Enzymatic Synthesis of Inulin-Containing Hydrogels

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The *Bacillus subtilis* protease Proleather FG-F catalyzed the transesterification of inulin with vinyl acrylate (VA) in dimethylformamide (DMF). The reaction conversion for different VA concentrations was greater than 57% after 96 h at 50 °C. The degree of substitution (DS, defined as the amount of acrylate groups per 100 inulin fructofuranoside residues) with acrylate moieties can be controlled by varying the molar ratio of VA to inulin. Reasonable yields were obtained (44−51%, 2 days) using a two-step purification methodology. Inulin derivatized with VA (Inul-VA) was characterized by gel permeation chromatography, and its structure was established by ^{1}H, ^{13}C, and $^{1}H-^{1}H$ correlation spectroscopy and $^{1}H-^{13}C$ heteronuclear multiple quantum coherence NMR. The main positional isomer was at the 6 position of the fructofuranoside residue and two other minor isomers were observed at the 3 and 4 positions. Thus, the enzymatic reaction was largely regioselective. Furthermore, the inulin fructose residues were monosubstituted. Gels with swelling ratios at equilibrium of up to ca. 20 were prepared by free radical polymerization of aqueous solutions of Inul-VA with different DS and monomer concentrations. Gel pore sizes were calculated from swelling experiments and range from 19 to 57 Å. To our knowledge, this work reports the first successful enzymatic modification of a polysaccharide solubilized in 100% DMF solution.

Introduction

Enzymes are effective catalysts for a wide range of reactions because of their high reaction specificity under mild conditions.[1] This is particularly evident in biotransformations catalyzed by hydrolases (e.g., proteases, lipases, etc.) wherein a variety of nucleophiles act as substrates for enzyme-catalyzed acyl transfer in nearly anhydrous organic solvents.[2−6] This reaction breadth has been extended to polymer synthesis. In particular lipases have been shown to catalyze polytransesterification[7,8] and ring opening polymerization[9,10] in organic solvents, and proteases have been used for regioselective synthesis of sugar polyesters in the nonaqueous milieu.[11,12]

Although the traditional uses of enzymes for synthetic applications involve small molecules, the benefits of enzyme technology have been extended to the modification of synthetic and natural polymers, particularly those that are soluble in organic solvents. For example, lipase from *Candida antarctica* was shown to catalyze the selective epoxidation of polybutadiene in organic solvents in the presence of hydrogen peroxide and catalytic quantities of acetic acid.[13] Unlike synthetic polymers, polyhydroxylated compounds such polysaccharides either are sparingly soluble in only the most polar organic solvents or are incompletely insoluble. Nevertheless, enzymatic derivatization of polysaccharides has been performed in either nonpolar organic solvents using insoluble polysaccharides with soluble[14] and suspended enzymes[15] or aqueous solution using insoluble polysaccharide and soluble enzyme.[16] The results obtained from nonaqueous enzymatic approaches showed that only surface chains could be enzymatically acylated whereas in aqueous enzymatic solutions it was impossible to control and characterize the reaction products. The rationale of the current work is to overcome these limitations.

Herein we report the first successful enzyme-catalyzed modification of an organic solvent-soluble polysaccharide, inulin, using anhydrous dimethylformamide (DMF) as the reaction medium. Inulin is composed by a mixture of oligomers and polymers containing 2−60 (or more) β-2,1 linked D-fructose molecules having a glucose unit as the initial residue.[17,18] While inulin is not digested in the upper gastrointestinal tract, it is hydrolyzed in the colon by intestinal flora. Thus, inulin-based materials may have use as drug delivery matrixes for colonic targeting. These site-specific delivery systems can be used in the treatment of colonic disorders such as Crohn's disease or colon carcinomas, reducing undesirable side effects caused by chemotherapeutic drugs.[17,19] For that purpose, we modified inulin with vinyl acrylate (Scheme 1) and then used free radical polymerization to yield cross-linked hydrogels. Hydrogels with different swelling and physical properties were obtained.

Experimental Section

Materials. Proleather FG-F, a crude serine protease from *Bacillus subtilis* (molecular weight of approximately 33.4

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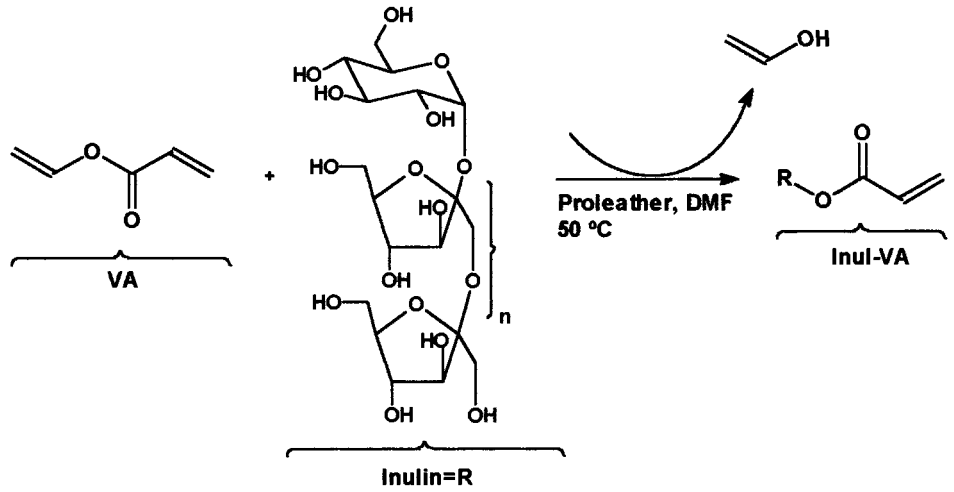
Scheme 1



KDa, as determined by electrophoresis assay) was a generous gift from Amano Enzyme Co. (Troy, VA). Chicory inulin (M_n = 3620 Da, M_w/M_n = 1.2, as determined by gel permeation chromatography (GPC) analysis) was purchased from Fluka Chemie AG (Buchs, Switzerland). Vinyl acrylate (VA), *N*,*N*-dimethylformamide (DMF), *N*,*N*,*N*',*N*'-tetramethylenethylenediamine (TEMED), and ammonium persulfate (APS) were obtained from Aldrich (Milwauke, WI). DMF was dried with 3 Å molecular sieves at least overnight before use. Regenerated cellulose dialysis tubes with a 1000 MWCO were purchased from Spectrum (CA, USA). All other chemicals and solvents used in this work were of the highest purity commercially available.

