

Exploring Chain Length Selectivity in HiC-Catalyzed Polycondensation Reactions

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Received November 9, 2009; Revised Manuscript Received January 3, 2010

Polyester synthesis activity of immobilized *Humicola insolens* (HiC) was systematically studied with three-series of substrates varying in (i) ω -hydroxyalkanoic acid (ω HA), (ii) α,ω -*n*-alkane diol, and (iii) α,ω -*n*-alkane diacid chain length. Covalent immobilization of HiC on Amberzyme oxirane (AO) resin (i.e., AO-HiC) was prepared. HiC-AO's activity for ω HA substrates with 6, 10, 12, and 16 carbons was C16 > C12, where C10- ω HA and C6- ω HA were not polymerized. In contrast, N435's activity for ω HA substrates was C16 = C12 > C10, where C6- ω HA was not polymerized. HiC-AO activity for copolymerization of sebacic acid (C10-diacid) with α,ω -*n*-alkane diols with 3-, 4-, 5-, 6-, and 8-carbon chain lengths was C8 > C6, where C3, C4, and C5 diols were not polymerized. N435's relative activity for diol substrates was C8 = C6 = C5 > C4 > C3. HiC-AO activity for copolymerizations of 1,8-octanediol with α,ω -*n*-alkane diacids with 6-, 8-, 9-, 10-, and 13-carbon chain lengths was C13 = C10, where HiC showed little activity for C6, C8, and C9 diacid copolymerization. N435 displayed similar activity for all these diacid chain lengths. Thus, N435 has a broader substrate promiscuity than HiC-AO. This is most apparent for shorter chain length ω HA, diol, and diacid monomers. These trends were similarly observed for a series of small molecule esterification reactions. Comparison of HiC-AO- and N435-catalyzed C16-HA homopolymerization at 8 h gave polymers with M_n 40.4 and 25.5 kg/mol, respectively. Furthermore, HiC-AO- and N435-catalyzed copolymerization of 1,8-octanediol/C13-diacid polymerizations at 8 h gave polymers with M_n of 11.0 and 9.6 kg/mol, respectively.

Introduction

As biocatalysis continues to mature and evolve, researchers seek to gain a better understanding of the potential range of reactions for which enzymes can be useful. Some well-known benefits of enzyme catalysis are the following: (i) circumvents the use of potentially toxic metal catalysts, (ii) enables reactions to be performed at much lower temperatures than with their chemical counterparts, (iii) often provides extraordinary chemo-, regio-, and stereoselectivity. In addition, immobilized enzymes offer catalyst recyclability.^{1–4} In the field of cell-free enzyme-catalyzed polyester synthesis, the enzyme most often studied due to its high catalytic activity for an extraordinarily diverse range of monomeric substrates is Lipase B from *Candida antarctica* (CALB).² This enzyme, immobilized on Lewatit PMMA beads (N435), is commercially available from Novozymes (Bagsverd, Denmark). Our laboratory is pursuing new enzyme families that contribute unique catalytic activities for use in polymer synthesis and modification reactions. With respect to polyester synthesis, reactions leading to polymers include step-condensation and ring-opening polymerization.^{3,5} One such enzyme that has proven interesting for polyester synthesis by ring-opening and step-condensation reactions is the cutinase from *Humicola insolens* (HiC).^{6,7}

Cutin, found in the cuticle of higher plants, is an insoluble lipid-polyester composed of hydroxy and epoxy fatty acids, including 16-hydroxyhexadecanoic acid, 10,16-dihydroxyhexadecanoic acid, and 9,10,18-trihydroxyoctadecanoic acid, and contains one to three hydroxyl groups.^{8–11} One main role of the plant cuticle is to function as a transpiration barrier. Cutin

is thought to play a key role in plant protection because it is a barrier against the entry of pathogens which feed on the plant leaf.^{11,12} Certain pathogens (fungi and bacteria) are able to secrete cutinases that degrade cutin by hydrolyzing the ester bonds, resulting in release of cutin monomers.

