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Chemo-enzymatic synthesis of disaccharide-branched copolymers with high molecular weight

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Abstract

Glycoconjugate polymers containing lactose or sucrose branches with high molecular weight were synthesized by radical polymerization from enzymatically prepared monomers, 6^{II}-*O*-vinylhexanedioyl-lactose (VHL) and 1^{Fru}-*O*-vinylhexanedioyl-sucrose (VHS). Lactose and sucrose were submitted to transesterification with divinyl hexanedioate in anhydrous pyridine catalyzed by an alkaline protease from *Bacillus subtilis* at 50 °C. VHL and VHS were obtained respectively without any blocking/deblocking steps. Homopolymer of vinyl sugar esters and copolymers of acrylic acid or acrylamide were prepared with $Fe^{2+}/K_2S_2O_8/H_2O_2$ as initiator. The resultant polymer was characterized by FTIR, NMR spectroscopy and GPC. The biodegradation of the glycoconjugate polymer was investigated using enzymatic method in aqueous buffer, and results showed that the polymers were biodegradable.

Keywords: Divinyl hexanedioate; Disaccharide; Copolymer; Chemo-enzymatic synthesis; Polymerization

1. Introduction

Synthetic polymers containing sugar branches have raised an increasing interest as artificial materials for a number of biological and biomedical uses (Gross, Kumar, & Kalra, 2001). This is mostly due to the expectation that polymers displaying complex functionalities, similar to those of natural glycoconjugates, might be able to mimic their performance in specific applications. Many studies have been published on the use of glycopolymers as drug delivery systems, artificial tissues, biocompatible device, hydrogels and surface modifiers (Miura, Ikeda, & Kobayashi, 2003a; Sihorkar & Vyas, 2001; Wang, Dordick, & Linhardt, 2002). Apart from polymer modification, glycopolymers have been so far prepared via ring opening, ring opening metathesis, and vinyl polymerization, the latter accounting for most of the publications in the field. However preparation of vinyl carbohydrate monomers via chemical routes is complicated and needs multi-step reactions including protection–deprotection steps of multiple hydroxyl groups. In these respects, enzymes are advantageous as catalysts to perform conversions and modifications of carbohydrates with high efficiency and high selectivity. A variety of esterases such as lipases and proteases have been reported to be effective in the biotransformation of carbohydrates (Pedersen, Wimmer, Matthiesen, Pedersen, & Gessesse, 2003; Raku, Kitagawa, Shimakawa, & Tokiwa, 2003; Therisod & Klibanov, 1986).

In the past few years, several groups have reported the enzyme-catalyzed synthesis of monosaccharide-functionalized vinyl monomers and their radical polymerization. For instance, Tokiwa et al. investigated enzymatic synthesis of vinyl adipoyl glucose (Tokiwa et al., 2000), vinyl adipoyl rhamnose and vinyl adipoyl fucose (Raku & Tokiwa, 2003), and their chemical polymerization with 1,1′-azoisobutyronitrile (AIBN) as initiator to give a monosaccharide branched polymer consisting poly(vinyl alcohol) main chain. The number average molecular weights of three glycopolymers were 11,000, 13,100, and 5600, respectively. Miura et al. reported the enzymatic synthesis of maltitol 6-vinyl sebacate, lactitol 6-vinyl sebacate (Miura, Ikeda,

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Wada, Sato, & Kobayashi, 2003b). Polymerization of these vinyl esters with hydrogen peroxide/ascorbic acid as initiator gave glucose-branched and galactose-branched polymers having a number average molecular weight of 12,000–28,000. To the best of our knowledge, little attention has been so far devoted to the preparation of copolymers containing disaccharide. Moreover, most of monosaccharide-carrying polymers reported had low molecular weight, and thus limited their application.

In this paper, we investigated the transesterification of divinyl hexanedioate with disaccharides (lactose and sucrose) catalyzed by an alkaline protease from *Bacillus subtilis*, and the vinyl disaccharide esters were homopolymerized or copolymerized with acrylic acid or acrylamide using $Fe^{2+}/K_2S_2O_8/H_2O_2$ as initiator to obtain a disaccharides-branched copolymers with high molecular weight.

