One-Step Biocatalytic Synthesis of Linear Polyesters with Pendant Hydroxyl Groups

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Abstract: The chemical synthesis of linear polyesters with various hydrophilic functional groups is difficult, involving a multistep reaction pathway and synthesis of a suitable monomer with a protected functional group. A straightforward conventional synthesis from a trifunctional compound, such as glycerol, and a diester or dicarboxylic acid to produce a polyester results in the formation of networks. An alternative approach to the one-step synthesis of aliphatic hydroxyl-substituted polyesters from divinyl adipate (DVA) and various triols has been found by utilizing the intrinsic specificity of an enzyme. The weight average molecular weights of the resulting polyesters vary according to the triol used and range from ∼3,000 to 14,000 Da. Analysis by MALDI-TOF mass spectrometry has confirmed the presence of a linear polyester with hydroxyl substituents, and there is no evidence for network formation. The pendant groups are 90−95% secondary and 5−10% primary hydroxyl groups. Experimental determination of hydroxyl number verifies that one hydroxyl group is present on each repeat unit of the substituted polyester. By the addition of increasing amounts of 1,4-butanediol to the reaction mixture of glycerol and DVA, predictable and sensitive control of the hydroxyl number can be accomplished.

Introduction

The impetus for research in the area of aliphatic polyesters over the past 20 years has centered on the need for efficient synthesis of functional polyesters. The presence of pendant groups such as carboxylic acid and hydroxyl functionalities imparts attractive characteristics to the polyester, including increased hydrophilicity, that are lacking in their unsubstituted analogues. The combination of water solubility and biodegradability for such polyesters would substantially widen the pool of potential applications and includes controlled release and drug delivery systems as well as other biomedical applications.[1] Furthermore, new routes to synthesis of novel comb, graft, or network polymers can be achieved through reaction with the pendant groups on the linear polyester.

Chemical synthesis of hydroxyl-,[2] carboxyl-,[3] and amino-substituted polyesters[4] has been documented; however, before polymerization can occur, synthesis of a suitable monomer with a protected functional group is necessary. Therefore, the overall chemical synthesis of the functional polyester involves a complex multiple-step reaction pathway from which several problems can arise. These difficulties include low yield of the monomer, unreliability concerning the fraction of deprotected pendant groups on the resultant polyester, and polymer cleavage as a consequence of the deprotection step. Jérôme and co-workers addressed some of these concerns in a recent paper in which they report the synthesis of a protected monomer, 5-ethylene ketal ϵ-caprolactone in one step with greater than 70% yield. Additionally, complete conversion of hydroxyl-substituted polyester from polyester chains with pendant acetal groups was accomplished.[2] However, the overall synthesis still requires multiple steps including synthesis of the monomer, copolymerization with ϵ-caprolactone, and deprotection (two steps) to obtain polyester with functional hydroxyl groups.

The necessity for protection of functional groups during reaction lies in the nonspecific nature of chemical synthesis. For example, network or cross-linked aliphatic polyesters result from the reaction of various aliphatic and aromatic dicarboxylic acids with multihydroxyl compounds such as glycerol and pentaerythritol because all the functional groups on the monomer are able to react.[5,6] In work reported by Tsutsumi, Nagata, and co-workers, a prepolymer is first prepared by melt polycondensation at 200 °C which is then polymerized at 230−310 °C. The resulting polymer film is transparent and insoluble in organic solvents. The degree of reaction was estimated by monitoring the change in the infrared absorption of the hydroxyl

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and methylene groups. The network polyesters based on glycerol achieved degrees of reaction ranging from 60 to 80% which were lower than those obtained for network polyesters synthesized from ethylene glycol and tetracarboxylic acids (87−95%).[6,7] The lower degree of reaction was attributed to the lower reactivity and compact nature of glycerol.[6]

