

CHEMOENZYMATIC SYNTHESIS OF BIODEGRADABLE POLY(1'-O-VINYLA DIP OYL-SUCROSE)

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Abstract A novel polymer containing the sucrose group was synthesized by radical polymerization from an enzymatically prepared monomer, 1'-O-vinyladipoyl-sucrose (VAS). Transesterification reaction of sucrose with divinyl adipate in anhydrous pyridine catalyzed by an alkaline protease from *Bacillus subtilis* at 60°C for 7 days gave VAS (yield 55%) without any blocking/deblocking steps. The vinyl sucrose ester could be polymerized with potassium persulfate and H₂O₂ as initiator to give poly(1'-O-vinyladipoyl-sucrose) with $M_n = 33,000$ and $M_w = 53,200$, $M_w/M_n = 1.61$. The polymer was biodegradable. After 6 days in aqueous buffer (pH 7), this alkaline protease could degrade poly(1'-O-vinyladipoyl-sucrose) to M_n of ca. 1080, $M_w/M_n = 3.30$ (37°C), and M_n of ca. 5200, $M_w/M_n = 2.44$ (4°C). The polymer containing the sucrose branch would be a functional material in various application fields.

Keywords Divinyladipate, Sucrose, Alkaline protease, Polymerization, Biodegradation

INTRODUCTION

Sucrose is the most abundant of all sugars presenting advantages due to its high world production and, consequently, low cost. The high hydrophilicity of the sucrose molecule suggests high biocompatibility levels, which confers upon it a potential for functionalization of polymers to prepare nontoxic, highly absorbent and biocompatible polymeric materials^[1]. However functionalization of polymers with sucrose is complicated because sucrose contains multiple hydroxyl groups and selective and single attachment of sucrose to polymers is difficult. A possible solution is the use of specific blocking/deblocking steps. Unfortunately, the process is tedious and expensive. Unlike chemical catalysts, enzymes are highly selective and have been used to modify sucrose in nonaqueous media. Specifically enzyme-catalyzed regioselective acylation of sucrose in organic media offers an attractive alternative to poorly selective chemical catalysts^[2]. Recently, the modification of sucrose with vinyl acrylate catalyzed by enzyme had been reported, and the resultant acryloyl sucrose was chemically polymerized to yield a sucrose-based polyacrylate^[2-5]. But the main chain of polyacrylate was not biodegradable. There are few reports about the sucrose branched polymer having biodegradability. Tokiwa *et al.* investigated enzymatic synthesis of 6-O-vinyladipoyl-D-glucose and its chemical polymerization to give a glucose branched polymer consisting poly(vinyl alcohol) main chain^[6]. Biodegradation studies indicate that the polymer is completely biodegradable^[7]. Copolymers of 6-O-vinyladipoyl-D-glucose and *N*-isopropyl acrylamide were thermosensitive materials^[8].

In our previous studies^[9], the alkaline protease from *Bacillus subtilis* efficiently catalyzed transesterification reactions of D-glucose with divinyl dicarboxylates and polymerizable vinyl glucose esters were prepared. In this paper, we investigated the regioselective acylation of sucrose with divinyladipate in pyridine catalyzed by the alkaline protease from *Bacillus subtilis*, and the vinyl sucrose ester was chemically polymerized to obtain a

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novel, biodegradable polymer containing sucrose branch.

EXPERIMENTAL

Materials

Alkaline protease (EC 3.4.21.62) from *Bacillus subtilis* was purchased from Wuxi Enzyme Co. Ltd. Lipase (EC 3.1.1.3) from *hog pancreas* and Lipozyme® immobilized from *mucor miehei* were purchased from Fluka. Lipase from *porcine pancreas* (Type II) was from Sigma. Divinyl adipate was prepared and purified as described in the patent^[10]. Pyridine was dried over 0.3 nm molecular sieves for 24 h prior to use. Sucrose and all other chemicals were of the highest purity commercially available.