2. Materials and methods

2.1. Materials

Alkaline protease from *Bacillus subtilis* (EC 3.4.21.62) was purchased from Wuxi Enzyme Co. Ltd (Wuxi, People's Republic of China). Divinyl hexanedioate was produced and purified as described in the patent (John, Henry, & Robert, 1960). Lactose, sucrose and all other chemicals were of analytical grade. Pyridine was dried over 0.3 nm molecular sieves for 24 h prior to use.

2.2. Analytical methods

The reaction process was monitored via analyses of samples using thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC). Analytical TLC was performed on silica gel 60 plates using an eluent consisting of ethyl acetate/methanol/water (17:4.5:1, by vol). Spots were detected by spraying with 10% (*V*/*V*) H_2SO_4 in MeOH and heating on a hot plate. HPLC was carried out by reverse-phase high performance liquid chromatography (HPLC) using a system equipped with a Waters 2690 Separations Module, a XTerraTM RP-18 Column (5 μm, 150×3.9 mm) and a Waters 2410 refraction index detector. Infrared spectra were measured with a Nicolet Nexus FTIR 670 spectrophotometer. The position of acylation in enzymatically prepared disaccharide esters was established by ^{13}C-NMR (Bruker AVANCE DMX 500). Me_2SO-d_6 or D_2O was used as a solvent and Me_4Si was used as an internal reference. Mass spectrometry data was obtained on Bruker Esquire-LC for electro-spray (MS-ES) measurements (solvent: methanol; positive mode). Elemental analysis was performed with CE EA1112 on the sample extensively dried in vacuo. Molecular weight analysis was performed by gel permeation chromatography (GPC) with refractive index detection (Waters 2410) and Ultrahydrogel (Waters) GPC columns of 12 and 2.5 nm in series. The GPC columns were standardized with narrow dispersity poly (ethylene glycol) in molecular weights ranging from 1.2×10^6 to 1000. The mobile phase was 0.1 M NaH_2PO_4 at a flow rate of 0.8 mL/min.

2.3. Enzymatic synthesis of 1^{Fru}-*O*-vinylhexanedioyl-sucrose (**1**)

A mixture of sucrose (6.84 g, $C_{12}H_{22}O_{11}$, 20 mmol), divinyl hexanedioate (15.8 g, 4 equiv.), alkaline protease from *Bacillus subtilis* (4 g, 20 mg/mL), 200 mL pyridine was shaken at 250 rev./min for 5 day at 50 °C. The reactions were terminated by filtering off the enzyme. The pyridine was evaporated. Formation of the disaccharide ester was confirmed by TLC. The product was isolated by silica gel chromatography with an eluent consisting of ethyl acetate/methanol/water (100:10:5, by vol) to give products (**1**). Yield: 5.46 g, 55%; yellow solid; $[\alpha]_D^{25}$ +58° (*c* 0.5, MeOH); IR (KBr): ν 3362 (ν_{O-H}), 1740 ($\nu_{C=O}$), 1648 ($\nu_{C=C}$); ^{1}H-NMR (D_2O): δ 7.15 (dd, 1H, $J_{a,b}$ 6.3, $J_{a',b}$ 14.0 Hz, –CH=), 5.36 (d, 1H, H-1 of sucrose), 4.92 (d, 1H, $J_{a',b}$ 13.9 Hz, CH_2=), 4.70 (br, HDO and the other H of CH_2=), 4.30–3.30 (br m, 13H, other H of sucrose), 2.43–2.35, 1.62 (m, 8H, 4 CH_2 of adipoyl part); ^{13}C-NMR (D_2O): δ 176.50 (CO), 174.51 (CO), 142.12 (–CH=CH_2), 100.26 (CH_2=CH–), 103.32 (C-2′), 93.61 (C-1), 82.54 (C-5′), 77.72 (C-3′), 74.43 (C-4′), 73.51 (C-3), 73.51 (C-5), 71.95 (C-2), 70.20 (C-4), 63.60 (C-1′), 63.04 (C-6′), 61.11 (C-6), 32.28, 32.14, 23.50, 23.31 (4CH_2); ESI-MS (*m*/*z*): 519 (M_1+Na^+), M_1 corresponding exactly to 1^{Fru}-*O*-vinyl-hexanedioyl-sucrose's molecular mass. Anal. Calcd for $C_{20}H_{32}O_{14}$: C, 48.39; H, 6.50. Found: C, 48.31; H, 6.52.