Methods. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Unity spectrometer (Palo Alto, CA) at 300 and 75 MHz, respectively. ^{1}H NMR spectra were recorded in D_2O (60−100 mg in 0.7 mL) using a pulse angle of 90° and a relaxation delay of 30 s. The water signal, used as reference line, was set at δ 4.75 ppm and was suppressed by irradiation during the relaxation delay. The number of scans in the spectra acquisition was set at 16. ^{13}C NMR spectra were recorded in D_2O using a pulse angle of 30° and relaxation delay of 1 s. *tert*-Butyl alcohol (tb) was used as reference, which was set at δ 31.2 ppm versus tetramethylsilane. Generally, the number of scans was set at 16 000. Bidimensional spectra were recorded on a Varian Unity 500 MHz spectrometer (Palo Alto, CA). ^{1}H−^{1}H correlation spectroscopy (COSY) spectra were collected as a 1024 × 416 matrix covering a 2500 Hz sweep width using 32 scans/increment. Before Fourier transformation, the matrix was zero filled to 2048 × 2048 and standard sine-bell weighting functions were applied in both dimensions. ^{1}H−^{13}C heteronuclear multiple quantum coherence (HMQC) spectra were collected as a 1024 × 256 matrix covering sweep widths of 2500 and 11 500 Hz in the first and second dimensions, respectively. Before Fourier transformation, the matrix was zero-filled to 1024 × 1024 and standard Gaussian weighting functions were applied in both dimensions.

The CP/MAS ^{13}C NMR spectra were recorded on a 360 MHz Chemagnetics spectrometer (90.5 MHz) equipped with CP-MAS (cross-polarization magic-angle-spinning) accessories at 25 °C. The sample (ca. 200−300 mg) was placed in a 7.5 mm Zirconia rotor (Chemagnetics PENCIL, Fort Collins, CO) and spun at 3 kHz. The contact time was 3 ms and a recycle time of 5 s was applied. The number of scans was set at 4000. The ^{13}C shifts were calibrated by substitution using external hexamethylbenzene.

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 44000 Da. The GPC chromatograms were obtained from samples dissolved in DMF over a concentration range of 2.1−2.4% (w/v).

In some cases (as stated in the text), the determination of degree of substitution (DS) was performed by titration based on a method described by Vervoort et al.[20] Inulin derivatives (50 mg) were dissolved in 0.1 N NaOH (4 mL) and stirred for 72 h at 20 °C to hydrolyze the ester. The molar consumption of NaOH was determined by back-titration with 0.1 N HCl after adding 2 drops of phenolphthalein solution as indicator. Underivatized inulin was used as blank.

Pretreatment of Proleather FG-F and Inulin. Proleather FG-F was "pH-adjusted" in the presence of 20 mM phosphate buffer at pH 8.0. After flash-freezing in liquid nitrogen, the sample was lyophilized on a Labconco freeze-drier (Labconco Corp., Kansas City, MO) for 48 h. Active site titration was performed before and after lyophilization according to the method of Schonbaum[21] using *N*-transcinnamoylimidazole as the titrant. The percentage of active enzyme in the commercial powder before and after lyophilization was 4.11 ± 0.09% and 2.89 ± 0.56% (average ± standard deviation, n = 3), respectively.

Thermally deactivated Proleather FG-F was prepared by suspending the enzyme in 250 mL of 20 mM phosphate buffer, pH 8.0, in a 500 mL round-bottomed flask fitted with a water-cooled condenser. The enzyme solution was refluxed for 5 h and then was allowed to cool to room temperature and lyophilized. The proteolytic activity of Proleather FG-F and its thermally deactivated preparation were determined

with casein as the substrate. The enzyme solution (0.1 mL, 80 mg/mL) was added to the reaction media formed by a mixture of 1 mL of 0.1 M phosphate buffer pH 8.0 with 5 mL of 1.0 % (w/v) casein solution. The mixture was incubated for 3 min at 37 °C, with magnetic stirring (200 rpm), and a 0.5 mL aliquot was taken and added to an equal volume of 0.4 M trichloroacetic acid. The resulting precipitate was removed by centrifugation (5000 rpm, 2 min) after standing for 25 min at 25 °C. The supernatant (0.5 mL) was placed in a test tube containing 5 mL of 0.4 M sodium carbonate and 0.5 mL of 5-fold diluted Folin's reagent. After thorough mixing, the solution was allowed to stand for 20 min at 37 °C, and the absorbance measured spectrophotometrically at 660 nm. The absorbance values were then converted to equivalent tyrosine concentration using a tyrosine calibration curve. One unit of protease activity (U) is defined as quantity of enzyme needed to produce the amino acid equivalent of 1 μg of tyrosine/min.

In some cases, as stated on the text, inulin was treated before reaction. Inulin (6.7%, w/v) was dissolved in 300 mL of DMF and further centrifuged at 4000 rpm for 5 min. The supernatant was precipitated in 500 mL of acetone, and the precipitate was dissolved in water and lyophilized for 48 h.

Enzymatic Synthesis of Inulin Ester Monomers. Preparative-scale reactions were performed in 60 mL of anhydrous DMF containing 0.017 M (6.7%, w/v) inulin and variable concentrations of VA. The reaction mixtures were shaken (250 rpm) at 50 °C in a temperature-controlled New Brunswick Scientific C24 orbital shaker (Edison, NJ) for 96 h. The reactions were terminated by removal of the enzyme (which is insoluble in DMF) by centrifugation at 4000 rpm for 10 min. The supernatants were precipitated in a 4-fold excess of acetone and further washed with the same solvent. The precipitate was subsequently dissolved in Milli-Q water and dialyzed using a regenerated cellulose dialysis tube with a 1000 MWCO for 2 days, at 4 °C, against water. Afterward, the aqueous solutions of Inul-VA were lyophilized for 48 h.

Time course reactions of inulin ester synthesis by Proleather FG-F (10, 20, and 30 mg/mL) were performed independently in 15 mL of anhydrous DMF containing 0.017 M (6.7%, w/v) inulin and 0.204 M VA (molar ratio of vinyl monomer to inulin fructofuranoside residues of 50%) at 250 rpm and 50 °C. The purification of the products was performed as before.