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 (17:4:1, by vol). Spots were detected by spraying with 10% (V/V) H₂SO₄ in MeOH and heating on a hot plate. HPLC was carried out by reverse-phase HPLC using a system equipped with a Waters 2690 Separations Module, a XTerra™ RP-18 Column (5 μ m, 150 $\times$ 3.9 mm) and a Waters 2410 refraction index detector. Methanol/water 40:60 (V/V) was used as mobile phase, flow rate 0.6 mL/min. Infrared spectra were measured with a Nicolet Nexus FTIR 670 spectrophotometer. The position of acylation in enzymatically prepared sucrose ester was established by ¹³C-NMR (Bruker AVANCE DMX 500). D₂O was used as a solvent. 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 (*M_w* range from 100 to 80,000 daltons). The GPC columns were standardized with poly(ethylene glycol) with molecular weights of 89,500, 20,000, 10,000, 6,000, 4,000, 2,000, 1,000, 600 and 400. The mobile phase was water at a flow rate of 0.8 mL/min.

Transesterification Reaction

Sucrose 6.84 g (20 mmol) was dissolved in 200 mL pyridine containing 9.90 g (50 mmol) divinyl adipate. The reaction was initiated upon addition of 1.0 g alkaline protease from *Bacillus subtilis* and the suspension was placed in a shaking incubator with a stirring speed of 250 rev./min for 7 days at 60 °C. The reaction was terminated by filtering off the enzyme. Pyridine was evaporated off. Formation of the sugar ester was confirmed by TLC. The product was isolated by silica gel chromatography with an eluent consisting of ethyl acetate/methanol/water (90:10:5, by vol) to give vinyl sucrose ester (**1**) (amorphous solid, yield 5.4 g, 55%).

Polymerization

The polymerization of vinyl sucrose ester (**1**) was carried out by dissolving 0.4 g of vinyl sugar ester in 2 mL H₂O, and the solution was sparged with N₂ for 10 min. Potassium persulfate (2 mg) and H₂O₂ (3 mg) were added, and the solution was stirred at 60 °C for 24h. The resulting product was precipitated in acetone, filtered, and dried under vacuum at 45 °C. The polymer (**2**) was obtained in 62% recovered yield (0.25 g).

Enzymatic synthesis of vinyl sucrose ester and its chemical polymerization was shown in Fig. 1.

RESULTS AND DISCUSSION

Synthesis and Characterization of 1'-O-Vinyl Adipoyl-Sucrose (**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 adipate in anhydrous pyridine at 60 °C for 3 days. The results are 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 because of its low cost and the possibility of industrial application.