2.4. Enzymatic synthesis of 6^{II}-*O*-vinylhexanedioyl-lactose (**2**)

Lactose (7.2 g, $C_{12}H_{22}O_{11}\cdot H_2O$, 20 mmol), divinyl hexanedioate (15.8 g, 4 equiv.), alkaline protease from *Bacillus subtilis* (4 g, 20 mg/mL), 200 mL pyridine, 50 °C, 5 day. Eluent: ethyl acetate/methanol/water (100:10:5, by vol). Yield: 4.56 g, 46%; yellow solid; mp 98–101 °C; Pure α anomeric product; $[\alpha]_D^{25}$ +38° (*c* 0.4, MeOH); IR (KBr): ν 3390 (ν_{O-H}), 1742 ($\nu_{C=O}$), 1647 ($\nu_{C=C}$); ^{1}H-NMR (Me_2SO-d_6): δ 7.22 (dd, 1H, $J_{a,b}$ 5.7, $J_{a',b}$ 13.1 Hz, –CH=), 6.33 (s, 1H, 1α-OH of lactose), 5.15 (s, 1H, 2′α-OH of lactose), 4.89 (m, 2H, H-1α of lactose and one H of CH_2=), 4.86 (s, 1H, 3′α-OH of lactose), 4.78 (s, 1H, 2α-OH of lactose), 4.65 (d, 1H, $J_{a,b}$ 5.5 Hz, CH_2=), 4.56 (d, 1H, $J_{4'\alpha\text{-OH},H\text{-}4'\alpha}$ 6.1 Hz, 4′α-OH of lactose), 4.43 (m, 1H, 3α-OH of lactose), 4.24 (m, 2H, 6α-OH and H-1′ of lactose), 4.18, 4.09 (2H, H_a-6′α and H_b-6′α of lactose), 3.73 (m, 2H, H-5α and H-4′α of lactose), 3.63, 3.58, 3.56 (m, 4H, H_a-6α, H_b-6α, H-5′α and H-3α of lactose), 3.33 (m, HDO and H-2′α of lactose), 3.39 (m, 1H, H-4α of lactose), 3.17 (m, 1H, H-2α of lactose), 2.45 (m, 2H, –CH_2–(CO_2–CH=CH_2)), 2.34 (m, 2H, –CH_2–(CO_2-lactose)), 1.57

