Biocatalytic Polytransesterification of Inulin with Divinyladipate

Lino Ferreira and Maria H. Gil

Departamento de Engenharia Química, Universidade de Coimbra, Pinhal de Marrocos, 3030 Coimbra, Portugal

Rui Carvalho and Carlos F. G. C. Geraldes

Departamento de Bioquímica, Universidade de Coimbra, Apartado 3126, 3000 Coimbra, Portugal

Dae-Yun Kim and Jonathan S. Dordick*

Department of Chemical Engineering, Rensselaer Polytechnic Institute, 102 Ricketts Building, Troy, New York 12180

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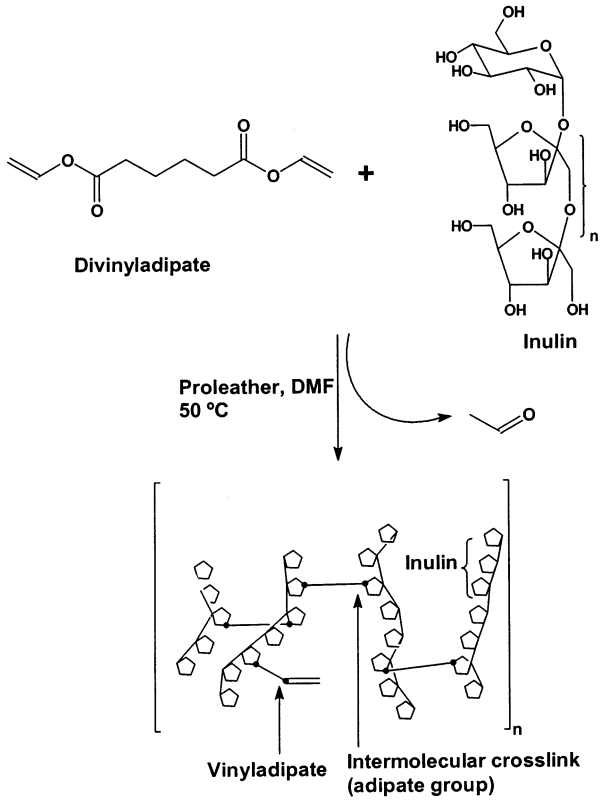
Enzymatic synthesis of polymers has attracted significant attention in recent years[1] because of high inherent selectivity under mild reaction conditions. A wide range of polymers has been synthesized using purely enzymatic means, including polyphenols,[2] polyesters,[3] and polycarbonates.[4] Although the vast majority of polymers have been prepared from rather simple monomers, enzymes offer the opportunity to incorporate complex polyfunctional compounds, such as sugars and polysaccharides, into polymer backbones,[5] thereby extending the synthetic repertoire of polymer chemistry.

In the current work, we report the enzyme-catalyzed polytransesterification of inulin with divinyladipate (DVA) in DMF to produce inulin polyesters. Inulin is composed by a mixture of oligomers and polymers containing 2 to 60 (or more) β 2−1 linked D-fructose molecules having a glucose unit as the initial residue.[6] Six proteases and five lipases, all commercially available, were tested for their abilities to catalyze the polytransesterification of inulin with DVA in anhydrous DMF (Scheme 1), at 50 °C, for 72 h (Table 1).[7] There was significant variation in the inulin conversion (based on consumption of native inulin) and molecular weight[8] obtained as a function of the enzyme, but in all cases the products were water soluble. Proleather, an alkaline protease from *Bacillus subtilis*, showed the highest conversion.[9]

Scheme 1. Schematic Representation of Enzyme-Polytransesterification of Inulin with DVA



The number average molecular weight (M_n) of the polymer formed was also influenced by the source of the enzyme, and this was mainly due to the extent of reaction conversion, an expected finding given the mechanism of AA−BB polycondensation reactions.[10] The relatively high polydispersities are expected with such a mechanism given the large size of the inulin "monomers" in the polymerization reaction. The polymer obtained using Proleather consisted of ca. 3−4 inulin molecules linked through adipate moieties, yet remained water soluble, indicating that it was not heavily crosslinked.