An alternate approach for synthesis is found through the use of enzymes. Specificity, stereoselectivity,[8] and regioselectivity[9−12] of different enzymes with various substrates has been well studied with considerable emphasis given to how these properties can be changed and controlled depending on the reaction parameters.[13] Here we are primarily concerned with the regiospecificity of the enzyme. Klibanov and co-workers have demonstrated that porcine pancreatic lipase (PPL) possesses a preference toward primary hydroxyl groups in the acylation of various glycols. Interestingly, only a very small percentage of the reaction product (0−2%) was diester.[9] The bias of PPL was then exploited in order to achieve the regioselective acylation of the primary hydroxyl group on various sugars.[10] The regioselectivity of certain enzymes toward specific hydroxyl groups was found to be tunable according to the hydrophobicity of the solvent in which the reaction is performed as well as the type of acylating agent.[11] The acylation of natural multihydroxyl compounds (sugars) by vinyl acetate catalyzed by *Candida antarctica* lipase B has also been investigated. The enzyme always acylated the primary hydroxyl groups on the various glycopyranosides studied. A maximum of two of the four hydroxyl groups was acylated, and the regioselectivity of the enzyme toward the remaining secondary hydroxyl groups depended on the identity of the aglycone as well as the stereochemistry of the glycosidic bond.[12] The enzymatic esterification and transesterification of glycerol to form mono- and distearin has also been studied using *C. antarctica* lipase B. Reactions performed in a low-polarity solvent showed formation of mono- and distearin in equal amounts, while reactions in more polar solvents favored production of the monostearin. Formation of 1,2-distearin did occur in significant and sometimes greater amounts than 1,3-distearin production.[14] The reaction of hydroxyl groups on a multifunctional compound such as glycerol is dependent on the relative reaction rate of primary and secondary groups, and it is well-known that primary hydroxyls are more reactive. However, to form linear functional polyester from a multihydroxyl compound, one of the hydroxyl groups must remain unreacted. It is our intent to utilize the inherent specificity of an enzyme to perform an efficient one-step synthesis of linear polyesters containing pendant hydroxyl groups.

Materials and Methods

Enzymes. Novozym 435 was provided as a kind gift from Novo Nordisk A/S (Bagsvaerd, Denmark). Novozym 435 is a triacyl glycerol lipase derived from *Candida antarctica* fraction B immobilized on a macroporous acrylic resin. The enzyme comprises approximately 10 wt % of the total weight of the enzyme−carrier resin bead. The activity as reported by Novo Nordisk is 7550 PLU/g. The activity was measured by a commercially available titrimetric assay (Sigma Chemical Co., St. Louis, MO) modified by performing the reaction of olive oil triglycerides to fatty acids and diglycerides in the presence of Novozym 435 at 50 °C for 2 h. The resulting activity was 53 U/mg of solid. Chirazyme L-9 (c.-f., dry), a lipase from *Mucor miehei* immobilized on a macroporous anion-exchange resin, was purchased from Boehringer Mannheim Biochemicals (Mannheim, Germany). Porcine pancreatic lipase was purchased from Sigma. All other enzymes used were purchased from Amano Pharmaceuticals, Ltd. (Lombard, IL), and include lipases from *Pseudomonas fluorescens*, *Aspergillus niger*, *Candida rugosa*, *Rhizopus Oryzae*, *Penicillium camemberti*, *Mucor Javanicus*, and *Pseudomonas cepacia*. All enzymes were dried under vacuum over phosphorus pentoxide for approximately 72 h to minimize hydrolysis in the reaction.

Chemicals. Tetrahydrofuran (HPLC grade) and standardized 1 N sodium hydroxide solution were purchased from Fisher Scientific (Pittsburgh, PA). Divinyl adipate, an activated ester of adipic acid, was obtained as a kind gift from Union Carbide Corporation (Danbury, CT). All other solvents, substrates, and analytical materials were purchased from Aldrich Chemical Co. (St. Louis, MO). All materials were used without further purification.

Reaction Procedure. Typically, divinyl adipate (0.991 g, 5.0 mmol) and an equimolar amount of the triol/glycol were added to a 7 mL glass vial. Because all the reactions were performed in a solvent-free environment, it was important to ensure that the substrates could form a well-dispersed mixture. DVA is a solid at room temperature and melts at approximately 30 °C. At temperatures above 30 °C, DVA and the various diols and triols form an immiscible liquid mixture which becomes well dispersed when it is vigorously agitated. Therefore, the vial was then placed in a New Brunswick Scientific Series 25 incubator/shaker at 50 °C until DVA melted. Enzyme (1 wt %) was added to the vial to initiate the reaction. The vial was kept in the incubator for 24 h at an agitation speed of 250 rpm and a temperature of 50 °C. The reaction was terminated by quenching the reaction contents with THF. The reaction solution was then filtered to remove the enzyme. The polymer was recovered by evaporating the THF. The product polyesters were analyzed without further purification. The resulting polyesters were soluble in organic solvents. In contrast to the white amorphous solid that is formed from the reaction of DVA and 1,4-butanediol, the polyesters produced from these reactions are clear, viscous liquids.