Preparation of Inul-VA Gels. Inul-VA gels were obtained by free radical polymerization of aqueous solutions of Inul-VA as a function of DS and monomer concentration. Inul-VA (100, 200, or 400 mg) was dissolved in 0.9 mL of 0.2 M phosphate buffer pH 8.0, and the polymerization reaction performed in 2 mL plastic Eppendorf tubes ($\cong$0.5 cm radius) was initiated by adding 50 μL of APS (80 mg/mL in 0.2 M phosphate buffer pH 8.0) and 50 μL of TEMED solution (13.6% (v/v) in water; adjusted to pH 8.0 with 12 N HCl) for 24 h at 25 °C. The gels were subsequently removed from the Eppendorf tubes and immersed in 100 mL of 0.010 M citrate−phosphate buffer pH 7.0, for 5 days at 25 °C, changing the buffer daily. At regular intervals, the swollen gels were removed, blotted with filter paper to remove surface water, weighed, and returned to the same container until weight stabilization was observed (5 days). The gels were then dried at room temperature, under vacuum, in the presence of phosphorus pentoxide, and weighed to determine the dried weight, W_d. The swelling ratio at equilibrium (SRE) was calculated according to eq 1.

$$\mathrm{SRE} = \frac{W_s - W_d}{W_d} \tag{1}$$

The molecular weight between cross-links ($\bar{M}_c$) was calculated with the model of Flory and Rehner,[22] modified by Peppas et al.,[23] according to the implicit eq 2

$$\frac{1}{\bar{M}_c} = \frac{2}{\bar{M}_n} - \frac{\left(\frac{\bar{v}}{V_1}\right)[\ln(1-\nu_{2,s}) + \nu_{2,s} + \chi_1(\nu_{2,s})^2]\left[1 - \frac{1}{\chi_c}\left(\frac{\nu_{2,s}}{\nu_{2,r}}\right)^{2/3}\right]^3}{\nu_{2,r}\left[\left(\frac{\nu_{2,s}}{\nu_{2,r}}\right)^{1/3} - 0.5\left(\frac{\nu_{2,s}}{\nu_{2,r}}\right)\right]\left[1 + \frac{1}{\chi_c}\left(\frac{\nu_{2,s}}{\nu_{2,r}}\right)^{1/3}\right]^2} \tag{2}$$

where $\bar{M}_n$ is number average molecular weight of the inulin used (3620 Da), ν is the partial specific volume of inulin (0.601 cm^3/g),[24] V_1 is the molar volume of water (18 cm^3/g), χ_1 is the Flory polymer−solvent interaction parameter (0.473 taken from dextran/water system[25]), χ_c is the number of links of the chain ($\chi_c = 2\bar{M}_c/M_r$, where M_r is the molecular weight of the inulin repeating unit, 162.14), $\nu_{2,r}$ is the polymer fraction of the gel after gel formation and $\nu_{2,s}$ is the polymer fraction at equilibrium swelling. $\nu_{2,r}$ and $\nu_{2,s}$ were calculated from the weight of the gels before exposure to the buffer solution and after equilibrium swelling, respectively, assuming volume additivity of water and inulin. The average mesh size, ξ, was calculated through the use of eqs 3 and 4[23]

$$\bar{r}_o^{\,2} = C_n \chi_c b^2 \tag{3}$$

$$\xi = \nu_{2,s}^{\,-1/3}(\bar{r}_o^{\,2})^{1/2} \tag{4}$$

where $\bar{r}_o^{\,2}$ represents the average end-to-end subchain length (in Å) when the gel is unswollen, C_n is the polymer rigidity factor, assumed to be 8.9 by analogy to polar poly(vinyl alcohol),[26] and b is the characteristic bond length of the polymer backbone (=1.54 Å, corresponding to the C−C bond length).

Results and Discussion

Synthesis of VA Derivatized Inulin. Recently we found that Proleather FG-F enzyme was able to acylate inulin with divinyl adipate in DMF (unpublished results),[27] from a group of 11 commercially available proteases and lipases, and therefore was chosen in this present work. VA was selected as an activated acrylate acyl donor that is known for its high reactivity in enzyme-catalyzed transesterification reactions.[28] The time-course reaction of inulin with VA, at 50 °C, at increasing concentrations of enzyme is shown in Figure 1. As expected, the initial reaction rate increases as a function of the enzyme concentration. Quantitative measurement of

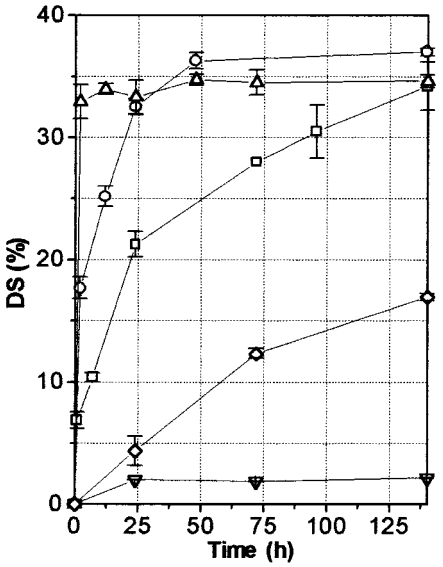


Figure 1. DS obtained as a function of time for the reaction of VA with inulin (molar ratio of VA to inulin fructofuranoside residues was 50%) in a concentration of 6.7% (w/v) in either the absence (▽) or presence of 10 mg/mL (□), 20 mg/mL (○), and 30 mg/mL (△) of Proleather as catalyst, or 20 mg/mL of thermally deactivated Proleather (◊), at 50 °C. Values were determined by titration (average ± standard deviation, $n = 3$).

acrylate incorporation onto the inulin backbone was possible in 2 h, when 30 mg/mL of Proleather was used. Further analysis of Figure 1 reveals that DS (corresponding to a conversion[29] of ca. 70%) of Inul-VA is practically unchanged after 50 h, for Proleather FG-F concentrations of 20 and 30 mg/mL, indicating that all the reactive sites on inulin have acrylate functionalities attached.

