionalized Sepharose supports reported herein, could be usefully employed as affinity matrices in future studies, e.g., in the isolation of pectin biosynthetic enzymes.

2. Results and discussion

2.1. Goals and strategy

Here, we describe the preparation and use of OGA-derivatized Sepharose supports combined with MALDI-TOF MS analysis as a new tool to monitor the addition of GalA residues onto an OGA chain using a solubilized α-1,4-galacturonosyltransferase and UDP-GalA. Several key issues had to be considered before carrying out this study: (i) the choice of solid-support, which has to be freely permeable to biosynthetic enzymes; (ii) the use of large OGA substrates (DP ≥ 10) of defined sizes;[12] (iii) the design of an immobilization strategy with pectin (i.e., OGA) fragments anchored to the matrix through the reducing-end in order to allow chain extension at the non-reducing end;[13] and (iv) the selection of a covalent, chemoselective and stable linkage that allows anchoring and subsequent release of the OGAs in solution under mild conditions.

Sepharose supports meet the first requirement and we selected them for the aforementioned reasons. As previously reported, we have developed an efficient method for immobilizing model pectin fragments through the reducing end by the formation of an oxime linkage, and have furthermore illustrated the effectiveness of a disulfide bridge linker in enabling release of attached OGAs with DTT.[24] This methodology has now been applied to immobilize long OGAs (DP = 13 or 14) purified by AEC onto aminooxy-terminated Sepharose supports that contained a disulfide cleavable linker (Scheme 1).

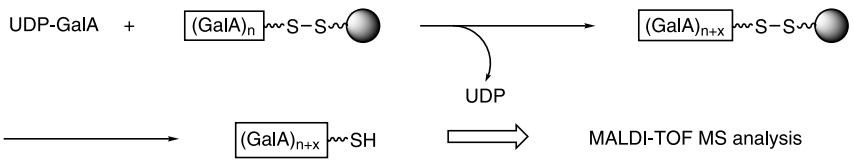
The OGA-derivatized supports were then subjected to enzymatic glycosylation using a solubilized GalAT from *Arabidopsis thaliana* (var. Columbia) and UDP-GalA. Following biosynthesis, the supports were treated with DTT, and the released OGAs were directly analyzed by MALDI-TOF MS as their thiol derivatives (Scheme 1).

Longer OGAs (DP ≥ 10) appear to offer important advantages over shorter ones for solid-supported biosynthetic reactions. On the one hand, they are potential substrates for the biosynthetic enzyme GalAT,[12] while on the other, their increased length positions the reaction site (the non-reducing end) further away from the anchoring point. Blixt and Norberg observed that an increased linker length had favorable effects on biosynthetic yields, and they argued that this was due to the reduced steric interference between enzyme and solid-phase, as well as greater conformational flexibility.[20]

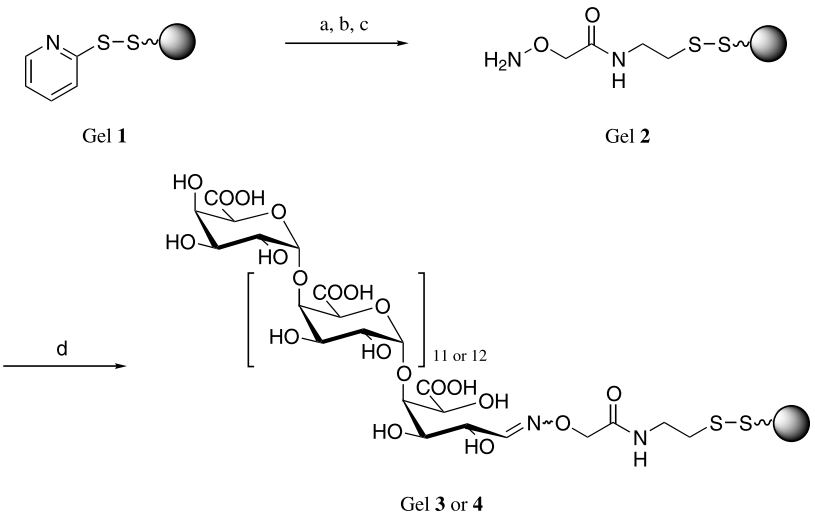
2.2. Preparation of OGA-derivatized Sepharose supports