Gel Permeation Chromatography. Molecular weights of the product polyesters were determined using a Waters 150CV gel permeation chromatograph which is equipped with a refractive index detector. THF is used as the mobile phase at a flow rate of 1.0 mL/min and a temperature of 35 °C. Three columns in series are installed in the instrument to achieve effective separation in the molecular weight range of 500−30 000 Da. The first two columns are PL-gel Mixed-E columns from Polymer Laboratories, which have a range of pore sizes. The third column is a Waters Ultrastyragel column with a 500 Å pore size. A calibration curve of the log of the molecular weight versus retention time was obtained using 11 polystyrene standards in the molecular weight range of 580−66,000 Da.

Matrix-Assisted Laser Desorption/Ionization−Time-of-Flight (MALDI-TOF) Mass Spectrometry. All analyses were performed using a PerSeptive Biosystems Voyager Elite MALDI-TOF mass spectrometer. The instrument was operated at a voltage of 20 kV in linear mode. The matrix preparation for different types of samples varies and must be optimized.[15] For polyester samples, a 10 μg/mL solution of sodium iodide in water is prepared and spotted on to the

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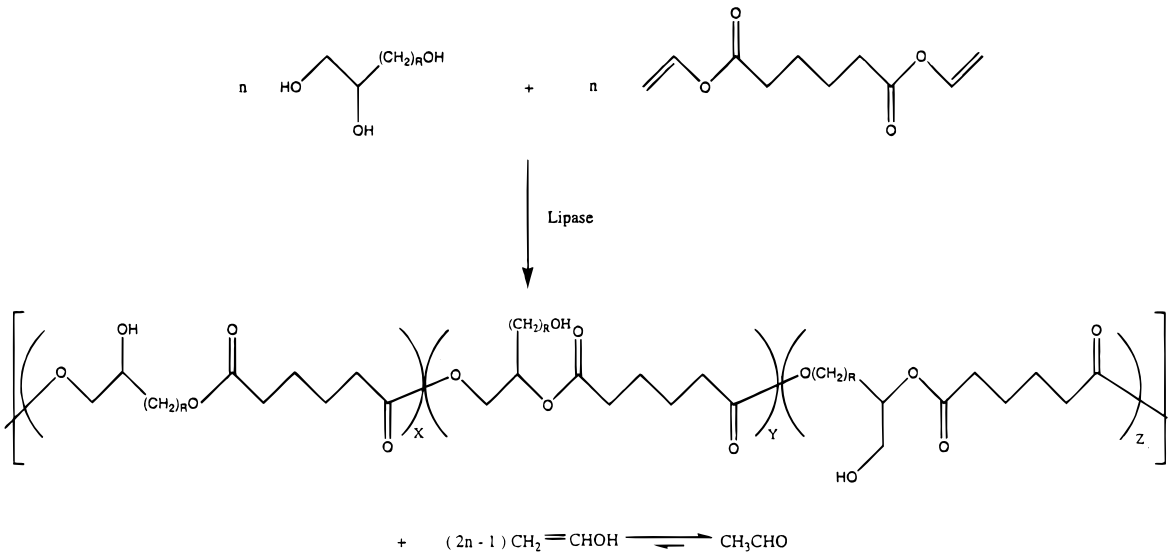

Figure 1. Lipase-catalyzed polycondensation between triols and divinyl adipate to form aliphatic polyester with pendant hydroxyl groups.

target plate prior to that of the polyester and matrix solution. A solution of the polyester and matrix is then prepared using 18 μL of dithranol (~5 mg/mL in THF) and 2 μL of the polyester (~20 mg/mL in THF). Approximately 2 μL is then spotted onto the target plate over the dried sodium iodide solution. The target plate can be loaded into the mass spectrometer when completely dry. The polyester spectra are calibrated using peptide standards obtained from PerSeptive Biosystems (Framingham, MA).