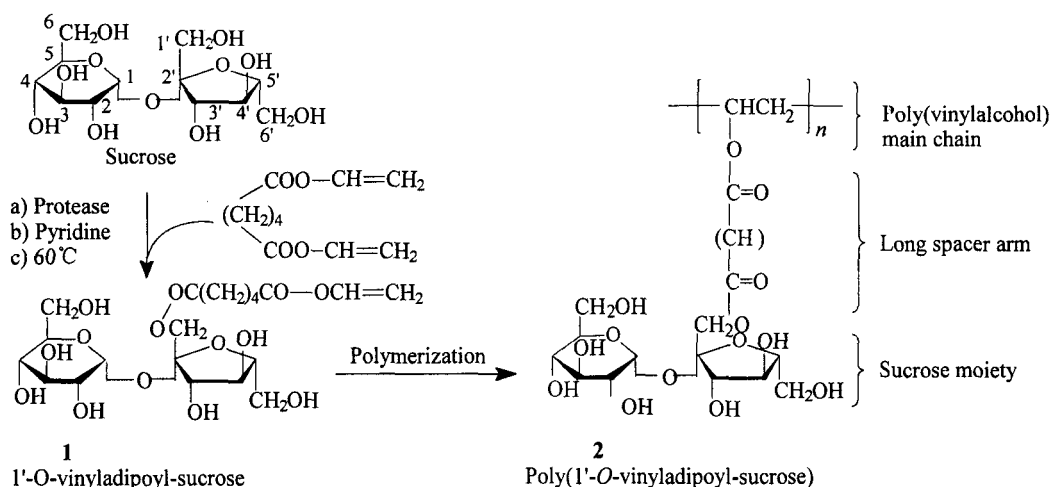


Fig. 1 Enzymatic synthesis of vinyl sucrose ester and its polymerization

Table 1. Enzyme screen for vinyl sucrose ester synthesis

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

^a Experimental conditions: 0.5 mmol sucrose, 1.5 mmol divinyl adipate, 20 mg/mL enzyme, 5 mL pyridine, 60°C, 3 days.

^b Sucrose conversion was determined by HPLC

The effect of temperature on vinyl sucrose ester formation from divinyl adipate and sucrose by the alkaline protease from *Bacillus subtilis* is illustrated in Fig. 2. The percentage conversion at 40, 50, 60 and 70°C was 30, 37, 47, and 32 respectively, in the 3-day reaction. The degree of esterification increased with temperature in the range of 40–60°C and decreased in the range of 60–70°C. This means that the optimum temperature for the vinyl sucrose ester synthesis reaction by the alkaline protease from *Bacillus subtilis* was 60°C and the protease was gradually inactivated above 60°C.

The effect of incubation time on the synthesis of vinyl sucrose ester by the alkaline protease from *Bacillus subtilis* is presented in Fig. 3. The conversion rate of sucrose increased with increasing reaction time and then reached a plateau.

The structure of vinyl sucrose ester was verified by ¹³C-NMR analysis, as shown in Table 2. The general strategy was the same as developed by Yoshimoto *et al.*^[11]. 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 ¹³C-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 to 63.6 and C-2' position shifted upfield from 104.5 to 103.3 compared with sucrose. This result implies that alkaline protease from *Bacillus subtilis* shows an effective regioselectivity in the

transesterification of sucrose with divinyl adipate in anhydrous pyridine. $^1\text{H-NMR}$ (D_2O , δ): 7.15 (1H, dd, $J = 6.3$, $J = 14.0$ Hz, $-\text{CH}=\text{}$), 5.36 (1H, d, H-1 of sucrose), 4.92 (1H, d, $J = 3.9$, $\text{CH}_2=\text{}$), 4.70 (br, HDO and the other H of $\text{CH}_2=\text{}$), 4.30–3.30 (13H, br m, other H of sucrose), 2.43–2.35, 1.62 (8H, m, 4 CH_2 of adipoyl part).

FTIR spectrum of 1'-*O*-vinyladipoyl-sucrose was shown in Fig. 4. Assignment was as follows: 3362 cm^{-1} ($\nu_{\text{O-H}}$), 1740 cm^{-1} ($\nu_{\text{C=O}}$), 1648 cm^{-1} ($\nu_{\text{C=C}}$). ESI-MS (m/z): 519 ($M_1 + \text{Na}^+$), M_1 corresponding exactly to 1'-*O*-vinyladipoyl-sucrose's molecular mass. Anal. Calcd for $\text{C}_{20}\text{H}_{32}\text{O}_{14}$: C, 48.39; H, 6.45; O, 45.16. Found: C, 48.18; H, 6.56; O, 45.26.

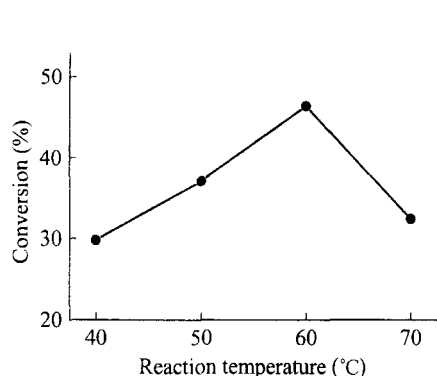


Fig. 2 Effect of temperature on transesterification catalyzed by alkaline protease from *Bacillus subtilis*. Experimental conditions: 0.5 mmol sucrose, 1.5 mmol divinyladipate, 20 mg/mL enzyme, 5 mL pyridine. The reaction was carried out for 3 days.