Cutinases have a molecular weight around 22 kDa with highly conserved stretches, which include four cysteines, forming two disulfide bridges.^{13,14} X-ray crystallography studies of *F. solani pisi* cutinase (FsC) revealed it belongs to the α/β hydrolase fold superfamily. It is one of the smallest members of the serine hydrolase family and contains a Ser-His-Asp catalytic triad.^{13,15} Cutinase activity for small molecule biotransformations has been explored. In addition to cutin, they hydrolyze insoluble triglycerides and soluble esters such as *p*-nitrophenyl butyrate (*p*NPB) as well as agrochemicals containing one or more chiral centers.^{14,15} An extraordinary property of cutinases is their hydrolytic activity on poly(ethylene terephthalate), a relatively rigid polyester used as a synthetic fiber.^{16,17} HiC, studied herein, was shown by Ronkvist et al.¹⁸ to catalyze the hydrolysis of 250 μ m thick PET films in aqueous media at 70 °C giving 97% weight loss in 96 h, corresponding to a loss in film thickness of 30 μ m per day.

Previous work by our laboratory found that HiC, immobilized by physical adsorption on Lewatit, shows promising activity for a broad range of condensation and lactone ring-opening polymerization reactions.⁶ Condensation reactions were performed with 1% w/w HiC for 48 h, under vacuum (10 mm of Hg), in bulk at 70 °C. Copolymerization of 1,4-cyclohexanedimethanol (1,4-CHDM) and sebacic acid (C10-diacid) gave poly(CHDM-*co*-sebacate) with M_n and M_w/M_n values were 19000 and 1.7, respectively. A limited range of diacid and diol substrates were studied at one reaction time (48 h). In another

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report⁷ was described a kinetic and mechanistic investigation of HiC-catalyzed ϵ -caprolactone (CL) ring-opening polymerizations (ROP).⁷ As above, HiC was physically immobilized on Lewatit. A linear kinetic plot of $\ln([M]_0/[M]_t)$ versus time ($r^2 = 0.99$) for bulk HiC-catalyzed CL ROP suggested chain termination did not occur and the propagation rate is first order with respect to monomer concentration. A linear plot of M_n versus fractional CL conversion to 90% is consistent with a chain-end propagation mechanism. Also, reactions occurred optimally at 70 °C, while lack of polymerizations at 80 and 90 °C suggest that denaturation occurred.

This paper builds on our communication that first described HiC's activity for polyester synthesis. As the above referenced work focused on HiC-catalyzed ROP reactions with an emphasis on kinetic and mechanistic aspects, this paper expands on HiC's activity for step-condensation polymerization reactions using a systematic series of monomers differing in length of methylene spacers between hydroxyl or acid reactive groups. Instead of physically immobilizing HiC on Lewatit as in previous work by our laboratory,^{6,7} covalent immobilization on resins with epoxide moieties was explored and a suitable carrier (Amberzyme oxirane, AO, beads) was identified. To define HiC specificity during step condensation polymerization reactions, three series of monomers were studied: (i) ω -hydroxyalkanoic acids (HAs) with 6, 10, 12, and 16 carbons, (ii) copolymerizations of sebacic acid (C10-diacid, C₁₀H₁₈O₄) with α,ω -*n*-alkane diols with 3-, 4-, 5-, 6-, and 8-carbon chain lengths, and (iii) copolymerizations of 1,8-octanediol with α,ω -*n*-alkane diacids with 6-, 8-, 9-, 10-, and 13-carbon chain lengths. HiC selectivity was evaluated by monitoring increases in polymer chain length versus reaction time as a function of monomer structure. Similarities and differences in activity and selectivity between CALB and HiC were determined by performing all reactions above using immobilized CALB catalysis (N435) under identical conditions (in bulk, in vacuo, 70 °C).