Hydroxyl Content Titration. The hydroxyl number of the polyesters was determined using a titrimetric technique developed by the Bayer Corp. (Miles' Quality Laboratories, Method Number 414-C). The polyester sample was reacted with an excess of phthalic anhydride in pyridine at ~182 °C for 35 min. The unreacted phthalic anhydride was manually titrated with a standardized 1.0 N solution of sodium hydroxide to its end point using phenolphthalein as the indicator. Larger scale reactions, using 20.0−33.0 mmol of DVA and an equimolar amount of the diol/triol, were performed to obtain a suitable amount of polyester for analysis. The amount of polyester used for hydroxyl number analysis varied according to the expected hydroxyl number and decreased as the hydroxyl content increased.

The presence of a significant amount of unreacted monomer in the product polyester could skew the results of the analysis. However, the product polyester was analyzed without purification, which can be justified by considering the mechanism of stepwise condensation. Step polymerization is characterized by the disappearance of monomer early in the reaction to the point that, for most step polymerizations, the amount of monomer in the reaction is less than 1% of the starting quantity by the time the average polymer chain contains 10 monomer residues.[16] The absence of monomer was confirmed by examining the GPC chromatograms for monomer peaks.

Results and Discussion

Figure 1 shows the general reaction of trihydroxyl compounds (glycerol, 1,2,4-butanetriol, and 1,2,6-trihydroxyhexane, where *R* in the reaction scheme is equal to 1, 2, and 4, respectively) with DVA to produce linear hydroxyl-substituted polyester. The byproduct of the reaction is vinyl alcohol, which tautomerizes to acetaldehyde. While this side reaction effectively shifts the equilibrium of the reaction in the forward direction, acetaldehyde has previously been reported to deactivate enzymes.[17] Therefore, a set of reactions was performed in the presence of several different lipases in an effort to discover which enzymes produced polymer. Only the immobilized forms of *Mucor miehei* and *Candida antarctica* lipase (commercially available as Chirazyme L-9, c.-f., dry, and Novozym 435, respectively) formed polyester, the latter enzyme being far more effective than the former. *C. antarctica* lipase has also exhibited an intrinsic stability toward acetaldehyde which is enhanced further via immobilization.[17] Novozym 435 has also been successfully used in the bulk reaction of 1,4-butanediol with DVA to produce high molecular weight polyester using low enzyme concentrations in short reaction times.[18] Therefore, we have assessed the feasibility of the reaction described in Figure 1 using this lipase. Three different repeat units potentially exist for the reaction shown in Figure 1, and the occurrence of these will depend on the relative reactivity of the primary and secondary hydroxyl groups as well as the regioselectivity of Novozym 435 under the conditions of the reaction.[19]

Table 1. Lipase-Catalyzed Polymerization To Produce Linear Hydroxyl-Substituted Polyesters

triol	reacn time (h)	M_w (GPC) (Da)	PDI	reacn extent[a] (%)
glycerol	4	1485 ± 202	3.10 ± 0.13	82.2
1,2,6-trihydroxyhexane	4	3354 ± 447	2.77 ± 0.05	90.6
glycerol	24	10391 ± 409	2.25 ± 0.04	96.2
glycerol	28	10025 ± 25	2.93 ± 0.15	96.0
1,2,4-butanetriol	28	3916 ± 373	2.22 ± 0.05	89.5
1,2,6-trihydroxyhexane	28	13219 ± 443	2.43 ± 0.02	96.4

[a] Extent of reaction is calculated on the basis of the equation $p = (X_w - 1)/(X_w + 1)$ developed by Flory.[16] X_w is the weight average degree of polymerization and is calculated by dividing the weight average molecular weight by the average molecular weight of the two monomers.