* To whom correspondence should be addressed. Phone: 518-276-2899. Fax: 518-276-2207. E-mail: dordick@rpi.edu.

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(7) The enzymes were "pH-adjusted" prior to use in the presence of 20 mM phosphate buffer at pH 8.0 (Proleather, Protease S, and subtilisin Carlsberg) or at pH 7.5 (Proteases A, N, and P, and Lipases A, AY, M, PS, and Porcine Pancreas) following the procedure by Klibanov (Klibanov, A. M. *CHEMTECH* **1986**, *16*, 354). After being flash-frozen in liquid nitrogen, the samples were lyophilized on a Labconco freeze-drier (Labconco Corp., Kansas City, MO) for 48 h. Enzymes were screened for their reactivity on inulin by adding 300 mg of lyophilized enzyme powder (130 mg for subtilisin) to 15 mL of anhydrous DMF containing 17 mM inulin (M_n = 3620 Da, M_w/M_n = 1.2, obtained from Fluka Chemie AG, Buchs, Switzerland) and 200 mM DVA (TCI, Portland, OR). The reaction mixtures were shaken (250 rpm) at 50 °C in a temperature-controlled New Brunswick Scientific C24 orbital shaker (Edison, NJ) for 72 h. The reactions were terminated by removing the enzyme (all enzymes were insoluble in DMF) by centrifugation at 4000 rpm for 10 min. The supernatants were precipitated in a 4-fold excess of acetone and the precipitates were subsequently dissolved in Milli-Q water and dialyzed using a regenerated cellulose dialysis tube with a 1000 MWCO (Spectrum, CA) for 2 days, at 4 °C, against water. Afterwards, the aqueous solutions of Inulin polyesters [poly(Inul-DVA)] were lyophilized for 48 h. The conversion was determined by back-titration with 0.1 N HCl using phenolphthalein as indicator.

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Table 1. Enzyme Screening for the Polytransesterification Reaction of Inulin[a] with DVA

entry	enzyme	origin	conv. (%)[d]	M_n[e]	M_w/M_n
1	Proleather FG-F[b]	*Bacillus subtilis*	56.8	14 310	2.5
2	Protease A[b]	*Aspergillus oryzae*	11.2	6130	1.8
3	Protease N[b]	*Bacillus subtilis*	8.4	5420	1.9
4	Protease P[b]	*Aspergillus melleus*	17.2	7590	2.1
5	Protease S[b]	*Bacillus stearothermophilus*	3.8	5560	1.1
6	Protease Subtilisin Carlsberg[c]	*Bacillus licheniformis*	6.2	5780	2.3
7	Lipase A[b]	*Aspergillus niger*	14.4	6640	2.2
8	Lipase AY[b]	*Candida rugosa*	36.4	9820	3.9
9	Lipase M[b]	*Mucor javanicus*	20.0	8170	2.3
10	Lipase PS[b]	*Pseudomonas cepacia*	21.6	8000	2.3
11	Lipase Porcine Pancreas[c]	*Porcine pancreas*	2.4	ND	ND

[a] The M_n and M_w/M_n of original Inulin were 3620 Da and 1.2, respectively. [b] Obtained from Amano Enzyme Co (Troy, VA). [c] Obtained from Sigma Chemical Co (St. Louis, MO). [d] Determined by titration. The conversion is defined as the percentage of DVA molecules incorporated into inulin taking into account the initial molar ratio of DVA to inulin fructose units in the reaction mixture. [e] The number average molecular weight was determined as described in ref 8. ND = Not determined.