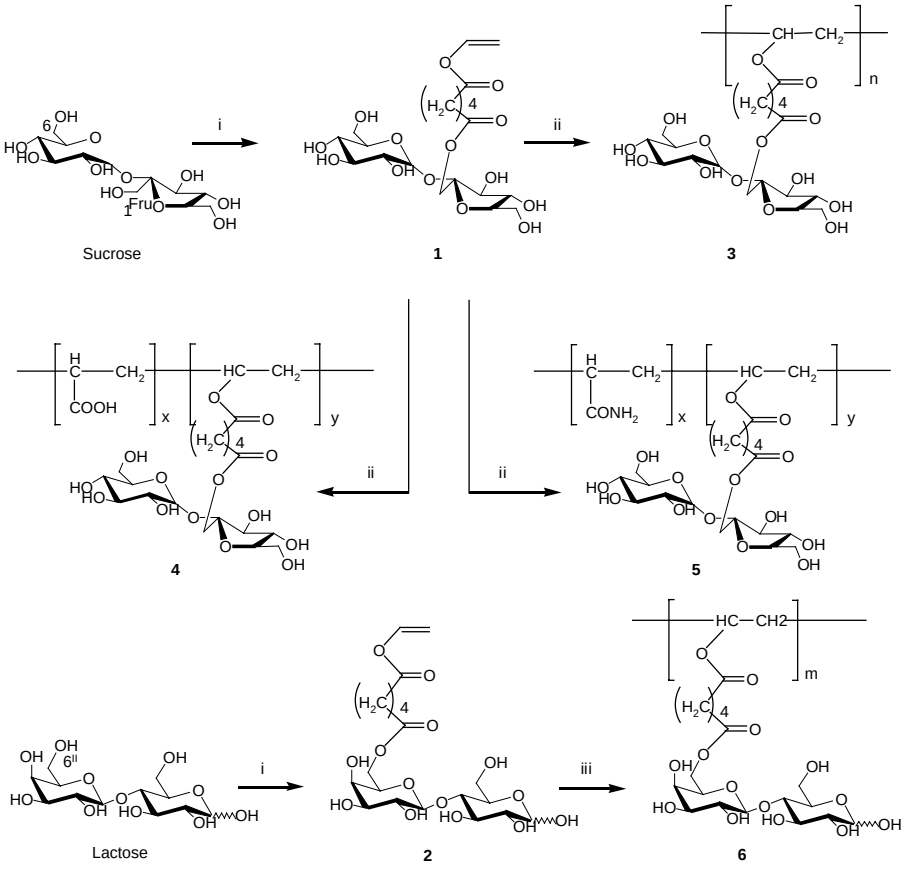

Fig. 1. Synthesis of disaccharide-branched polymers via enzymatic transesterification of disaccharide and subsequent radical polymerization. *Conditions*: (i) divinyl hexanedioate, pyridine, 50 °C, alkaline protease from *Bacillus subtilis*; (ii) radical initiator $Fe^{2+}/K_2S_2O_8/H_2O_2$; (iii) radical initiator $K_2S_2O_8/H_2O_2$.

(m, 4H, other 2 CH_2 of adipoyl part); ^{13}C-NMR (Me_2SO-d_6): δ 172.21 (CO), 169.85 (CO), 140.80 (–CH=CH_2), 97.60 (CH_2=CH–), 103.12 (C-1′), 91.55 (C-1α), 80.70 (C-4α), 72.34 (C-3′), 71.92 (C-5′), 71.71 (C-5α), 70.79 (C-3α), 69.83 (C-2′), 69.23 (C-2α), 67.82 (C-4′), 62.95 (C-6′), 60.01 (C-6α), 32.44, 32.21, 23.14, 23.01 (4CH_2); ESI-MS (*m*/*z*): 519 (M_2+Na^+), M_2 corresponding exactly to 6^{II}-*O*-vinylhexanedioyl-α-lactose's molecular mass.

2.5. Polymerization

Poly(1^{Fru}-*O*-vinylhexanedioyl-sucrose) (**3**: Poly(VHS)) synthesis was carried out by dissolving 400 mg of vinyl sucrose ester in 1 mL H_2O. The solution was degassed (freeze/pump/thaw cycles), and 0.3% $K_2S_2O_8$/0.3% H_2O_2/0.2% Fe^{2+} (w/w) was added (Chen, Johnson, Dordick, & Rethwisch, 1994). The polymerization was continued for 24 h at 20 °C. The resulting product was precipitated in acetone, filtered, and dried under vacuum at 45 °C. The polymer (**3**) was obtained in 60% recovered yield (0.24 g).