Experimental Section

Materials. 10-Hydroxydecanoic acid (C10-HA), 12-hydroxydodecanoic acid (C12-HA), adipic acid (C6), brassylic acid (C13), propanediol (C3), and butanediol (C4) were purchased from Sigma-Aldrich, while 16-hydroxyhexadecanoic acid (C16-HA), sebacic acid (C8), azelaic acid (C9), 1,5-pentanediol (C5), and 1,6-hexanediol (C6) were purchased from Acros. 1,8-Octanediol (C8-diol) and sebacic acid (C10-diacid) were purchased from TCI and 6-hydroxyhexanoic acid was synthesized in our lab from ϵ -caprolactone following a literature procedure.¹⁹ Novozym 435 (specific activity 10500 PLU/g), abbreviated as N435, was a gift from Novozymes (Bagsvaerd, Denmark) and consists of *Candida antarctica* Lipase B (CALB) physically adsorbed within the macroporous resin Lewatit VPOC 1600 [poly[methyl methacrylate-co-butyl methacrylate], supplied by Bayer). N435 contains 10 wt % CALB that is located on the outer 100 μ m of 600 μ m average diameter Lewatit beads.^{18,20} HiC was a kind gift from Novozymes (Bagsvaerd, Denmark) received in liquid media. Amberzyme oxirane (AO) resin was a kind gift of Rohm & Haas/Ion Exchange Resins (Philadelphia, PA). Resindion EC-EP was a kind gift of Resindion (Binasco, Italy). All other solvents and reagents were obtained commercially at the highest purity available and used without further purification.

Covalent Immobilization of HiC on Amberzyme Oxirane (AO) Beads. HiC was first purified by several cycles of ultrafiltration [Millipore model 2000 high-performance Ultrafiltration Cell] through a 10 kDa membrane and lyophilized. Enzyme purity, determined by SDS-PAGE, showed it was >95% pure. Lyophilized HiC (50 mg) was dissolved in 20 mL of potassium phosphate buffer (0.1 M, PH 7.8). Various epoxy-based resins (500 mg) were first activated with EtOH

and then added to the 20 mL of enzyme solution 2.5 mg/mL HiC. Immobilization was performed in 50 mL polypropylene tubes at room temperature, with shaking (150 rpm), for 48 h. Subsequently, the supernatant was removed, beads were washed 3 times with 10 mL of potassium phosphate buffer (0.1M, PH 7.8) to remove loosely bound protein, and the resin was dried by lyophilization for 24 h. From aliquots taken both prior to and after HiC immobilization, HiC concentration was measured by the BCA method to determine loading on the beads. Below, HiC immobilized on AO resin will be abbreviated as HiC-AO.

Synthetic Methods. Homopolymerization of ω -Hydroxyalkanoic Acids (HAs). HA (300 mg) and diphenylether (1.2 mL) were transferred to reaction tubes (100 \times 15 mm) that were placed in a Radley Greenhouse Plus Parallel Synthesizer (Brinkmann). Contents of reaction tubes were maintained at 100 °C for 30 min with magnetic stirring to obtain a monophasic solution. Subsequently, the temperature was reduced to 70 °C and immobilized catalyst (HiC-AO or N435, 1% w/w protein relative to monomer) was added. After 2 h, vacuum (10 mg/Hg) was applied. Aliquots were removed after 15 min, 30 min, 1, 2, 4, 8, and 24 h and prepared for GPC analysis. Reaction products were isolated by addition of chloroform, removing immobilized enzyme by filtration and rotoevaporation of solvent.

General Method for Diol/Diacid Condensation Copolymerizations. Diol (2 mM) and diacid (2 mM) were transferred to reaction tubes (140 \times 25 mm) that were placed in an Eyela Process Station PPS-5510 (Tokyo Rikakikai Co., Ltd.). Contents of reaction tubes were maintained at 115 °C for 30 min with magnetic stirring to obtain a monophasic solution. Subsequently, the temperature was reduced to 70 °C and immobilized catalyst (HiC-AO or N435, 1% w/w protein relative to monomer) was added. After 2 h, vacuum (20 mg/Hg) was applied. Aliquot removal, preparation of samples for GPC, and isolation of products follows exactly as described above for poly(HA) synthesis.

General Method for Small Molecule Esterification Reactions. Alcohol (2 mM), acid (2 mM), and diphenylether (1:1 ratio to monomer weight) were transferred to reaction tubes (100 \times 15 mm) that were placed in a Radley Greenhouse Plus Parallel Synthesizer (Brinkmann). Contents of reaction tubes were maintained at 70 °C for 30 min with magnetic stirring to obtain a monophasic solution. Subsequently, immobilized catalyst (HiC-AO or N435, 1% w/w protein relative to monomer) was added. Aliquots were removed after 30 min and 24 h and prepared for gas chromatography (GC)-mass spectrometry (MS) analysis.