An aminooxy derivative, Boc–Aoa–NH–$(CH_2)_2$–S–S–Pyr, was synthesized in solution from Boc–Aoa–OH and *S*-(2-pyridylthio)cysteamine hydrochloride.[31–34] Thiopropyl Sepharose supports (gel **1**; Scheme 2) were deprotected with DTT at pH 8.4, and the resultant thiol-functionalized gel was reacted with the protected aminooxy derivative via a disulfide exchange reaction in methanol (Scheme 2). A yellow color was observed, indicating release of pyridine-2-thione in solution. Final deprotection of Boc-groups afforded aminooxy-terminated supports **2** that contained a disulfide bridge linker. Typically, OGAs (DP of 13 or 14) were immobilized onto gel **2** through the reducing end. Oxime formation took place under mild conditions, in H_2O–DMF (4:1) at pH 4.8 and 40 °C over 24 h, to give either $(GalA)_{13}$- or $(GalA)_{14}$-functionalized supports (gels **3** & **4**, respectively; Scheme 2).

In contrast to previous work, in which short OGAs were added in excess in model studies in order to push reactions to completion,[24] a 7- to 8-fold excess of support was used here for the larger OGAs. We have previously developed a method for direct determination of the OGA loading on PEGA supports.[24] However, due to the acid-lability of Sepharose supports, this method could not be adapted here. Instead we relied on an indirect procedure for determination of the loading. Hence, the amount of OGA recovered after



Scheme 1. General strategy for solid-supported biosynthesis and analysis of OGA reaction products. n is the initial DP and $(n+x)$ represents the resulting DP.



Scheme 2. Preparation of $(GalA)_{13}$- and $(Gal\ A)_{14}$-functionalized Sepharose supports (gels **3** & **4**, respectively). (a) DTT, 0.3 M aq $NaHCO_3$ containing 1 mM disodium ETDA, pH 8.4, rt, 40 min; (b) Boc–Aoa–NH–$(CH_2)_2$–S–S–Pyr, MeOH, rt, 2 h; (c) TFA–H_2O (1:4), rt, 1 h; (d) OGA ($n = 13$ or 14), H_2O–DMF (4:1), pH 4.8, 40 °C, 24 h.

loading was subtracted from the initial amount applied to the gel. The immobilization reactions were quantified by the *m*-hydroxybiphenyl (*m*-HDP) assay, specific for uronic acids,[35,36] and the degree of functionalization of the gels with $(GalA)_{13}$ and $(GalA)_{14}$ was determined as ≈ 2 μmol OGA per mL drained gel.

2.3. Solid-phase biosynthetic reactions

$(GalA)_{14}$-derivatized Sepharose gels **4** (1 equiv OGA) were incubated with a partially purified solubilized GalAT from *Arabidopsis thaliana* in the presence of a limiting amount of the glycosyl donor UDP-GalA (0.20 μmol, approx 0.5 equiv) and 6.5 mM $MnCl_2$ (Schemes 1 and 2) for various times. The reaction buffer used was the same as that recommended by Doong and co-workers,[10] and Scheller and co-workers.[13] The pH was adjusted to 7.8 and the temperature was maintained at 30 °C during the reactions in order to ensure maximal GalAT activity.[10] After termination of the reactions, the gels were treated with DTT at pH 8.0 in order to reduce the disulfide linkage between the OGAs and the resin, and thereby release the OGAs into solution.

Fig. 1 shows the MALDI-TOF MS spectra recorded during a solid-supported time course experiment. The released OGAs were detected as their thiolated derivatives (i.e., carrying H–Aoa–NH–$(CH_2)_2$–SH at their reducing ends). At time 'zero', only the original OGA 14-mer was detected as its thiolated derivative ($m/z =$ 2612.2). At all other times, two thiolated OGA derivatives ($m/z = 2612.2$ and 2789.6) were observed, namely the starting $(GalA)_{14}$ and a new product, extended by one GalA residue, i.e., $(GalA)_{15}$. Judging by visual inspection of the relative series of the $(GalA)_{14}$ and the $(GalA)_{15}$ peaks, conversion of the former to the latter was complete within 30 min. That the reaction appeared to stop after this time is understandable, given the limiting amounts of UDP-GalA that were supplied in relation to the number of support-immobilized $(GalA)_{14}$ molecules available. No additional peaks corresponding to e.g., intermediate, side, or degradation products were detected in any of these samples within the *m*/*z* scan range of 200–10,000 Da.