The results of the reactions of various triols with DVA are shown in Table 1 and agree with the formation of polymer according to the mechanism of step polycondensation. After 4 h, the extent of reaction ranges between approximately 80 to 90% for glycerol and 1,2,6-trihydroxyhexane reactions. While these extents could be considered high under other circumstances, high molecular weight polymer is formed only at extremely high reaction extents for stepwise condensations. As a result, the molecular weight of the polyesters after 4 h of

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(19) For symmetric compounds, like glycerol, only two potential repeat units exist. *R* is equal to 1 and the repeat units denoted by Y and Z in Figure 1 would be structurally identical.

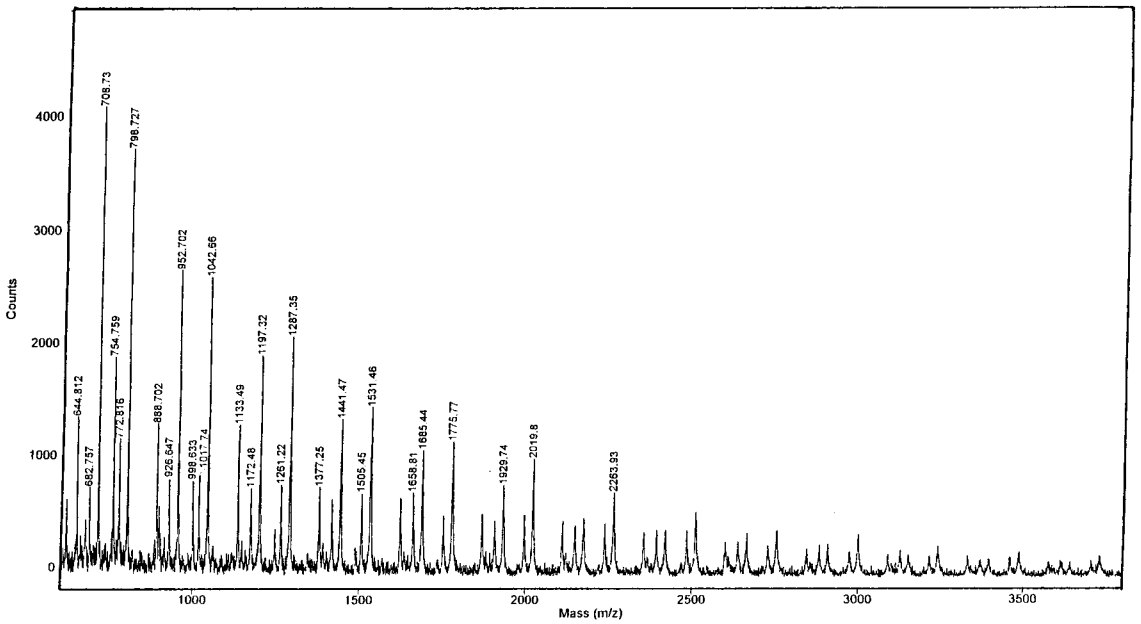

Figure 2. MALDI-TOF spectrum for polyester synthesized from lipase-catalyzed reaction of 1,2,6-trihydroxyhexane and divinyl adipate. (Matrix: dithranol + sodium iodide).