In parallel to these time-course reactions with Proleather, control reactions in the absence or with thermally deactivated enzyme were also carried out (Figure 1). In the absence of added active enzyme no significant (DS ca. 2%) inulin derivatization occurred in 140 h of reaction. However, unexpectedly, the addition of Proleather thermally deactivated for 5 h at 100 °C did catalyze a noticeable transesterification of inulin with VA to give a DS of ca. 17% after 140 h. To assess whether this conversion could be a result of nonspecific reactions due to nucleophilic functionalities in the protein preparation, or due to a true intrinsic catalytic residual enzymatic activity, the proteolytic activity of the heat-treated enzyme preparation was measured using casein as substrate. It was found that active Proleather FG-F and its deactivated preparation had activity values of 104.9 ± 7.9 and 10.3 ± 0.3 U/mL of enzyme solution, respectively, and this corresponded to a similar ratio of reactivities using 20 mg/mL Proleather on inulin. Thus, it may be concluded that the residual inulin activity present in the heat-treated Proleather was due to intrinsic activity, and this further suggests that the enzyme is highly thermostable. Furthermore, we found that some enzymes did not present any activity on the polytransesterification reaction of inulin with divinyl adipate,[27] further corroborating the absence of nonspecific reactions due to their external amino acids (not involving the catalytic site). On the basis of these results, we performed preparative-scale synthesis of inulin esters for 96 h in the presence of 20 mg/mL Proleather at 50 °C.

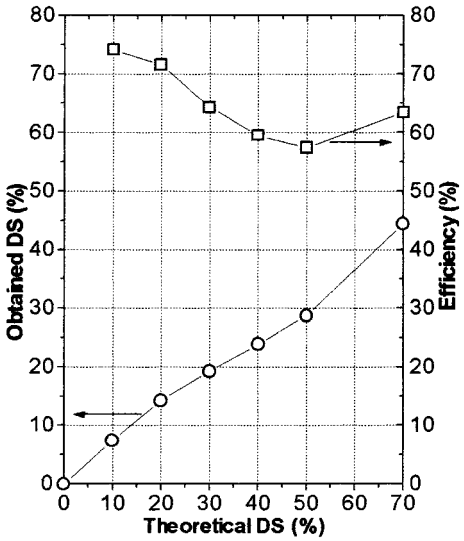


Figure 2. Relationship between the theoretical and the obtained DS for Inul-VA as determined by ^{1}H NMR. The efficiency was calculated as the ratio of the obtained DS to the theoretical DS.

Figure 2 shows the relationship between the molar ratio of VA to inulin fructose units in the reaction mixture (theoretical DS) and the degree of substitution of the products, as determined by ^{1}H NMR (obtained DS). From these results, Inul-VA can be obtained with different DS, ranging the concentration of the acyl donors. The efficiency of the coupling reaction (calculated as the ratio of the obtained DS to the theoretical DS) was above 57.4%.

The two-step purification procedure adopted in this work, based on a precipitation with acetone followed by dialysis against water, was revealed to be an efficient way to obtain ester products (isolated yield of 44−51%, except for Inul-VA DS 44.4%, which had an isolated yield of 27.7%) with no impurities as detected by ^{1}H NMR spectroscopy and further confirmed by elemental analysis (nitrogen content was below 0.5%). Yet due to the easy removal of Proleather enzyme from the reaction mixture (insoluble in DMF), the purification protocol is faster than the one presented by Vervoort et al.[20] which employed an extensive dialysis process for 10 days to remove the catalyst 4-(dimethylamino)pyridine in the methacrylation of inulin.

GPC Analysis. GPC analysis of Inulin and Inul-VA derivatives showed different elution profiles. Representative chromatograms of these polymers are presented in Figure 3. The inulin GPC chromatogram shows a single peak corresponding a M_n of 3620 Da ($M_w/M_n = 1.2$) and an average degree of polymerization of 25. The same profile was not observed for Inul-VA samples with different DS values. Chromatograms B and C present a major peak (B1 or C1) and two other minor peaks (B2 and B3, M_n of 13 450 and 35 160 Da, respectively; C2 and C3, M_n of 12 180 and 31 640 Da, respectively). The major inulin peak in those samples is shifted to higher molecular weight from M_n 4100 Da to M_n 4440 Da when DS values increase. This is likely due to introduction of acrylate groups in the inulin backbone. The minor peaks in chromatogram B and C (representing 12.4% and 19.7% of the sample, respectively) may be from high molecular weight polymers in the inulin which were not originally soluble in DMF but soluble after derivatization

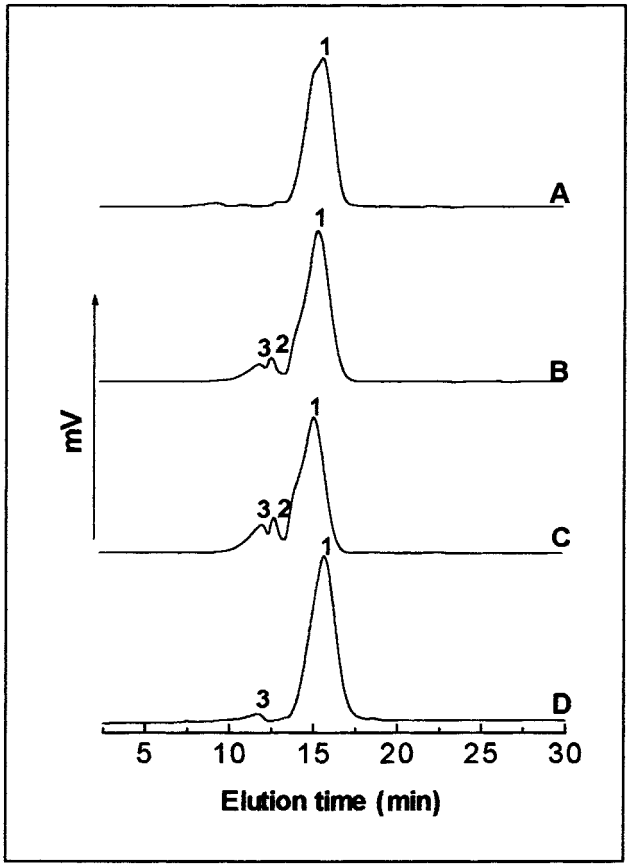


Figure 3. GPC chromatograms of inulin (A) and Inul-VA (B, C, D). Inul-VA samples were obtained from either original inulin (B, C) or acetone-precipitated inulin (D) as starting polymers for the transesterification reaction. The DS for Inul-VA samples was 21.2% (B), 34.2% (C), and 17.7% (D).