The above experiment demonstrated that the product resulting from in vitro biosynthetic reaction between an immobilized OGA acceptor and soluble UDP-GalA donor catalyzed by solubilized GalAT from *Arabidopsis*, is the immobilized OGA extended at the non-reducing end by one GalA residue. The non-processive action of GalAT observed here is in accordance with earlier observations from our laboratories on the action of a GalAT solubilized from tobacco membranes.[10,12,13] Using soluble $(GalA)_{15}$ as an acceptor and UDP-[^{14}C]GalA as the glycosyl donor, the product isolated by HPAEC was identified on the basis of retention time as [^{14}C]$(GalA)_{16}$, labeled at the non-reducing end.[12]

In view of the non-processive action of *Arabidopsis* GalAT, we reasoned that controlled synthesis of immobilized OGAs of desired length might be achieved by modifying the molar UDP-GalA/OGA ratio. To test this, we incubated a $(GalA)_{13}$-linked support (gel **3**) with GalAT in the presence of $\approx$ 5-fold excess of UDP-GalA (i.e., over the immobilized OGA) for 45 min at 30 °C. The MALDI-TOF MS spectrum we recorded is presented in Fig. 2.

In addition to the starting $(GalA)_{13}$, longer thiolated OGAs with DPs of 14, 15, and 16 were detected. Clearly, under the conditions applied, solubilized *Arabidopsis* GalAT had non-processively transferred GalA units from UDP-GalA onto the exposed non-reducing end