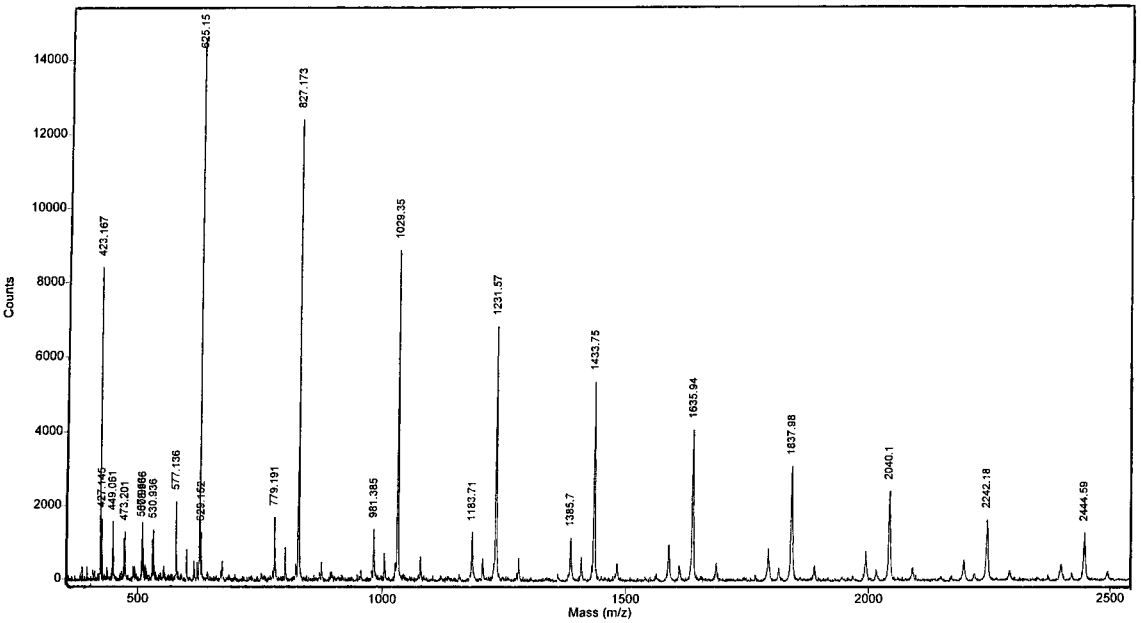

Figure 3. MALDI-TOF spectrum for polyester synthesized from lipase-catalyzed reaction of glycerol and a 50% excess of divinyl adipate. (Matrix: dithranol + sodium iodide).

reaction are low. As expected, the 24 h reaction results in polyesters with increased molecular weights. The extent of reaction and molecular weight of the polyester did not vary significantly when the reaction time was increased from 24 to 28 h. Also, the 1,2,4-butanetriol/DVA reaction showed a decreased reaction extent in comparison to that of the glycerol and 1,2,6-trihydroxyhexane/DVA reactions indicating that the rate of reaction is slower for this triol.

Bulk reactions of various triols with DVA in the presence of Novozym 435 yields polyester, which we analyzed by matrix-assisted laser desorption/ionization−time-of-flight (MALDI-TOF) mass spectrometry.[20] Figure 2 shows a representative spectrum for polyester produced from the reaction of 1,2,6-trihydroxyhexane and DVA. Several series of peaks are apparent, indicating that the polyester chains are terminated with various groups. Each peak within any given series is separated from the next peak by ∼244 Da. (*m*/*z*), which is the molecular weight of the repeat unit for linear hydroxyl-substituted polyester from 1,2,6-trihydroxyhexane and DVA. For example, we have assigned the peak at *m*/*z* 708.73 as a polyester chain with two repeat units and vinyl terminal groups. Other major peak series on the spectrum include hydroxyl−vinyl (799 + 244*n*), hydroxyl−hydroxyl (645 + 244*n*), vinyl−acid (683 +244*n*), and hydroxyl−acid (773 +244*n*) terminated chains.[21] Since MALDI-TOF analysis has yielded results confirming the presence of linear hydroxyl-substituted polyester, it is important to stress that a complete analysis of nonenzymatic polymerizations with glycerol and dicarboxylic acids has been published by Nagata

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(21) $n = 1, 2, 3, \ldots$ and corresponds to the number of repeat units in a polyester chain.

Table 2. Hydroxyl Number Analysis for Unsubstituted and Hydroxyl-Substituted Polyesters

diol/triol	diester	M_w (Da)	no. of repeat units[a]	hydroxyl no. (mg of KOH/ g of sample)	no. of hydroxyl groups per repeat unit (equiv/mol)
glycerol	divinyl adipate	10 060	23	236	0.83
glycerol	divinyl adipate	9 990	22	250	0.89
1,2,4-butanetriol	divinyl adipate	3 540	7.0	260	1.04
1,2,6-trihydroxyhexane	divinyl adipate	12 780	22	222	0.95
1,2,6-trihydroxyhexane	divinyl adipate	13 660	23	255	1.11
1,4-butanediol	divinyl adipate	12 670[b]	45[b]	19.3	0.07
commercial unsubstituted hydroxyl-capped polyester		6 700	13	47.0	0.17

[a] The number of repeat units of each polyester was calculated from the number average molecular weight. [b] As the expected hydroxyl number of the polymer decreases, more sample is necessary to procure an accurate titration. Therefore, five different polyester samples were combined to obtain one hydroxyl number and, consequently, the weight average molecular weight and number of repeat units are averages of these five samples. The error among the five samples is less than 5%.