Instrumental Methods. Gel Permeation Chromatography (GPC). The number- and weight-average molecular weights (M_n and M_w , respectively) were determined by size exclusion chromatography using the identical HPLC system described elsewhere.⁷ Trisec GPC software version 3 was used for calculations. Chloroform was used as eluent with a flow rate of 1.0 mL min⁻¹ at room temperature. Sample concentrations of 5 mg/mL and injection volumes of 35 μ L were used. Molecular weights were determined on the basis of a conventional calibration curve generated by narrow molecular weight polystyrene standards obtained from Aldrich Chemical Co.

GC-MS Analyses. Conversions of small molecule esterification reactions were determined by GC-MS. Analyses were performed at 70 eV using a ThermoFinnigan TraceGC Ultra gas chromatograph coupled with a Trace DSQ mass spectrometer. GC-MS analyses were performed with injector, ion source, and interface temperatures of 200, 250, and 280 °C, respectively. Samples in hexane (1 μ L) were injected in PTV split mode and run on a capillary column (Varian CP8944 VF-5MS, 0.25 mm \times 0.25 μ m \times 30 m). The oven temperature was programmed at 120 °C for 1 min increasing to 300 at 20 °C/min and then maintained at 300 °C for 4 min. Standard esters were used to construct standard curves that allowed quantification of percent conversion values. These compounds were synthesized in an Eyela Process Station PPS-5510 (Tokyo Rikakikai Co., Ltd.) at 90 °C with N435 catalysis, in toluene, following the general method for small molecule esterification reactions given above. Ester standards were purified by silica column chromatography using a 10:1 mixture of *n*-hexane and ethyl ether as eluent.

Table 1. Physical and Chemical Characteristics of Various Resins Used for HiC Immobilization

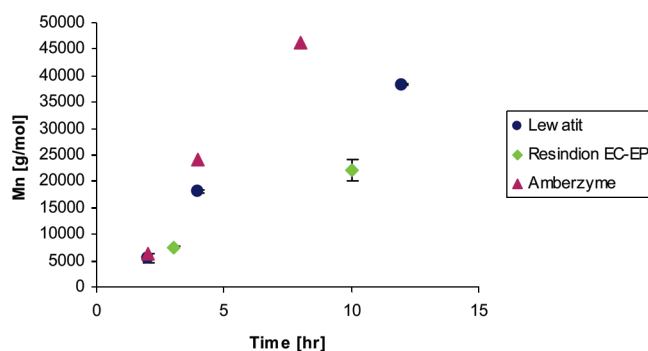
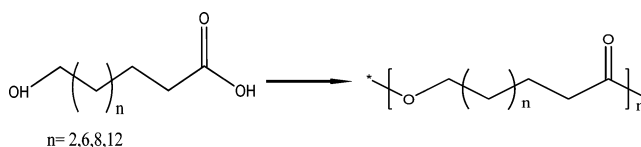
parameters	Lewatit POC 1600	Resindion EC-EP	Amberzyme oxirane
matrix polymeric material	methacrylate	methacrylate	acrylate
active group	none	epoxy	epoxy
particle size range (μm)	400–600	150–300	220
average pore radius (nm)	140–170	>30	100
enzyme loading (% by wt)	10.0	14.0	9.14

Results and Discussion

Immobilization of HiC. Resins bearing epoxy functionalities for covalent immobilization of HiC were investigated. Prior to immobilizations, HiC in >95% purity was obtained by multiple ultrafiltration cycles. Immobilizations were performed in potassium phosphate buffer (0.1 M, pH 7.8) for 48 h. Epoxy resins were first activated with ethanol and the ratio of resin to HiC was fixed at 10:1 w/w. Bead loading with HiC was determined by changes in protein content in media both prior to and after immobilization. Physical and chemical features of macroporous supports Lewatit VPOC 1600, Lewatit VPOC is shown in Table 1.

Activities of immobilized HiC preparations were assessed by their catalysis of ω -hydroxyhexadecanoic acid homopolymerizations (see Figure 1). Reactions were performed in diphenylether at 70 °C with 1% by wt HiC relative to monomer. All three resins, two of which possessed epoxy functionalities for covalent immobilization of HiC, resulted in active immobilized HiC preparations. Increase in M_n as a function of time was most rapid using Amberzyme oxirane (AO) and was slowest using Resindion Sepabeads. Thus, HiC-AO, which provides covalent immobilization of HiC, was more active than HiC physically immobilized on Lewatit. Covalent relative to physical immobilization of an enzyme is expected to better retain the enzyme (i.e., less enzyme leaching) over multiple reaction cycles. Given the above, HiC immobilized on AO (HiC-AO) was used for all substrate selectivity studies described below.