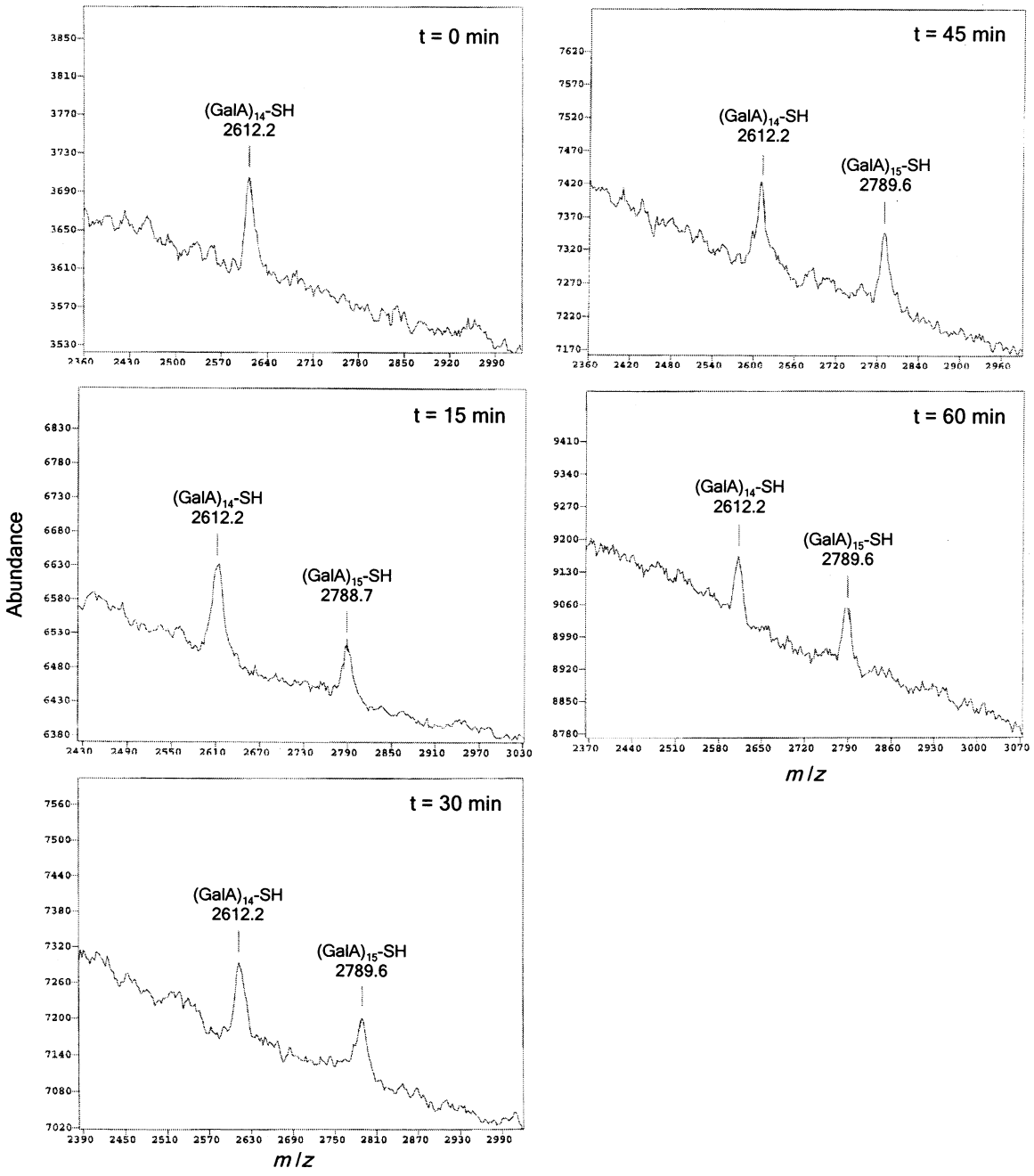


Fig. 1. MALDI-TOF MS analysis of thiolated OGAs released from Sepharose supports by reductive cleavage following solid-phase biosynthesis using $(GalA)_{14}$-derivatized Sepharose supports and limiting amounts of the glycosyl donor, UDP-GalA. $(GalA)_{14}$- and $(GalA)_{15}$-SH represent the thiolated $(GalA)_{14}$ and $(GalA)_{15}$, respectively (i.e., derivatized with H–Aoa–NH–$(CH_2)_2$–SH at their reducing end).

of immobilized OGA products produced in a prior catalytic cycle. Our findings here are consistent with those recently reported by Akita and co-workers who demonstrated the glycosyl transfer activity of GalAT solubilized from pollen tubes of *Petunia axillaris*.[14] For example, when using a 66-fold excess of UDP-GalA and HPAEC analysis, these authors showed that a fluorogenic pyridylaminated-$(GalA)_{14}$ could be extended by up to 13 GalAs, i.e., up to the 27-mer. In our study (Fig. 2), the largest synthesized GalA oligomer detected by MS was the starting $(GalA)_{13}$ extended by three GalA units. It is likely that by raising the fold of UDP-GalA

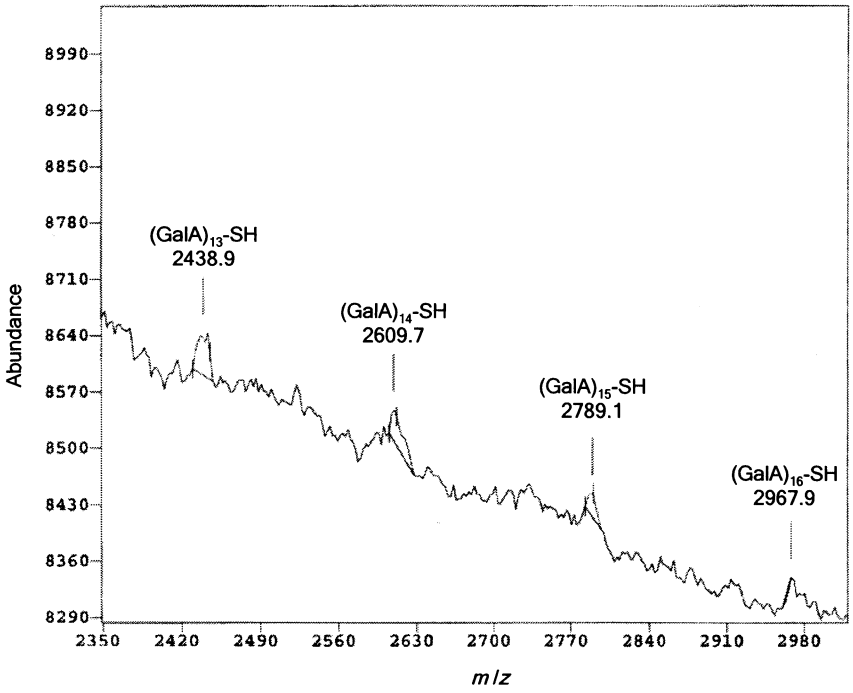


Fig. 2. MALDI-TOF MS analysis of thiolated OGAs released from Sepharose supports by reductive cleavage following solid-phase biosynthesis using $(GalA)_{13}$-linked supports and a $\approx$ 5-fold molar excess of the UDP-GalA donor. As in Fig. 1, $(GalA)_n$-SH represents the thiolated $(GalA)_n$.

excess one or two orders of magnitude further, much longer OGAs could be synthesized on the solid-supports.

3. Conclusions

This study is the first report of the use of uronic acid oligomers anchored to a solid matrix as glycosyl acceptors for solid-supported oligosaccharide biosynthesis. The solid-phase strategy we have developed offers several key advantages over previously reported approaches for studying oligo- or polysaccharide biosynthesis. First, long OGA structures do not require chemical modification, such as derivatization or use of protecting groups. Second, the use of labeled nucleotide sugars, e.g., UDP-[^{14}C]GalA,[11,13] is circumvented. Third, no sample conditioning is required after biosynthesis, allowing the OGAs to be analyzed directly, in contrast to previous comparative methods based on co-elution of synthesized OGAs and standards in HPAEC analysis.[11–13] Finally, the disulfide linker employed in this study allows for release of OGAs under mild reducing conditions and for the production of thiolated pectin fragments, which may find novel applications in biophysical studies.