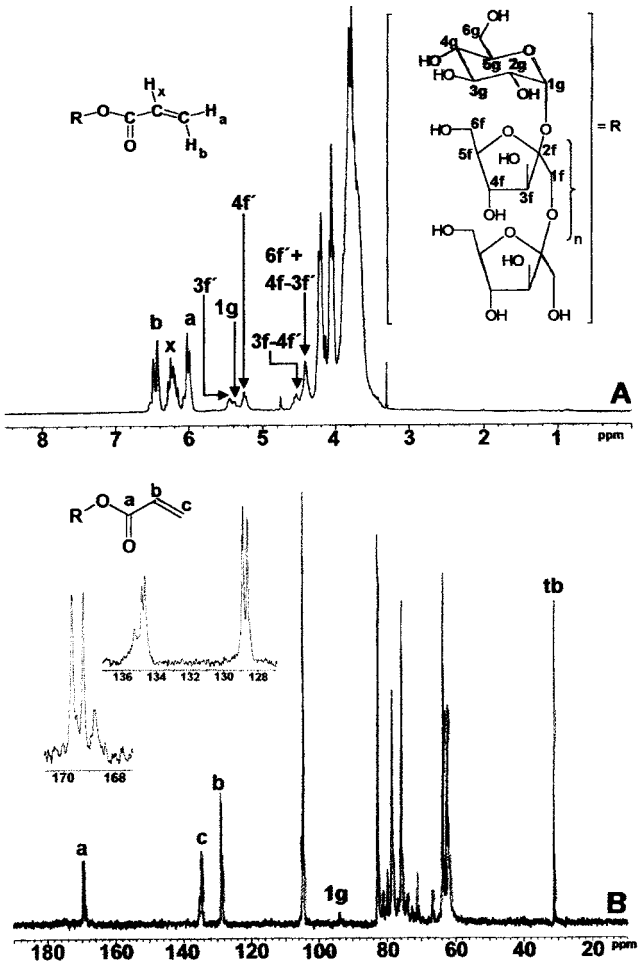


Figure 4. ^{1}H (A) and ^{13}C (B) NMR spectra of Inul-VA (DS = 28.7%) in D_2O, at 25 °C.

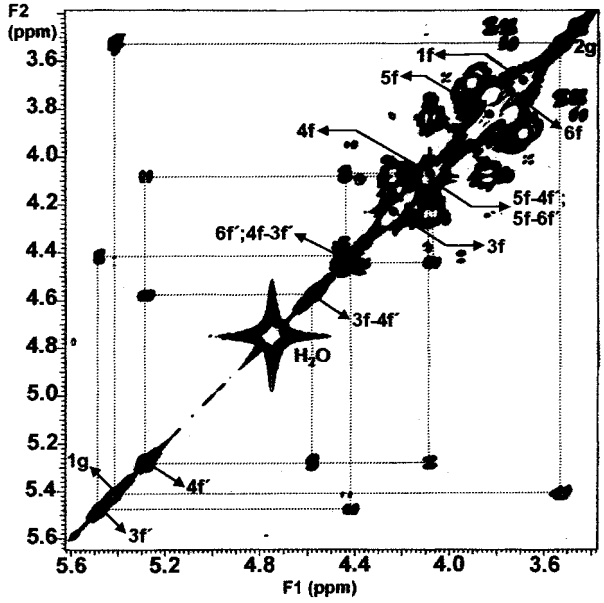


Figure 5. $^{1}H-^{1}H$ COSY spectrum of Inul-VA (DS = 19.3%) in D_2O at 25 °C, showing the $^{1}H-^{1}H$ correlations important for the assignment (see text for more details).

with VA. It is noteworthy to mention that traces of original inulin were not completely soluble in DMF, and therefore its GPC analysis (chromatogram A), using DMF as eluent, shows just the soluble moiety. As the transesterification reaction proceeds, high molecular weight polymers may become soluble in DMF due to modification with the relatively hydrophobic VA. This was further verified by removing the DMF-insoluble fraction of inulin by centrifugation and precipitating the supernatant in acetone. The precipitated inulin was reacted with VA in the presence of Proleather and the reaction product characterized by GPC. The GPC chromatogram obtained (D) shows that one of the minor peaks is totally removed (corresponding to B2 and C2) and the other one is partially removed (corresponding to B3 and C3). Even if the removal of minor peaks was not complete the results suggest that those peaks are related to high molecular weight polymers. Similar results showing small amounts of high molecular weight polymers on inulin were described by Verraest et al.[30] by GPC analysis using 0.1 M $NaNO_3$ as eluent.

Characterization by NMR spectroscopy. The structure of Inul-VA was analyzed by NMR spectroscopy. Figure 4 displays ^{1}H (A) and ^{13}C (B) NMR spectra of Inul-VA. In the ^{1}H NMR spectrum (spectrum A) the intense peaks between δ 3.38 and 4.23 ppm are attributed to protons of unreacted inulin, including the anomeric proton at δ 5.42 ppm belonging to the D-glucopyranosyl units. The assignments of each proton signal are clearly shown in the $^{1}H-^{1}H$ COSY displayed in Figure 5. Furthermore, from the ^{1}H NMR spectrum of Inul-VA signals from the acrylate groups are observed at δ 6.4 ppm (H_b, $^{3}J_{bx}$ = 17.21 Hz, $^{2}J_{ba}$ = 1.47

Table 1. ^{13}C NMR Assignments of the Fructofuranosyl Ring Carbons (δ, ppm) on Inul-VA with DS 28.7%

		Inul-VA					
	inulin	6-substituted		4-substituted		3-substituted	
carbon	obsd signal	obsd signal	$\Delta\delta$	obsd signal	$\Delta\delta$	obsd signal	$\Delta\delta$
1	62.4	62.1	−0.3	61.9	−0.5	61.7	−0.7
2	104.8	104.7	−0.1	105.0	+0.2	105.3	+0.5
3	78.6	78.4	−0.2	76.9	−1.7	80.6	+2.0
4	75.8	76.1	+0.3	78.2	+2.4	74.6	−1.2
5	82.6	80.0	−2.6	81.2	−1.4	82.4	−0.2
6	63.7	66.8	+3.1	63.5	−0.2	63.9	+0.2

Hz), δ 6.2 ppm (H_x, $^3J_{xa}$ = 10.38 Hz, $^3J_{xb}$ = 17.21 Hz), δ 6.0 ppm (H_a, $^3J_{ax}$ = 10.38 Hz, $^2J_{ab}$ = 1.47 Hz).