Poly(1^{Fru}-*O*-vinylhexanedioyl-sucrose-co-acrylic acid) (**4**: Poly(VHS-co-acrylic acid)) was performed under conditions identical to those of **3**. A solution of 250 mg of VHS monomer and 150 mg acrylic acid in 1 mL H_2O was added. The solution was degassed (freeze/pump/thaw cycles), and 0.3% $K_2S_2O_8$/0.3% H_2O_2/0.2% Fe^{2+} (w/w) was added. The polymerization was continued for 24 h at 20 °C. Precipitating the polymer in acetone terminated the reaction, and the white precipitate was filtered, and dried under vacuum at 45 °C to yield 340 mg (85% yield) of the polymer.

Poly(1^{Fru}-*O*-vinylhexanedioyl-sucrose-co-acrylamide) (**5**: Poly(VHS-co-acrylamide)) was performed under conditions identical to those of **4**. The polymer (**5**) was obtained in 90% recovered yield (0.36 g).

Poly(6^{II}-*O*-vinylhexanedioyl-lactose) (**6**: Poly(VHL)) synthesis was carried out by dissolving 0.4 g of vinyl lactose ester in 2 mL H_2O, and the solution was sparged with N_2 for 10 min. Potassium persulfate (2 mg) and H_2O_2 (3 mg) were added (Patil, Dordick, & Rethwisch, 1991), and

Table 1
Enzyme screen for vinyl sucrose ester synthesis[a]

Enzyme	Sucrose conversion[b] (%)
Control, no enzyme	0
Alkaline protease from *Bacillus subtilis*	47
Lipase from *hog pancreas*	17
Lipozyme® immobilized from *mucor miehei*	4
Lipase from *porcine pancreas*	15

[a]Experimental conditions: 0.5 mmol sucrose, 1.5 mmol divinyl hexanedioate, 20 mg/mL enzyme, 5 mL pyridine, 50 °C, 3 days.
[b]Sucrose conversion was determined by HPLC.

Table 2
Polymerization of 1^{Fru}-*O*-vinylhexanedioyl-sucrose (VHS) and 6^{II}-*O*-vinylhexanedioyl-lactose (VHL)

Entry	Monomer	Polymer	Initiator	Yield (%)	Mn (10^6)	Mw (10^6)	Mw/Mn
1	VHS	Poly(VHS)	$Fe^{2+}/K_2S_2O_8/H_2O_2$	62	2.15	6.50	3.0
2	VHS + AA	Poly(VHS-co-acrylic acid)	$Fe^{2+}/K_2S_2O_8/H_2O_2$	85	6.0	9.1	1.5
3	VHS + AM	Poly(VHS-co-acrylamide)	$Fe^{2+}/K_2S_2O_8/H_2O_2$	90	4.11	7.1	1.7
4	VHS	Poly(VHS)	$K_2S_2O_8/H_2O_2$	48	0.033	0.053	1.6
5	VHS	Poly(VHS)	AIBN(DMF)	42	0.011	0.021	2.0
6	VHL	Poly(VHL)	$K_2S_2O_8/H_2O_2$	77	0.021	0.033	1.6

the solution was stirred at 60 °C for 24 h. The resulting product was precipitated in acetone, filtered, and dried under vacuum at 45 °C. The polymer **6** of 6^{II}-*O*-vinylhexanedioyl-lactose was obtained in 77% recovered yield (0.30 g).

3. Results and discussion

3.1. Enzymatic synthesis of vinyl disaccharide ester monomers

Enzymatic synthesis of vinyl disaccharide ester was shown in Fig. 1. Enzymes derived from a variety of sources including bacteria, yeast, and molds exhibit different activity and specificity. Four commercially available enzymes were tested for the transesterification of sucrose with divinyl hexanedioate in anhydrous pyridine at 50 °C for 3 days. The results were compared and presented in Table 1. The percent conversion of the substrate catalyzed by the four enzymes ranged from 4–47%. The best result was obtained by the alkaline protease from *Bacillus subtilis*. In the absence of enzyme, no sucrose conversion was obtained. To make this synthesis more practical, we employ the alkaline protease from *Bacillus subtilis*, a crude enzyme which is also an efficient catalyst in the transesterification for its low cost and the possibility of industrial application.