Using the disclosed solid-phase approach, we have obtained additional data on the non-processivity of GalAT to support that obtained in earlier studies carried out in free solution with GalATs from different sources and using less sensitive analytical methods than MALDI-TOF MS.[12–14] Further work is required to gain new insight into the mode of action and reaction kinetics/properties of GalAT enzymes, as well as the influence of solid-phase attachment of the acceptor on such properties. Armed with this information, and using controlled batchwise feeding of appropriate amounts (i.e., perhaps equimolar) of glycosyl donor to the immobilized acceptor, one could envisage that it should be possible to generate, at will, OGAs of precisely defined length, requiring no purification following release from the support.

4. Experimental

4.1. General methods

Thiopropyl Sepharose 6B support (gel **1**) was purchased from Amersham Biosciences (Uppsala, Sweden) and nitrocellulose (NC) was obtained from BioRad (Hercules, CA, USA). All other reagents were supplied by Sigma-Aldrich A/S (Milwaukee, WI, USA, St. Louis, MO, USA, or Vallensbæk Strand, Denmark). Water used for the synthesis of Boc–Aoa–NH–$(CH_2)_2$–S–S–Pyr was distilled and purified to 18.2 MΩ cm^{-1} in a Milli-Q apparatus. In all other cases, water was deionized using an Ultrafiltration water system (US Filter, Alpharetta, GA, USA). Highly pure ($>99\%$) $(Gal\ A)_{13}$ and $(GalA)_{14}$ were prepared from pectin lyase digested

pectin using a recently described AEC-based fractionation protocol.[23]

Solid-phase derivatization reactions were performed in 10 mL plastic syringes equipped with a polypropylene filter and a rubber cap. The syringes were placed on a Teflon Domino block (Torviq, Tucson, AZ, USA) suitable for parallel solid-phase synthesis, and connected to a vacuum line. Free thiol-terminated supports were stored overnight as a suspension in 0.1 M aq AcOH containing 0.5 M NaCl and 1 mM disodium EDTA, under inert atmosphere (He) at 4 °C. Protected thiol-terminated gels were kept in H_2O–EtOH (4:1) at 4 °C.

Thin-layer chromatography (TLC) was performed on Merck Silica gel 60 F_{254} plates. Spots were visualized under UV light and by heating after dipping into a solution of 2% cerium (IV) sulfate and 5% phosphomolybdic acid hydrate in 2 M aq H_2SO_4. ^1H and ^{13}C NMR spectra were recorded on a Varian Mercury 300 instrument equipped with a 4-nuclei probe operating at 300.06 and 75.45 MHz, respectively. ES-MS analyses were conducted on a Micromass LCT mass spectrometer (Manchester, UK) equipped with an AP-ESI probe. MALDI-TOF MS analyses were performed on a G2025A LD-TOF Hewlett-Packard mass spectrometer (Palo Alto, CA, USA). All the MALDI-TOF MS spectra were acquired in the negative ion mode. The instrument was operated at an accelerating voltage of 28 kV, an extractor voltage of 7 kV, and a pressure of approx 10^{-7} torr. The spectrometer was externally calibrated using: (i) a peptide mixture of insulin chain A (1 mg mL^{-1}), somatostatin (1 mg mL^{-1}), and angiotensin (1 mg mL^{-1}); and (ii) a saturated solution of sinapinic acid in CH_3CN–H_2O (1:1) containing 0.1% TFA as the matrix.