Table 2. DS_{Total}, DS_{Vinyl}, M_n, and M_w/M_n of Poly(Inul-DVA) as a Function of Initial Concentration of DVA Added to the Reaction[a]

entry	theoretical DS[b](%)	obtained DS_{total}[c](%)	obtained DS_{vinyl}[c](%)	efficiency[d] (%)	M_n[e]	M_w/M_n
1	10	8.5	1.7	85.0	6690	2.6
2	20	17.5	2.1	87.5	8760	3.1
3	30	25.0	4.1	83.3	11 360	3.3
4	40	39.1	7.7	97.8	14 610	3.5
5	50	45.8	8.6	91.6	>14 610[f]	

[a] Reactions were performed in 30 mL of anhydrous DMF containing 17 mM inulin and a calculated amount of DVA. The reaction mixtures were shaken at 250 rpm and 50 °C for 140 h, after which they were purified as before[7] (isolated yields: 44−69%). [b] Calculated from the initial molar ratio of DVA to inulin fructofuranoside residues. [c] Degree of substitution of the products (determined by ^{1}H NMR). [d] Calculated as the ratio of the obtained DS_{total} to the theoretical DS. [e] The number average molecular weight was determined as described in ref 8. [f] Higher than the exclusion limit of the GPC column, circumventing any precise determination of M_n.

Poly(Inul-DVA) synthesized by Proleather was further analyzed by NMR (^{1}H, ^{13}C NMR, and 2-dimensional ^{1}H−^{1}H COSY and ^{1}H−^{13}C HMQC NMR; see Supporting Information). The calculation[11] of DS_{total} (defined as the number of DVA molecules incorporated into inulin through single or double ester bonds per 100 inulin fructofuranoside residues) and DS_{vinyl} (defined as the number of DVA incorporated to inulin by single ester bonds, and hence retaining a vinyl ester moiety, per 100 inulin fructofuranoside residues) yielded 45.8 and 8.6% (the initial molar ratio of DVA to inulin fructofuranoside residues was 0.5), respectively, which means that most of the DVA is incorporated into the inulin through double ester bonds. Hence, adipate esters were incorporated as inter- or intramolecular cross-links on the inulin structure.

The structure of poly(Inul-DVA)[12] revealed one predominant positional isomer in the fructofuranoside residue at the 6-position and two minor isomers at the 3 and 4 positions (24.3:11.0:10.5, at the 6, 4, and 3 positions, respectively), showing the enzyme's preference for primary hydroxyl groups.[13] Furthermore, the ^{1}H−^{1}H COSY NMR experiment indicated that the reacted fructose residues are monosubstituted as no cross-peaks were shared by the three positional isomers. Hence, the intramolecular cross-links were between different fructose residues on the same inulin chain.[14]

Encouraged by these results, we proceeded to study the effect of DVA concentration on DS_{total}, DS_{vinyl}, and M_n of Poly(Inul-DVA) (Table 2). In all cases, water-

(8) Gel permeation chromatography (GPC) was performed with a Shimadzu LC-10AT (Columbia, MD) equipped with a Waters 410 refractive index detector (Milford, MA). The eluent was DMF at a flow rate of 0.5 mL/min. Waters 500 and 100 Å Ultrastyragel (7.5 × 300 mm), and Styragel HR 5E (4.6 × 300 mm) were installed in series to achieve effective separation of polymers. Calibration was made with polystyrene standards of narrow polydispersity in the molecular weight range from 762 to 44 000 Da. Thus, the reported molecular weights are more accurately described as apparent molecular weights. The GPC chromatograms were obtained from samples dissolved in DMF over a concentration range of 2.1−2.4% (w/v).

(9) Two controls were performed: in the absence of enzyme <7% conversion was obtained, whereas with the use of thermally deactivated Proleather (i.e., boiled for 5 h followed by lyophilization) in place of the active enzyme (50 °C, 24 h), conversions of ca. 5% were obtained. These results indicate that the polytransesterification reaction proceeded through enzymatic catalysis.

(10) A plot of M_n as a function of reaction conversion for the different enzymes studied is given in the Supporting Information. The high linearity is strongly indicative of an AA−BB polycondensation reaction catalyzed by the different enzymes.

(11) On the basis of the ^{1}H NMR assignments, the DS_{total} was calculated as $DS_{total} = (7z/4y) \times 100$, and DS_{vinyl} was calculated from $DS_{vinyl} = (7w/y) \times 100$, where w is the integral of the vinyl proton at δ 7.15 ppm, z is the average integral of the protons from adipate group in the range of δ 2.45−1.63 ppm, and y is the integral of all inulin protons between δ 5.38−5.05 ppm and δ 4.50−3.38 ppm (see Supporting Information).