The structure of vinyl sucrose ester (**1**) was verified by ^{13}C-NMR analysis. The general strategy was the same as developed by Yoshimoto, Itatani, and Tsuda (1980). As established by them, acylation of a hydroxyl group of sugar results in a downfield shift of the peak corresponding to the *O*-acylated carbon and an upfield shift of the peak corresponding to the neighboring carbon. Characterization of the sucrose ester (**1**) by ^{13}C-NMR revealed that vinyl sucrose ester was substituted at C-1′ position of sucrose. Thus δ signals for C-1′ of sucrose shifted downfield from 62.3 (C-1′ of sucrose) to 63.6 and C-2′ position shifted upfield from 104.5 (C-2′ of sucrose) to 103.3 compared with sucrose. Characterization of the lactose ester **2** by ^{13}C NMR revealed that vinyl lactose ester was substituted at C-6′ position of lactose. Thus signals for C-6′ of lactose shifted downfield from 61.9 to 64.6 ppm and C-5′ position shifted upfield from 76.2 to 73.5 ppm compared with lactose. These results imply that Alkaline protease from *Bacillus subtilis* shows an effective regioselectivity in the synthesis of vinyl disaccharide esters.

3.2. Polymerization of vinyl disaccharide esters

Glycopolymer containing disaccharide branches were prepared using free radical initiation. Drodick et al. have extensively studied the radical polymerization of sucrose acrylate monomer using several azo-type or redox initiators in aqueous media or organic media (Chen et al., 1994). They found the $Fe^{2+}/(NH_4)_2S_2O_8/H_2O_2$ initiated system could give a substantial improvement in molecular weight. We carried out the polymerization of VHS with $Fe^{2+}/K_2S_2O_8/H_2O_2$, $K_2S_2O_8/H_2O_2$ and AIBN, respectively. The comparison results were showed in entry 1, 4, 5 of Table 2. The polymerization using $Fe^{2+}/K_2S_2O_8/H_2O_2$ initiated system provided Poly(VHS) with Mn of over 2,000,000. The number molecular weight of Poly(VHS) was the lowest when using AIBN as initiator.

Disaccharide-branched polymers were analyzed by FTIR and NMR and, as expected, the double bonds present in the vinyl disaccharide ester monomer and acrylic acid or acrylamide were absent in the polymer. Bands assigned, respectively, to the sucrose ester and acrylic acid or acrylamide appeared in the IR (Fig. 2) and NMR spectra (Fig. 3). In addition, inspection of the corresponding

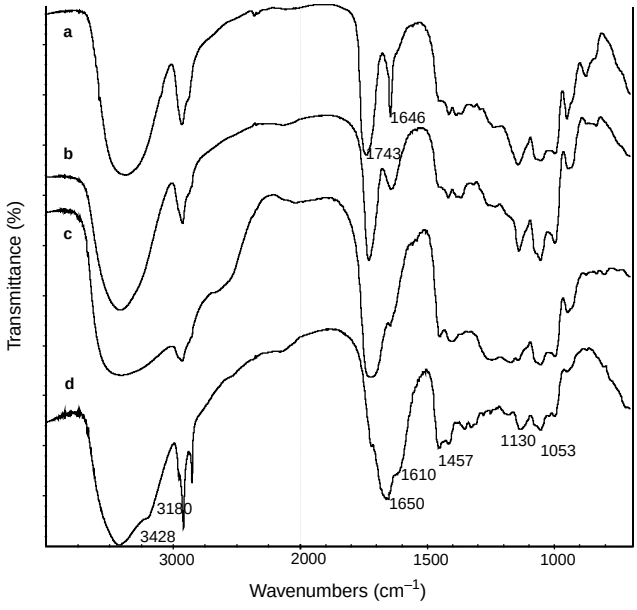

Fig. 2. FTIR spectra of 1^{Fru}-*O*-vinylhexanedioyl-sucrose (VHS) and its polymer. (a) VHS; (b) Poly(VHS); (c) Poly(VHS-co-acrylic acid); (d) Poly(VHS-co-acrylamide).