The formation of Inul-VA is also confirmed by its ^{13}C NMR spectrum (Figure 4, spectrum B). The fructofuranosyl and acrylate carbons are displayed in the range of 62.0−105.2 and 128.6−169.7 ppm, respectively. Except for carbon α (denoted as b in Figure 4) of the double bond (duplicate: 128.9 and 128.6 ppm), all other signals are in triplicate (C_a: 169.7, 169.2, and 168.8 ppm; C_c: 135.3, 134.9, and 134.7). This indicates the presence of three different positional isomers in the Inul-VA product.

The ester positions on the fructofuranosyl ring were assigned based on the additional signals presented in ^{13}C NMR spectrum (Figure 4) of Inul-VA ranging from δ 105.2 to 62.0 ppm. According to the literature[31] chemical shifts of acylated carbons suffer a downfield shift and the respective adjacent carbons a concomitant upfield shift. The chemical shifts of the other carbon atoms are hardly affected. As shown in the ^{13}C NMR spectrum there is no upfield shift of C-2 carbons, which appears to indicate no positional isomer at position 3. Therefore, the two acylated isomers in the main inulin backbone are at positions 6 and 4 in the fructofuranosyl ring. The respective ^{13}C NMR assignments are presented in Table 1. An $^1H-^{13}C$ HMQC NMR experiment was performed to correlate those ^{13}C signals with ^1H signals (Figure 6). In this spectrum the ^{13}C peaks at δ 66.8 ppm (modification at 6 position) and δ 78.2 ppm (modification at 4-position) are correlated with ^1H signals at δ 4.41 ppm (6f') and δ 5.24 ppm (4f'), respectively. From $^1H-^1H$ COSY (Figure 5) the signal at δ 5.24 ppm has two cross-peaks at 4.58 and 4.08 ppm corresponding to the vicinal protons at positions 3 (denoted as 3f−4f') and 5 (5f−4f'), while the signal at δ 4.41 ppm has a single correlation with a peak at 4.09 ppm corresponding to a vicinal proton at position 5 (5f−6f').

However, still remaining is the assignment of the third isomer. In the ^1H NMR spectrum of Inul-VA there is a small signal at δ 5.45 ppm that overlaps with the D-glucopyranosyl anomeric proton at 5.42 ppm, the latter correlating in the $^1H-^{13}C$ HMQC spectrum with a ^{13}C signal at δ 80.6 ppm. This signal corresponds to an acylated carbon at position 3 (Table 1). This is further confirmed by the $^1H-^1H$ COSY spectrum, which shows a single cross-peak for this signal at 4.42 ppm corresponding to a vicinal proton at position 4 (4f−3f'). Interestingly, as previously mentioned, there is no upfield shift in the ^{13}C NMR spectrum corresponding to the C-2 position, as would be expected according to the literature.[31] This might be due to the absence of protons attached to that carbon, thereby mitigating the observed upfield shift.

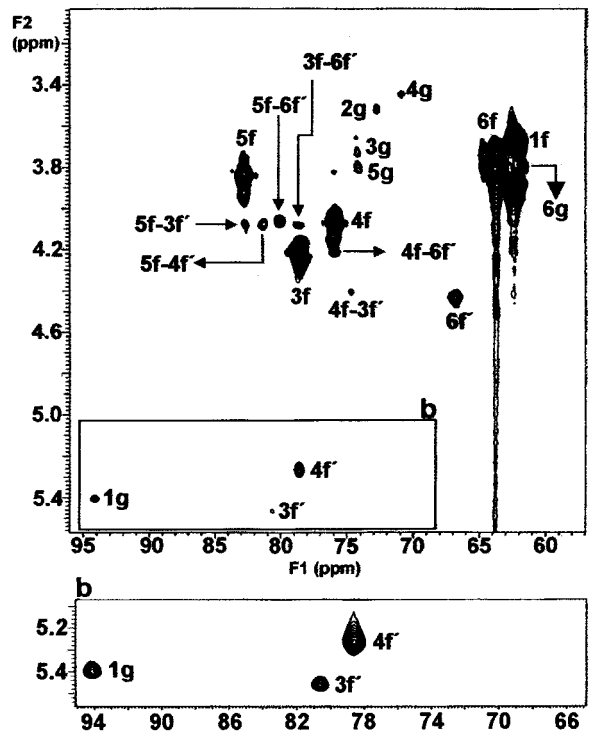


Figure 6. $^1H-^{13}C$ HMQC spectrum of Inul-VA (DS = 19.3%) in D_2O at 25 °C, showing the $^1H-^{13}C$ correlations (see text for more details).

On the basis of the ^1H NMR assignments, the DS was determined using eq 5

$$DS = (7x/y)*100 \quad (5)$$

where x is the average integral of the protons from vinyl group (δ 6.0−6.4 ppm) and y is the integral of all inulin protons.

Distribution of the Acrylate Substituents. On the basis of the ^1H NMR assignments (Figure 4, spectrum A) the relative DS (DS_i) of modified individual hydroxyl groups attached to the C-3, C-4, and C-6 carbons have been estimated from the following equations

$$DS_3 = [x(DS)]/y \quad (6)$$

$$DS_4 = [z(DS)]/y \quad (7)$$

$$DS_6 = [(x + z) - DS]/y \quad (8)$$

where x and z are the integral of the proton signals at δ 5.45 and δ 5.24 ppm, respectively; y is the average integral of the protons from vinyl group (δ 6.0−6.4 ppm); and DS is the total degree of substitution calculated from eq 5. By use of these equations the distribution of substituents in Inul-VA samples with different DS was calculated and presented in Figure 7. The results obtained indicate that the reactivity of hydroxyl groups toward acylation reaction decreases in the order C6*f* > C4*f* > C3*f*, where *f* indicates the fructosyl moiety. Since $^1H-^1H$ COSY clearly indicates that all fructose units substituted are monosubstituted (no cross-peaks are shared by the three positional isomers), the relative reac-