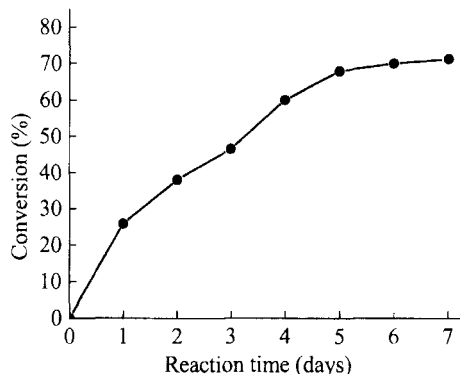


Fig. 3 Time course of the enzymatic synthesis of vinyl sucrose ester catalyzed by alkaline protease from *Bacillus subtilis*. Experimental conditions: 0.5 mmol sucrose, 1.5 mmol divinyladipate, 20 mg/mL enzyme, 5 mL pyridine. The reaction was carried out at 60°C .

Table 2. Chemical shifts of $^{13}\text{C-NMR}$ (D_2O) of sucrose and sucrose ester

Carbon	Sucrose	Sucrose ester (1)	Carbon	Sucrose	Sucrose ester (1)
C-2'	104.5	103.3	C-1'	62.3	63.6
C-1	92.9	93.6	C-6'	63.2	63.0
C-5'	82.2	82.5	C-6	61.1	61.1
C-3'	77.4	77.7	$-\text{CH}_2-$		34.3, 34.0
C-4'	74.9	74.4			24.6, 24.3
C-3	73.4	73.5	$\text{C}=\text{O}$		176.5
C-5	73.2	73.5			174.5
C-2	71.9	71.9	$-\text{CH}=\text{CH}_2$		142.1
C-4	70.1	70.2			100.3

Polymerization and Characterization of Sucrose-Branched Poly(vinyl alcohol) (2)

The vinyl sucrose ester was polymerized with potassium persulfate and H_2O_2 as initiator to give poly(1'-*O*-vinyladipoyl-sucrose) with $M_n = 33,000$, $M_w = 53,200$, $M_w/M_n = 1.61$ (Fig. 5a). IR spectroscopy (Fig. 4) of the polymer revealed that vinyl group absorption disappeared. The spectroscopic comparison result of vinyl sucrose ester monomer and the resulting polymer confirmed vinyl group free radical polymerization. The polymer with sucrose branch consists of: the main chain, a long spacer arm and the sugar moiety. The use of various divinyl dicarboxylates as a spacer arm changes the distance between the main chain and sugar moiety as well as hydrophobicity. Such modification is of significance in the development of new materials. A number of potential applications of these novel sugar containing polymers including water-absorbent materials, biocompatible polymers and hydrogels, are being investigated in some groups^[4-7].

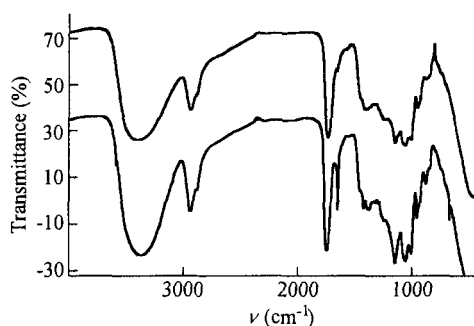


Fig. 4 FTIR spectra of 1'-O-vinyladipoyl-sucrose and its polymer

Biodegradation Studies

The resulting polymer poly(1'-O-vinyladipoyl-sucrose) consists of the main chain, a long spacer arm and sucrose branch. The main chain is poly(vinyl alcohol) which is known as the biodegradable polymer^[12]. The sucrose moiety is linked to the main chain with ester bonds which are highly susceptible to microbial hydrolysis. To test this hypothesis, Tokiwa *et al.* investigated the biodegradation of poly(6-O-vinyladipoyl-D-glucose) by a biochemical oxygen demand (BOD) test using soil or activated sludge at 25°C and GPC analysis. Their result indicated that the polymer was completely biodegradable^[7].