Although previous studies by our laboratory found 70 °C to be a preferred temperature to conduct polymerizations with HiC immobilized on Lewatit, activity as a function of temperature was reinvestigated for HiC-AO. Studies performed using a 1,8 octanediol/sebacic acid copolymerization in bulk at temperatures ranging from 60 to 90 °C showed that optimal HiC-AO activity is attained at 70–75 °C. HiC-AO showed little or no activity

**Figure 1.** Comparison of different immobilized HiC preparations for catalysis of ω -hydroxyhexadecanoic acid homopolymerizations conducted in diphenyl ether at 70 °C with 1%-by-wt HiC relative to monomer.**Scheme 1.** HiC-AO- and N435-Catalyzed Homopolymerization Reactions of ω -Hydroxyalkanoic Acids

when the reaction temperature was increased to 80 °C. Therefore, in this study, 70 °C was selected as the temperature at which HiC-AO polymerizations were performed. For comparison with HiC-AO, N435-catalyzed polycondensation reactions were also performed at 70 °C.

The discussion below describes three sets of experiments comprised of variation in chain length of either the diol, diacid, or HA monomer. The corresponding chain length selectivity and activity for HiC-AO and N435 are compared. With the exception of ω -hydroxyalkanoic acid (HA) polymerizations that were performed in diphenyl ether, all other studies were performed without solvent addition.

Homopolymerization of ω -Hydroxyalkanoic Acids (HAs).

In the first set of experiments, ω -HAs with 6, 10, 12, and 16 carbons were used as monomers for HiC-AO- and N435-catalyzed homopolymerization reactions (See Scheme 1).

Figure 2a shows that HiC-AO is highly active for polymerizations of C16- ω HA. For polymerizations of C12- ω HA, a lag period for up to 2 h was observed. However, by 6 and 10 h reaction times, poly(C12- ω HA) and poly(C16- ω HA) reached similar M_n values (at 10 h, 32.1 and 40.3 kg/mol, respectively). Polydispersity (M_w/M_n) values were likewise similar at 1.7 and 1.4, respectively. By further decreasing the monomer chain length by only two carbons (C10- ω HA), polymer formation was not observed. Similarly, HiC-AO was not active for polymerizations of C6- ω HA. Figure 2b displays the results of N435-catalyzed polycondensation reactions with the same set of ω HA monomer substrates. N435-catalyzed polymerization of C16- ω HA and C12- ω HA occur at similar rates giving polyesters with nearly identical M_n values (25.5 kg/mol and 24.5 kg/mol, respectively, at 8 h). Again, polydispersity was likewise similar at 2.0 and 2.3, respectively. Furthermore, whereas HiC-AO was inactive for C10- ω HA polymerizations, N435 accepts C10- ω HA as a substrate, forming poly(C10- ω HA) with M_n values of 5.0 and 11.1 kg/mol at 1 and 8 h, respectively (PDI 1.4 for both time points). However, as was observed with HiC-AO, N435 was inactive for polymerization of C6- ω HA. Thus, HiC-AO's activity for ω HA substrates was as follows: C16 > C12, where C10- ω HA and C6- ω HA were not polymerized. In contrast, N435's activity for ω HA substrates was as follows: C16 = C12 > C10, where C6- ω HA was not polymerized. In a set of control experiments where no catalyst was used, M_n values of under 1 kg/mol were attained for each of the substrates.

Copolymerization of Sebacic Acid with Diols of Varying Chain Lengths.

For this second set of experiments, polymerizations were performed in bulk. Sebacic acid and diols with chain lengths of 3, 4, 5, 6, and 8 carbons were used as monomers for HiC-AO- and N435-catalyzed copolymerization reactions (see Scheme 2a).

Figure 3a shows that HiC-AO is active for copolymerizations of both C6- and C8-diols, although activity is higher for the latter.