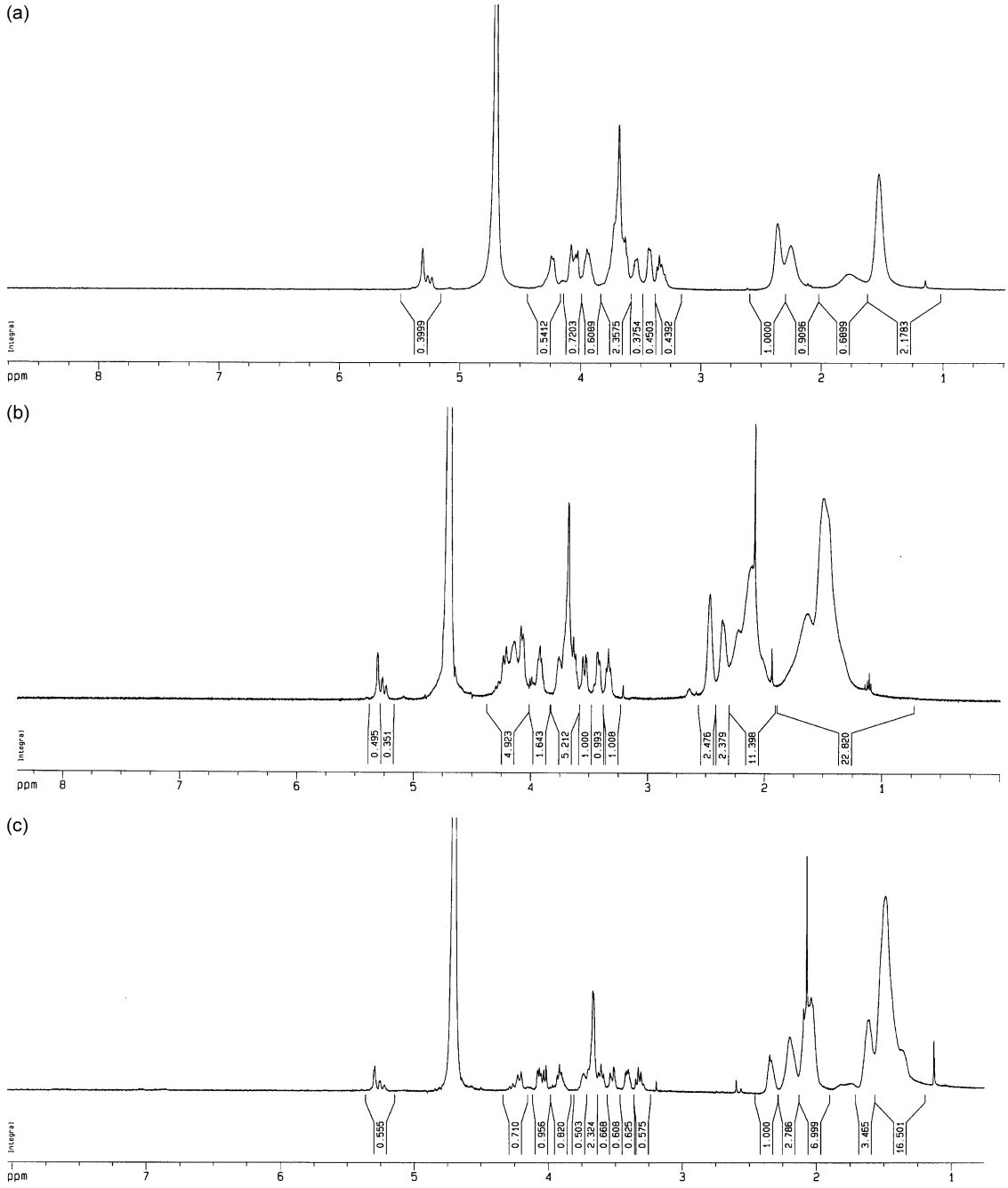

Fig. 3. ^{1}H-NMR spectra of sucrose-branched polymers. (a) Poly(VHS); (b) Poly(VHS-co-acrylic acid); (c) Poly(VHS-co-acrylamide).

resonances showed that the integrity of the sucrose moiety was maintained under the reaction conditions used. The composition of the copolymers was calculated from their ^{1}H NMR according to the ratio between the integral area of sucrose protons (δ 3.3–5.4 ppm) and the integral of methylene and methyne protons (δ 1.1–2.5 ppm).