(12) Poly(Inul-DVA) ^{1}H NMR results (δ, D_2O, ppm): δ 7.15 (dd, 1H, H_x), 5.38 (m, 2H, H_{1g} and $H_{3f'}$), 5.16 (m, 2H, $H_{4f'}$ and H_{1g}), 4.94 (dd, 1H, H_b), 4.69 (dd, 1H, H_a), 4.43 (d, 1H, $H_{3f-4f'}$), 4.23 (m, 3H, $H_{6f'}$ and $H_{4f-3f'}$), 4.20 (d, 1H, H_{3f}), 4.04 (t, 1H, H_{4f}), 3.90−3.50 (m, 5H, H_{5f}, H_{6f} and H_{1f}), 2.45 (s, 4H, adipate), 1.63 (s, 4H, adipate). Poly(Inul-DVA) ^{13}C NMR results (δ, D_2O, ppm): δ 177.4−174.1 (C═O), 142.7 (H*C*═CH_2), 104.7 (C_{2f} and $C_{2f-3f'}$), 100.7 (HC═*C*H_2), 94.3 (C_{1g}), 82.6 (C_{5f}), 81.3 ($C_{5f-4f'}$), 80.2 ($C_{3f'}$), 79.9 ($C_{5f-6f'}$), 78.8 ($C_{4f'}$), 78.5 (C_{3f}), 76.8 ($C_{3f-4f'}$), 76.6 ($C_{4f'}$), 75.8 (C_{4f}), 74.3 ($C_{4f-3f'}$), 66.5 ($C_{6f'}$), 63.6 (C_{6f}), 62.4 (C_{1f}), 34.8 and 34.6 (−*C*H_2−CH_2−CH_2−*C*H_2−, adipate), 25.2 and 24.9 (−CH_2−*C*H_2−*C*H_2−CH_2−, adipate). Inulin ^{13}C NMR results (δ, D_2O, ppm): δ 104.8 (C_{2f}), 82.6 (C_{5f}), 78.6 (C_{3f}), 75.8 (C_{4f}), 63.7 (C_{6f}), 62.4 (C_{1f}). The shifts observed from that of inulin are a downfield shift in C_{6f}, C_{4f}, and C_{3f}. This indicates the acylation of C_{6f}, C_{4f}, and C_{3f} (denoted as $C_{6f'}$, $C_{4f'}$, and $C_{3f'}$) according to Yoshimoto et al. (Yoshimoto, K.; Itatani, Y.; Tsuda, Y. *Chem. Pharm. Bull.* **1980**, *28*, 2065).

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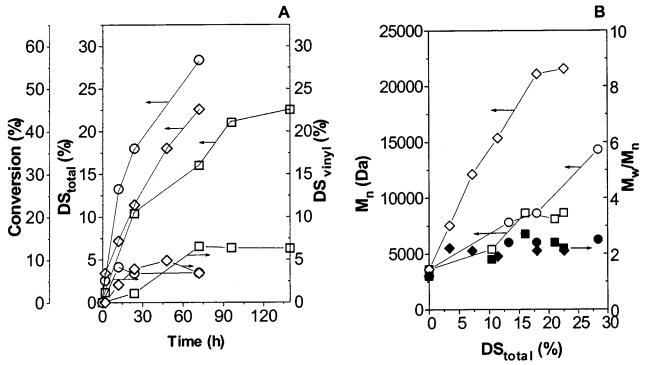

Figure 1. (A) DS_{total} and DS_{vinyl} obtained as a function of time for the reaction of DVA with Inulin (molar ratio of DVA to Inulin fructofuranoside residues was 0.5) at a concentration of 6.7% (w/v) in the presence of 10 mg/mL (□), 20 mg/mL(○), and 40 mg/mL (◇) Proleather shaken at 250 rpm at 50 °C. DS values were determined by ^{1}H NMR (see text for details). (B) M_n and M_w/M_n as a function of DS_{total}.

soluble derivatized inulin polymers were obtained with different DS_{total}, depending on the concentration of the acyl donor, and with a coupling reaction efficiency >83%. Furthermore, the ratio of the $DS_{adipate}$ to DS_{vinyl} is relatively constant as a function of the molar ratio of DVA to inulin employed. This indicates that the reactions of diester formation and monoester formation proceed independently. Finally, increasing the DVA concentration resulted in poly(Inul-DVA) with higher M_n, such that at 40% theoretical DS, 3−4 inulin monomers are cross-linked together.