and co-workers. Their detailed study demonstrated that no linear hydroxyl-functional polyesters can be synthesized in this manner.[6]

To determine whether increasing the stoichiometric ratio of DVA/triol would generate network formation or reaction at all hydroxyls, reactions were also performed at constant glycerol concentration with various concentrations of DVA. As expected, when the reaction deviated from equimolarity, the molecular weight of the resulting polymer decreased significantly.[22] Figure 3 shows a MALDI-TOF spectrum of a polyester produced from the reaction of glycerol and excess DVA, giving a repeat unit of 202 Da. In this particular reaction, the hydroxyl content is equivalent to the ester content. The MALDI-TOF spectra indicate that linear hydroxyl-substituted chains were still formed, but a larger percentage possessed vinyl end groups ($423 + 202n$). A new peak series also appeared at ($577 + 202n$), which can be attributed to a linear hydroxyl-substituted polyester in which one glycerol residue in the entire polyester chain reacted with three DVA's. No further evidence of network formation was found, and further analysis of this chain is in progress to determine the location of the fully reacted glycerol residue. We suggest that this residue most likely appears on the end of the polyester chain and was formed near the end of the reaction when the rate of linear chain extension is very slow. The possibility does exist that a network polymer is forming, but the MALDI-TOF instrument is not able to detect it; however, the polyesters formed from these reactions remain fully soluble in organic solvents indicating that networks are not produced.

Because there are three potential sites for reaction on a triol, identifying which hydroxyl groups react would be beneficial in that the structure of the polyester would be more defined, and if necessary, attempts could then be made to control the regioselectivity of the polymerization. Hence, DVA was reacted with a series of diols containing primary and secondary hydroxyls. The degrees of polymerization of the resulting polymers were then compared to that of the polyester formed from the reaction of triols with DVA. Figure 4a,b depicts the results of the comparisons and clearly indicates that the diols with secondary hydroxyls react at a much slower rate than those with only primary hydroxyl groups. Furthermore, if the assumption is made that the secondary hydroxyl groups on the triols possess the same reactivity as those on the diols, it can be concluded that the triols react essentially through the primary hydroxyl groups. Indeed, we estimate that both glycerol/DVA and 1,2,6-trihydroxyhexane/DVA polyesters consist of approximately 90−95% of the repeat unit denoted by X in Figure 1.

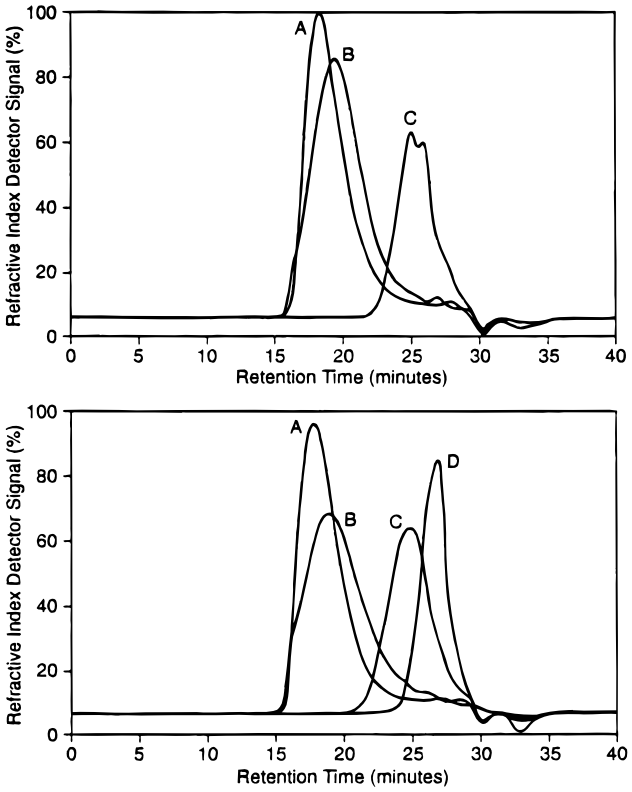

Figure 4. (a) Top: Gel permeation chromatograms for polyesters formed from lipase-catalyzed reactions between divinyl adipate and 1,3-propanediol (A), glycerol (B), and 1,2-propanediol (C). Degrees of polymerization for A−C are 91, 70, and 6.0, respectively. (b) Bottom: Gel permeation chromatograms for polyester formed from lipase-catalyzed reactions between divinyl adipate and 1,6-hexanediol (A), 1,2,6-trihydroxyhexane (B), 1,5-hexanediol (C), and 1,2-hexanediol (D). Degrees of polymerization for A−D are 101, 72, 7.0, and 3.5, respectively.