In this work, biodegradability of the polymer containing sucrose branch was examined by a biodegradation test in a shake flask using enzymatic hydrolysis at different temperature and GPC analysis. 10 mg/mL polymer was dissolved in phosphate buffer, pH 7, 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 as shown in Fig. 5. GPC analysis showed that the polymer was degraded from $M_n = 33,000$, $M_w/M_n = 1.61$ (Fig. 5a) to M_n of ca. 1080, $M_w/M_n = 3.30$ (37°C) (Fig. 5b), and M_n of ca. 5200, $M_w/M_n = 2.44$ (4°C) (Fig. 5c). It was confirmed that the biodegradability of sucrose branched polymer was dependent upon the temperature; the biodegradation at 37°C is faster than at 4°C. In general, hydrolysis activity of enzymes is greater at 37°C than at 4°C. On the other hand, high temperature is favorable to chemical hydrolysis of polymers.

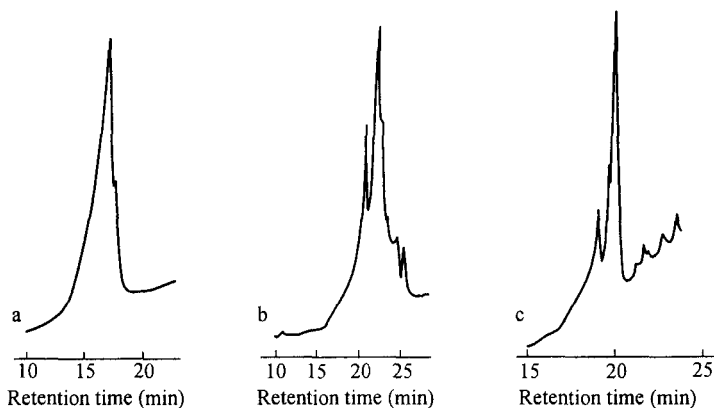


Fig. 5 GPC profiles of poly(1'-O-vinyladipoyl-sucrose) before and after the biodegradation test

a) $M_n = 33000$, $M_w/M_n = 1.61$ (the polymer before the biodegradation test)

b) $M_n = 1080$, $M_w/M_n = 3.30$ (after the biodegradation test at 37°C)

c), $M_n = 5200$, $M_w/M_n = 2.44$ (after the biodegradation test at 4°C)

CONCLUSION

Novel polymer containing sucrose branch was synthesized by radical polymerization from an enzymatically prepared monomer, 1'-O-vinyladipoyl-sucrose (VAS). VAS was regioselectively synthesized by transesterification reaction of divinyl adipate and sucrose using the alkaline protease from *Bacillus subtilis* in pyridine at 60°C for 7 days without any blocking/deblocking steps. The optimum temperature for the vinyl sucrose ester synthesis reaction by the alkaline protease was 60°C. The alkaline protease from *Bacillus subtilis* showed high regioselectivity toward multiple groups of sucrose. The vinyl sucrose ester could be polymerized with potassium persulfate and H₂O₂ as initiator to give poly(1'-O-vinyladipoyl-sucrose) with $M_n = 33,000$ and $M_w = 53,200$, $M_w/M_n = 1.61$. Biodegradability of the polymer was examined. After 6 days in aqueous buffer (pH 7), this alkaline protease could degrade poly(1'-O-vinyladipoyl-sucrose) to M_n of ca. 1080, $M_w/M_n = 3.30$ (37°C), and M_n of ca. 5200, $M_w/M_n = 2.44$ (4°C).

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