The time-course reactions of inulin with DVA, at 50 °C, with different enzyme concentrations, are shown in Figure 1A. Taking into account the DS_{total}, the initial incorporation of DVA into inulin molecules (reaction times ≤2 h) increases with increased enzyme concentration: 1.2, 2.6, and 3.5% for 10, 20, and 40 mg/mL Proleather, respectively. However, the rate of adipate incorporation into the inulin structure changes significantly at later times. Furthermore, the observed reactivity at an enzyme concentration of 40 mg/mL was *lower* than that with 20 mg/mL. This unusual behavior may be explained by the presence of a competing reaction that results in the hydrolysis of DVA. Such a competing reaction has been observed in other polycondensation reactions performed in organic media, where traces of water associated with the enzyme promote the hydrolysis of the highly activated divinyl esters such as DVA.[15] The water content of the freeze-dried Proleather was 5.6% (w/w);[16] hence, sufficient amounts of water are present in the reaction mixture, and this water content would be expected to increase as the enzyme concentration is increased, thereby resulting in lower yields of polycondensation product. Finally, the incorporation of DVA molecules by a single ester moiety (DS_{vinyl}) followed almost the same trend for the different concentrations of enzyme (Figure 1A).

The variation of M_n and the polydispersity of poly(Inul-DVA) versus DS_{total} (Figure 1B) was studied for the same set of experiments described in Figure 1A. As expected, the M_n of poly(Inul-DVA) increased with DS_{total}. Interestingly, there is a strong dependence of enzyme concentration on M_n; larger polymers are formed in the presence of higher enzyme concentrations. The reason for this enzyme concentration dependence is not clear.

The acylation of inulin with DVA could be conducted chemically, and this provides us with an opportunity to directly compare the enzymatic and chemical approaches. To that end, we followed a chemical synthesis procedure.[17] The incorporation efficiency of DVA in the inulin backbone by the chemical approach (53.4%) was similar to the results achieved for the enzymatic reaction (56.7%; Proleather concentration of 20 mg/mL, reaction time of 72 h); however, poly(Inul-DVA) obtained chemically had an M_n of 9580 Da ($M_w/M_n = 2.1$), ca. 50% lower than that generated enzymatically. Thus, the enzymatic transformations yield higher molecular weight polymers than are achieved chemically. It is possible that the high degree of regioselectivity achieved enzymatically favors the formation of higher-molecular-weight inulin-based polymers, and our continuing work on this subject is underway.

In summary, we have demonstrated the enzyme-catalyzed polycondensation of a low-molecular-weight polysaccharide. To our knowledge, this is the first report dealing with enzyme-catalyzed polycondensation reactions using a polysaccharide as a monomer. These polymers may have commercial significance as polymeric drug carriers,[18] as carriers for magnetic resonance imaging contrast agents such as Gd^{III} chelates,[19] and as hydrogels.[20,21]

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Supporting Information Available: Plot of M_n as a function of reaction conversion for the different enzymes and NMR spectra of poly(Inul-DVA) (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(21) We have used the free vinyl moieties that are present on the enzymatically derivatized inulin as "monomers" for free radical polymerization. Two aqueous solutions of Poly(Inul-DVA) present DS_{vinyl} of 8.6% and 18.7% gel after ca. 10 min. The swelling ratio of these hydrogels in 0.01 M citrate-phosphate buffer pH 7.0 (at 25 °C, for 5 days) was 34.71 and 10.83 for Poly(Inul-DVA) DS_{vinyl} 8.6% and 18.7%, respectively. Furthermore, under this pH, inulin hydrogels undergo partial ester hydrolysis as confirmed by FT-IR. Therefore, these inulin hydrogels are attractive networks for designing drug delivery systems or matrix for tissue engineering.