4.2. Boc–Aoa–NH–$(CH_2)_2$–S–S–Pyr

The starting materials, Boc-aminooxyacetic acid (Boc–Aoa–OH) and *S*-(2-pyridylthio)cysteamine hydrochloride, were prepared according to established literature procedures.[31–34] *S*-(2-Pyridylthio)cysteamine hydrochloride (500 mg, 2.24 mmol) and *N*-[(1H-Benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HBTU, 850 mg, 2.24 mmol) were dissolved in CH_3CN (20 mL). NEt_3 (710 μL, 5.09 mmol) was pipetted into a solution of Boc–Aoa–OH (390 mg, 2.04 mmol) in CH_3CN–H_2O (25:1, 10.4 mL) and the mixture was then added to the *S*-(2-pyridylthio)cysteamine hydrochloride-HBTU cocktail. The reaction was completed within 3 h at room temperature (rt), according to TLC. The mixture was subsequently diluted in EtOAc (50 mL), washed with 1 M aq HCl (3 × 20 mL), saturated aq $NaHCO_3$ (3 × 20 mL), and brine (3 × 20 mL), dried over $MgSO_4$, and concentrated in vacuo to give a transparent oil, identified as the title compound (504 mg, 70%). ^1H NMR (acetone-d_7) δ 1.45 (s, 9 H, CH_3, Boc), 3.01 (t, 2 H, CH_2–S–S, *J* 6.6 Hz), 3.59 (m, 2 H, NH–CH_2), 4.25 (s, 2 H, NH–O–CH_2), 7.24 (m, 1 H, H-5′, Pyr) 7.81 (m, 2 H, H-3′, H-4′, Pyr), 8.31 (s, 1 H, N*H*), 8.48 (m, 1 H, H-6′, Pyr), 9.46 (s, 1 H, N*H*). ^{13}C NMR (acetone-d_7) δ 27.71 (CH_3, Boc), 37.75 (CH_2–S–S), 38.10 (NH–CH_2), 75.61 (NH–O–CH_2), 119.84, 121.30, 137.81, 149.73 (Pyr), 157.78 (*C*(O), Boc), 168.88 (CH_2–*C*(O)–NH). ES-MS, Calcd for $C_{14}H_{21}N_3O_4S_2$: 359.10. Found: *m*/*z* 382.09 $[M+Na]^+$, 360.08 $[M+H]^+$, 304.01 $[M-{}^t Bu+H]^+$, 260.04 $[M-Boc+H]^+$.

4.3. Preparation of aminooxy-terminated Sepharose supports containing a disulfide bridge linker (gel 2)

Washing of the gel was typically performed in 10 mL portions. Dry thiopropyl Sepharose 6B gel **1** (400 mg) was weighed out in a 10 mL syringe and swelled with 8 mL water. The swollen matrix was subsequently washed in situ with H_2O (80 mL) for about 15 min. DTT (50 mg) was dissolved in 0.3 M aq $NaHCO_3$ (5 mL) containing 1 mM aq disodium EDTA (2 mg) and the pH was adjusted to 8.4 with 0.1 M KOH. An aliquot (1.6 mL) of this solution was applied to the gel bed and the syringe was gently shaken at rt for 40 min. The solvents and excess reagents were filtered off under vacuum and the gel was washed with 160 mL of 0.1 M aq AcOH containing 0.5 M NaCl and 1 mM disodium EDTA. After overnight storage at 4 °C under inert atmosphere, the free thiol gel was washed with MeOH (80 mL). Boc–Aoa–NH–$(CH_2)_2$–S–S–Pyr (50 mg, 0.14 mmol) was dissolved in MeOH (1.5 mL), loaded onto the gel and the syringe was then shaken at rt for 2 h. The gel was drained and washed consecutively with 80 mL portions of MeOH and then H_2O. Deprotection of Boc groups was performed by TFA–H_2O (1:4, 1.6 mL) at rt for 1 h. Reagents and solvents were filtered off, affording 1.2 mL of drained gel, which was finally washed with H_2O (100 mL) and used immediately for immobilization of OGAs.

4.4. General method for the immobilization of OGAs (DP = 13 or 14) via oxime formation. Preparation of $(GalA)_{13}$- and $(GalA)_{14}$-functionalized Sepharose supports (gels 3 & 4, respectively)

Washing of the gel was typically performed in 10 mL portions. Aminooxy-terminated support (gel **2**; 1.2 mL drained gel) was washed with 100 mL H_2O. $(GalA)_{14}$ (10.0 mg, 4 μmol) was weighed into a 10 mL plastic vial. Water (1.3 mL) was added and the pH of the solution was reduced from 5.0 to 4.8 with 0.1 M HCl. The solution was applied to the gel. Dimethylformamide (DMF, 0.3 mL) was added and the syringe was gently shaken at 40 °C for 24 h. The solvents were filtered off and the filtrate was collected. The gel was washed with 4

mL of H_2O and the collected water wash was combined with the previous filtrate, stored at 4 °C, and then freeze-dried until quantitation. The freeze-dried filtrate (recovered OGA) was used for an indirect method to estimate the amount of OGA coupled to the support and thus the loading. The amount of recovered OGA, determined by a *m*-HDP assay as described below, was subtracted from the original amount of OGA applied to the aminooxy-functionalized gel. The protected thiol-terminated gel (**4**) was finally washed with H_2O (100 mL) and EtOH–H_2O (1:4, 50 mL), and stored as a suspension in the latter solvent mixture until further use.