The molecular weight of copolymers was determined and shown in Table 2. As shown in the GPC profile, the copolymers have high molecular weight and narrow polydispersity. This is a substantial improvement in molecular weight with $Fe^{2+}/K_2S_2O_8/H_2O_2$ initiated system. Some deviations were existed at molecular weights that were extrapolated beyond the range of the calibration data. Despite their large size, the polymers remained completely water-soluble.

3.3. Biodegradation studies

The resulting homo-polymers of vinyl disaccharide esters consist of the main chain, a long spacer arm and disaccharide branch. The main chain is poly(vinyl alcohol) which is known to be biodegradable polymer (Suzuki,

1976). Disaccharide moiety is linked to the main chain with ester bonds, which are highly susceptible to microbe hydrolysis. In this work, biodegradability of the polymer containing disaccharide branch was examined by a biodegradation test in a shake flask using enzymatic hydrolysis at different temperature and GPC analysis. In phosphate buffer pH 7, 10 mg/mL polymer was dissolved and 2 mg/mL protease was added and the reaction was shaken with a stirring speed of 250 rev./min at 37 °C or 4 °C for 6 days. GPC is a convenient method for evaluating the degradation of a polymer. The reaction mixtures before and after the biodegradation test were directly analyzed by GPC. GPC analysis showed that the Poly(VHS) was degraded from an $M_n = 33{,}000$, $M_w/M_n = 1.61$ to an M_n of ca. 1080, $M_w/M_n = 3.30$ (37 °C), and an M_n of ca. 5200, $M_w/M_n = 2.44$ (4 °C). Poly(VHL) was degraded from an $Mn = 21{,}200$, $Mw/Mn = 1.56$ to an Mn of ca. 2100, $Mw/Mn = 2.56$. Results of biodegradation studies indicated that the polymers were completely biodegradable.

4. Conclusion

Novel disaccharide-branched polymers with high molecular weight were synthesized by radical polymerization from an enzymatically prepared monomer, 6^{II}-*O*-vinylhexanedioyl-lactose (VHL) and 1^{Fru}-*O*-vinylhexanedioyl-sucrose (VHS). VHL and VHS were regioselectively synthesized by transesterification reaction of divinyl hexanedioate and the corresponding disaccharide using the alkaline protease from *Bacillus subtilis* in pyridine at 50 °C without any blocking/deblocking steps. The alkaline protease from *Bacillus subtilis* showed high regioselectivity toward multiple groups of disaccharide. Structure analysis showed that VHL was substituted at C-6′ position of lactose and VHS was at C-1′ position of sucrose. Several initiator systems including $Fe^{2+}/K_2S_2O_8/H_2O_2$, $K_2S_2O_8/H_2O_2$ and AIBN were compared. GPC results displayed $Fe^{2+}/K_2S_2O_8/H_2O_2$ initiated system could give a substantial improvement in molecular weight. Homo-polymer of vinyl disaccharide esters and copolymers of acrylic acid or acrylamide were prepared with $Fe^{2+}/K_2S_2O_8/H_2O_2$ as initiator. The resultant polymer was characterized by FTIR, NMR spectroscopy and GPC. Biodegradability of the polymer was examined. After 6 days in aqueous buffer (pH 7), this alkaline protease could effectively degrade Poly(VHS) and Poly(VHL). A potential application of these disaccharide-branched polymers as bio-polyelectrolytes in nano-encapsulation of drug microparticles for targeting drug delivery systems is being investigated in our groups.

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