One concern with the chemical synthesis of functional polyesters, as stated previously, is the uniformity of the pendant group. To determine the number of hydroxyl groups per repeat unit that the biocatalytically synthesized polyesters possessed, a hydroxyl number analysis was performed, and the results are shown in Table 2 for various triol/DVA polyesters. These values were then compared to those for unsubstituted polyesters. The equivalent hydroxyl groups per mole was calculated from

(22) The equimolar reactions of DVA and glycerol produced polymer with weight average molecular weights of approximately 6400 Da. Reactions with excess DVA in which the amount of vinyl groups and hydroxyl groups were equivalent produced oligomers with molecular weights of about 1000 Da.

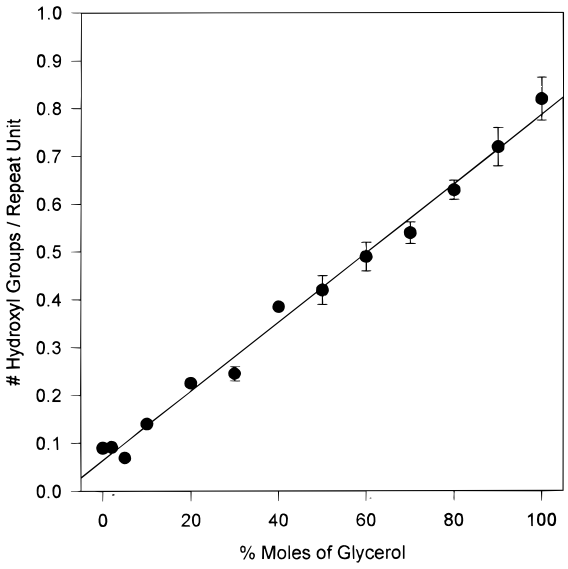

Figure 5. Effect of glycerol concentration on the degree of functionality of the polyester.

the hydroxyl number and then divided by the number of polyester repeat units. For a linear triol/DVA polyester with every repeat unit having a pendant hydroxyl group, this ratio should be 1 hydroxyl group per repeat unit of polyester, whereas, for the diol/DVA polymers, the value should approach 0 hydroxyl groups per repeat unit of polyester. In each case, any terminal hydroxyl groups will result in subtle variations in the hydroxyl number. Glycerol/DVA polyesters show a mildly depressed value in comparison to the other triol/DVA polymers which could be due to reaction of some of the functional hydroxyl groups with DVA as described above. The values obtained for 1,2,4-butanetriol/DVA and 1,2,6-trihydroxyhexane/DVA polyesters confirm that they possess approximately 1 hydroxyl group per repeat unit.

Remarkably, the degree of functionality of the substituted polyester can also be controlled by changing the triol concentration in the reaction maintaining equimolar concentrations of monomers by adding an appropriate amount of a diol. Figure 5 shows the results of a set of reactions in which glycerol concentration was varied from 0 to 100% of the total glycerol/1,4-butanediol concentration. Clearly, the enzyme-catalyzed reaction enables exquisite and predictable control of hydroxyl number in a manner which cannot be achieved by conventional approaches.

Conclusions

We have exploited the inherent specificity of enzymes to achieve the straightforward synthesis of linear polyester with pendant hydroxyl groups. The pendant groups are substantially secondary hydroxyls, since the reaction takes place principally at the primary hydroxyl groups. This work constitutes the most efficient synthesis of functional polyester to date in terms of the number of reaction steps. Furthermore, the hydroxyl contents of the resulting polyesters are consistent with one hydroxyl group per repeat unit and attempts to control the degree of functionality with addition of diol were successful.

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