In a similar fashion, $(GalA)_{13}$ (9.9 mg, 4.3 μmol) was immobilized onto aminooxy-terminated supports (gel **2**; 1.5 mL drained gel) via the reducing end, to give $(GalA)_{13}$-linked Sepharose matrices (gel **3**).

4.5. Determination of immobilization efficiency by the *m*-HDP assay

The OGA content of the filtrate collected after the immobilization reaction was quantified by the *m*-HDP assay, which was modified for use in ELISA plates.[35,36] The standard stock solution contained 2.0 mg of $(GalA)_{14}$ dissolved in 2 mL of H_2O.

4.6. Solubilization of GalAT from *Arabidopsis* membranes

Suspension-cultured *Arabidopsis thaliana* (cv Columbia) cells derived from leaf calli were grown in the dark for 10 days in Gamborg's B-5 basal medium containing 58.4 mM sucrose, 9 μM 2,4-dichlorophenoxyacetic acid, and 0.23 μM kinetin. The 'dark-grown' *Arabidopsis* cells (700 g) were homogenized in 1.2 L of homogenization buffer (50 mM HEPES, pH 7.3, 0.25 mM $MnCl_2$, 25 mM KCl, 50% (v/v) glycerol and 0.1% β-mercaptoethanol) at 4 °C using a Parr bomb. The homogenate was filtered through two layers of nylon mesh and the filtrate was centrifuged at 28,000*g* for 5 h at 4 °C. The supernatant was decanted and the top layer of the microsomal pellet was resuspended in 9 mL of 50 mM HEPES, pH 6.8, 0.25 mM $MnCl_2$, 25 mM KCl, and 25% (v/v) glycerol. To the resuspended pellet, Triton X-100, NaCl, EDTA, and NaOAc were added to final concentrations of 4% (v/v), 0.2 M, 2 mM, and 0.1 M, respectively, and the mixture was centrifuged at 150,000*g* for 1.5 h at 4 °C. The supernatant containing proteins that were soluble in 4% (v/v) Triton X-100 was removed and diluted with 4 volumes of 50 mM HEPES, pH 7.3, 0.25 mM $MnCl_2$, 2 mM EDTA, 25% (v/v) glycerol, and 0.25% (v/v) Triton X-100. The diluted fraction, subsequently called solubilized enzyme, was filtered through a 0.2 μm nylon filter and either used immediately or stored at −80 °C until use.

The specific activity of GalAT of 94.5 pmol GalA incorporated min^{-1} mg^{-1} protein was determined as described. A 10 μL fraction of solubilized enzyme (5 mg mL^{-1} protein) was incubated with 50 mM HEPES, pH 7.8, 200 mM sucrose, 25 mM KCl, 0.05% bovine serum albumin (BSA), 0.25 mM $MnCl_2$, 80 μg OGAs (DP = 7–23), 100 μM cold UDP-GalA, and 1 μM UDP-[^{14}C]GalA in a final reaction volume of 30 μl for 15 min at 30 °C. The reaction was terminated by the addition of 10 μL of 200 mM NaOH and the amount of radioactivity incorporated into product was determined using a filter-scintillation assay (Lemons, Forkner, Sterling, and Mohnen, in preparation).

4.7. Solid-supported biosynthetic reactions

$(GalA)_{13}$- and $(GalA)_{14}$-functionalized gels **3** and **4** (see above) were recovered from the storage solvent by filtration, and washed extensively, first with H_2O (10×10 mL) for 15 min and then with washing buffer (8×2 mL) for 10 min. The washing buffer was composed of 50 mM HEPES (pH 7.8), 0.2 M sucrose, 0.05% BSA, 25 mM KCl, and 0.25 mM $MnCl_2$. A 2 mL aliquot of five times strength washing buffer was added onto the gel. Portions of resuspended gels were transferred into 1.5 mL Eppendorf tubes and centrifuged briefly (approx 10,000 rpm for 1 min), before removing the supernatants. These latter steps were repeated to yield either 200 μL of functionalized drained $(GalA)_{14}$ gel (**4**) or 100 μL of $(GalA)_{13}$-support (gel **3**).