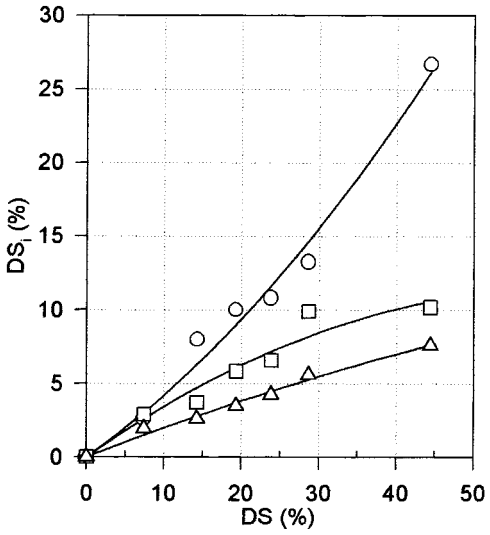


Figure 7. Variation of DS at hydroxyl groups (DS_i) in positions 6 (○), 4 (□), and 3 (△) with the total degree of substitution in Inul-VA (DS).

tivities of the hydroxyl groups are not influenced by substitution of other positions in the unit. Furthermore, as shown in Figure 7, the relative reactivities of the hydroxyl groups are independent of the DS, which demonstrates that the acylation of a particular hydroxyl group follows an independent trend.

The substitution pattern achieved in the Inul-VA samples with different DS shows the expected enzyme's preference for primary hydroxyl groups and agreement with results described in the literature showing that enzymatic acylation of small nucleophiles, including sugars, occurs preferentially at the primary hydroxyl groups.[2−4] However, there is also the derivatization of secondary hydroxyl groups at positions 3 and 4, albeit to a lesser degree. Interestingly the enzymatic derivatization spectrum of inulin, which gives only single ester moieties, is distinct from the chemical derivatization of inulin reported in the literature. In fact, disubstituted fructofuranoside residues were found in inulin when molar ratios of acylating agent and inulin reported in this work were used in carboxymethyl and cyanoethylation reactions.[32]

Preparation and Characterization of Inul-VA Gels. The acrylate groups in Inul-VA were polymerized to form a cross-linked network. The polymerization proceeded quickly, and within ca. 5 min the solution started to gel. The minimal DS values necessary to gel 40, 20, and 10% (w/v) Inul-VA solutions in the presence of a free radical initiator were 7.4, 14.3, and 23.8%, respectively.

To follow the polymerization reaction, CP/MAS ^{13}C NMR spectroscopy was performed (more conventional FTIR spectroscopy could not be used due to the overlapping inulin bands with vinyl monomer bands at 1635 cm^{-1} (stretching of C═C bond) and ca. 811 cm^{-1} (twisting of CH bond, from vinyl group) (data not shown)). CP/MAS ^{13}C NMR spectra of unreacted Inul-VA and after 24 h of polymerization are displayed in Figure 8. Upon polymerization the carbon from the carbonyl group is shifted from δ 169.3 to δ 177.8 ppm as a result of a hybridization change in the adjacent carbons (vinyl carbons) from sp^2 to sp^3. Furthermore, the tertiary methine carbon from the vinyl group shifts from δ 139.1 to

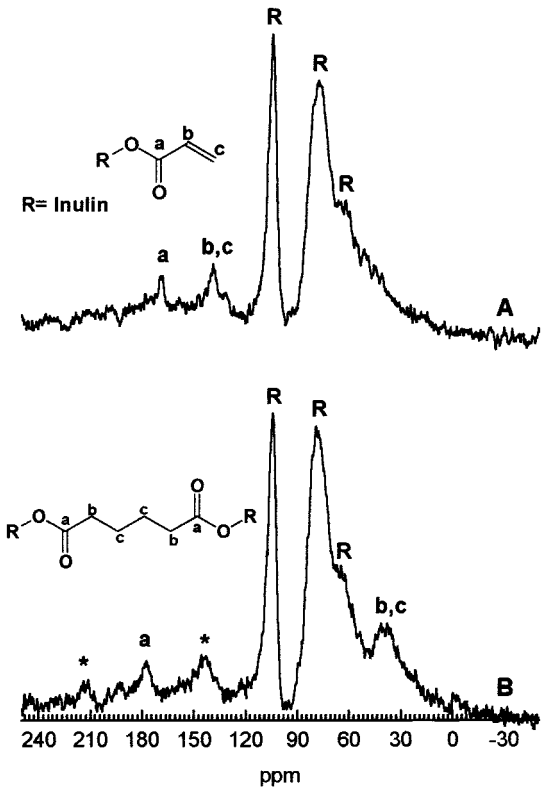


Figure 8. Solid-state CP/MAS ^{13}C NMR spectra of Inul-VA (DS = 28.7%) in the beginning (A) and after 24 h (B) of the polymerization reaction. The peaks labeled with an asterisk represent spinning sidebands.

δ 39.8 ppm, while the secondary methylene carbon at 131.7 ppm is shifted to around 55 ppm,[20] overlapped by inulin carbons. Even if the degree of conversion of the acrylate groups could not be quantitatively determined by CP/MAS ^{13}C NMR, the results confirm the polymerization of vinyl monomers attached to inulin.

The determination of structural properties of cross-linked structures is crucial for gel characterization. The determination of the polymer volume fraction before and after swelling allows the calculation of the molecular weight between cross-links, $\bar{M}_c$, according to eq 2. This equation, developed by Peppas et al.,[23] describes the swelling of a highly cross-linked, moderately swollen polymeric network. This approach takes into account the small average chain length between cross-links (fewer than 100 repeating units), which deviates from a Gaussian distribution.[23] Another critical parameter of gels is their average mesh size, ξ, which is important to assess the transport properties of solutes. ξ was calculated from $\bar{M}_c$ by eqs 3 and 4.