In studies with the $(GalA)_{14}$-linked Sepharose supports **4**, 40 μL volumes of washing buffer were added to the gels, followed by 6.7 μL of 30 mM UDP-GalA, 20 μL of 6.5 mM $MnCl_2$, 98.3 μL of H_2O and finally, 35 μL of solubilized *Arabidopsis* GalAT. The final concentrations of HEPES, sucrose, BSA, KCl, $MnCl_2$, and UDP-GalA were 5 mM, 20 mM, 0.005%, 2.5 mM, 350 and 502 μM, respectively. The reaction cocktails (approx 400 μL) were briefly mixed with a Teflon stick and then gently rotated in an oven set at 30 °C for various times (0, 15, 30, 45 and 60 min). The enzymatic reactions were terminated by addition of 1 mL 1 M NaCl. The suspensions were then gently mixed at rt for 5 min, centrifuged before removing the supernatant, and the resulting 'modified' support resuspended in 1 mL 1 M NaCl. The modified supports were washed five times by repeated resuspension in 1 mL 1 M NaCl and centrifugation, and then washed a further 10 times with 1 mL H_2O. The gel was stored as a suspension in 500 μL H_2O at 4 °C, in readiness for the cleavage step (see below). In these experiments, the UDP-GalA:immobilized-OGA molar ratio was $\approx$1:2.

In a subsequent experiment with the $(GalA)_{13}$-functionalized support (gel **3**), the glycosyl donor UDP-GalA was supplied in a 5-fold molar excess (5.7 mM final concentration) relative to the immobilized OGA.

Only one reaction time was investigated (i.e., 45 min) and the composition of the reaction cocktail was: 100 μL of washed Sepharose gel **3**; 20 μL of reaction buffer; 11.7 μL of 98.1 mM UDP-GalA; 10 μL of 6.5 mM $MnCl_2$; 40.8 μL H_2O; and 17.5 μL of solubilized *Arabidopsis* GalAT. All the other conditions (i.e., final concentration of common reagents, temperature, termination and washing regimes) were the same as those detailed above for experiments with the $(GalA)_{14}$-derivatized supports (gel **4**).

4.8. Cleavage of OGAs from the support

The suspended gel containing the original or biosynthetically modified OGAs was centrifuged and the supernatant was removed. The gel was washed four times with 1 mL 50 mM Tris–HCl buffer (pH 8.0) for a duration of 5 min for each wash. A solution of DTT (4.5 mg mL^{-1}, 200 μL) in 50 mM Tris (pH 8.0) was added to the gel and the suspension was mixed gently with the tip of a pipette before shaking gently at rt for 3 h. After this time, the sample was centrifuged and the supernatant collected in a separate vial. The gel was finally washed with H_2O (5×500 μL $\times 5$ min) and the supernatant fractions were combined.

4.9. MALDI-TOF MS analysis

The Dowex 50WX8 resin employed in this study was preconditioned by 4 M HCl, stored in EtOH–H_2O (1:1), and washed three times with three column volumes of H_2O. After cleavage from the support, each OGA sample was micropurified over a mini Dowex 50WX8-column (containing approx 2 ml of Dowex 50WX8 resin), freeze-dried, redissolved in water (50 μL), and finally diluted 10 (experiments with $(GalA)_{13}$) or 1000 (experiments with $(GalA)_{14}$) times with H_2O. The matrix was prepared as follows: 2,4,6-Trihydroxyacetophenone (THAP) was first dissolved in MeOH to a concentration of 200 mg mL^{-1} (solution A). NC was dissolved in acetone (30 mg mL^{-1}) and the resulting solution was further diluted with propan-1-ol to a concentration of 15 mg mL^{-1} (solution B). A and B were mixed immediately prior to use in a 4:1 ratio. The THAP–NC matrix (0.2 μL) was applied to the MS probe tip, followed by 20 mM dibasic ammonium citrate (0.2 μL) and finally the OGA sample (0.2 μL). The matrix-sample mixture was immediately evaporated under vacuum to form homogeneous crystals. The sample was desorbed/ionized from the probe tip with a 10 ns pulse of a nitrogen laser, set to deliver approximately 10.5–11 μJ per pulse. Spectra were recorded over a mass-to-charge ratio (m/z) range of 200–10,000 Da and 200–500 individual spectra were averaged for each mass determination.

Acknowledgements

We wish to thank Dr Kudzai E. Mutenda and Kerry Hosmer for advice and helpful discussions regarding MALDI-TOF MS analyses. We thank Stefan Eberhard for the suspension-cultured *Arabidopsis* cells. We are indebted to the Otto Mønsted Fund for funding part of FG's stay at the CCRC, the Lundbeck foundation, and the Danish Technical Science Research Council for fiscal support through a 'Centerkontrakt' grant. Further, we also gratefully acknowledge the financial assistance provided by NRI competitive USDA award 2001-35318-11111 and the DOE-funded center grant DE-FG02-93-ER20097.

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