SRE, $\bar{M}_c$, and ξ from Inul-VA gels were determined as a function of monomer concentration and degree of substitution of the monomers and are given in Table 2. SRE, $\bar{M}_c$, and ξ decrease as the monomer concentration increases from 10 to 40% (w/v), while maintaing a constant DS of Inul-VA monomer (28.7%). SRE and ξ decreases from 19.80 to 2.73 and from 56.83 to 19.11 Å, respectively. Furthermore, maintaining a constant monomer concentration (40% w/v) the SRE, $\bar{M}_c$, and ξ decrease as monomer DS increases in the starting polymerizing solutions. In this case, SRE and ξ decreases from 6.05 to 2.71 and from 34.03 to 19.00 Å,

Table 2. Network Properties of Inul-VA Gels as a Function of the Initial Monomer Concentration and the DS

gel	W_0[a] (%, w/v)	DS[b] (%)	SRE[c]	$\nu_{2,s}$[d]	$\bar{M}_c$[e] (g/mol)	ξ[f] (Å)
1	10	28.7	19.80 ± 0.09	0.048 ± 0.001	1640.6 ± 1.6	56.83 ± 0.09
2	20	19.3	12.03 ± 0.30	0.077 ± 0.002	1535.2 ± 12.4	47.04 ± 0.36
3	20	23.8	8.04 ± 0.21	0.111 ± 0.003	1268.6 ± 20.8	37.86 ± 0.29
4	20	28.7	5.64 ± 0.01	0.151 ± 0.001	956.4 ± 1.1	29.65 ± 0.01
5	40	14.3	6.05 ± 0.13	0.142 ± 0.003	1209.9 ± 18.1	34.03 ± 0.21
6	40	19.3	3.59 ± 0.06	0.218 ± 0.003	769.3 ± 13.8	23.53 ± 0.11
7	40	23.8	3.31 ± 0.11	0.232 ± 0.006	709.4 ± 24.5	22.12 ± 0.19
8	40	28.7	2.73 ± 0.15	0.269 ± 0.010	583.7 ± 31.2	19.11 ± 0.25
9	40	44.4	2.71 ± 0.03	0.270 ± 0.002	579.3 ± 6.7	19.00 ± 0.05

[a] Initial monomer concentration. [b] Degree of substitution, i.e., the amount of vinyl groups per 100 fructose units, determined by ^{1}H NMR. [c] Swelling ratio at equilibrium (average ± SD, $n = 3$). [d] Polymer fraction at equilibrium swelling (average ± SD, $n = 3$). [e] Molecular weight between cross-links (average ± SD, $n = 3$). [f] Average mesh size (average ± SD, $n = 3$).

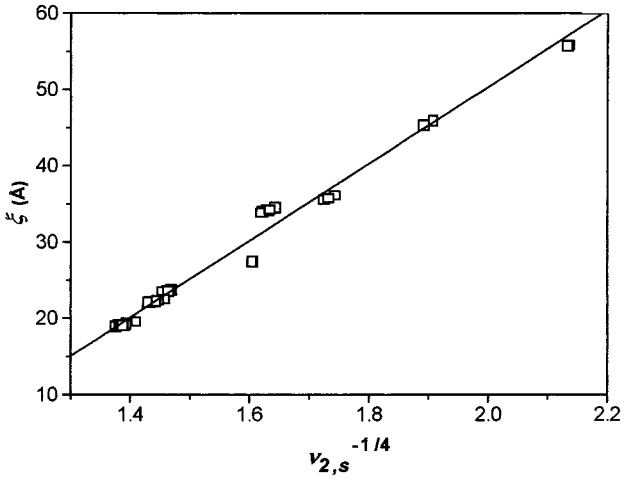


Figure 9. Relationship between the mesh size (ξ) and the equilibrium polymer volume fraction ($\nu_{2,s}$) for all Inul-VA gels prepared in this work. The straight line indicates the linear regression of the data with $r^2 = 0.9923$.

respectively. Both results can be explained by the increasing of cross-linking (intermolecular cross-links and polymer chain entanglements) formed at higher monomer (Inul-VA) concentrations and as monomer DS increases, which restricts network expansion upon swelling.

The calculation of both $\nu_{2,s}$ and ξ for Inul-VA gels allows us to establish a correlation among these parameters. Since $\nu_{2,s}$ can be easily determined by the swelling of the cross-linked network, a correlation between $\nu_{2,s}$ and ξ, it was important to determine which solute could be applied in these gels. According to deGennes[33] for semidilute polymer solutions ($\nu_{2,s} \leq 0.01$), ξ is related to $\nu_{2,s}$ by a power-law exponent of -0.75. At high polymer concentrations ($\nu_{2,s} > 0.01$) power-law exponents of -0.5 [34] and -1[26] were reported on the literature. Inul-VA gels were analyzed using eq 9, using a linear regression with a predetermined exponent n

$$\xi = k_1 + k_2 \nu_{2,s}^{\ n} \tag{9}$$

For the data of Inul-VA hydrogels with $\nu_{2,s}$ between 0.048 and 0.270, eq 9 with $n = -0.25$, $k_1 = -50.4$, $k_2 = 50.4$ gives a good correlation ($r^2 = 0.9923$) (Figure 9). It is noteworthy that all hydrogel samples were prepared from different initial polymer and cross-linker concentrations, and the correlation obtained seems to extend to all gels prepared. The average mesh size range achieved for Inul-VA gels suggests that they may have applications in controlled release of compounds with low molecular weight (high cross-linked gels) or macromolecular compounds such as proteins (low cross-linked gels). In this case, globular proteins with a molecular weight of 30 000 Da which have a diameter[35] of ≅42 Å could be administered through gels 1 and 2 (Table 2).

Conclusions

This work reports the first successful enzyme-catalyzed modification of a soluble polysaccharide, in this case inulin, in anhydrous DMF. Incorporation of vinyl groups in the inulin backbone was accomplished by transesterification of inulin with vinyl acrylate catalyzed by Proleather. The efficiency of the transesterification reaction and the isolated yield were above 57.4 and 44.0%, respectively. The structure of inulin esters revealed one predominant positional isomer in the fructofuranoside residue at the 6 position and two minor isomers at the 3 and 4 positions. Upon free radical polymerization of aqueous solutions of Inul-VA, hydrogels were obtained, which may have properties ideal for use as colon-specific drug delivery systems. The calculated values of $\bar{M}_c$ varied between 579.3 and 1640.6 g/mol, which corresponded to an average mesh size of 19.00−56.83 Å. A correlation was established between ξ and $\nu_{2,s}$. The exponent of this correlation was found to be -0.25, and it allows definition of which drug solutes may be loaded in the inulin gels by the simple determination of their swelling characteristics.

The enzymatic process described herein can be envisioned as a new method for the modification of polymers in nonaqueous media. We are presently using this enzymatic approach to derivatize other polysaccharides and hydroxylated polymers.

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