Indeed, by 2 h, M_n values for poly(hexylsebacate) and poly(octylsebacate) are 1.7 and 6.6 kg/mol, respectively (PDI 1.5 and 1.7, respectively), and at 8 h, M_n values are 6.5 and 10.2 kg/mol, respectively (PDI 1.9 and 2.9, respectively). By

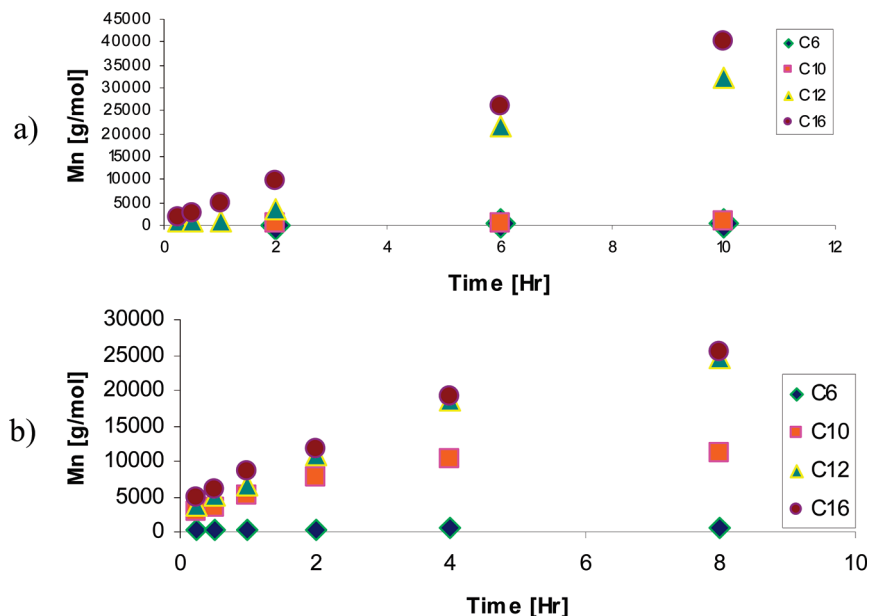
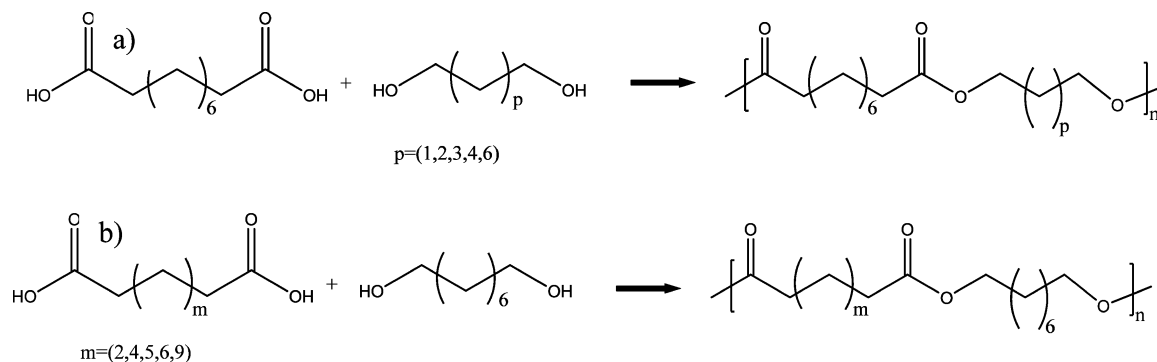


Figure 2. Enzyme-catalyzed polyesterification of linear aliphatic ω -hydroxyacids (HA; 70 °C, in diphenylether, 1 wt %/wt enzyme HiC-to-monomer, with application of vacuum after 2 h): (a) HiC-AO catalysis, extent of M_n as a function of time; (b) N435 (immobilized CALB) catalysis, M_n as a function of time.

Scheme 2. HiC-AO- and N435-Catalyzed Copolymerizations of (a) Sebacic Acid and α,ω -*n*-Alkane Diols of Varying Chain Lengths, (b) 1,8-Octanediol with α,ω -*n*-Alkane Diacids of Varying Carbon Chain Lengths



decreasing the diol carbon chain length by only one methylene unit, from C6 to C5, HiC-AO no longer converted these substrates to polyesters. Similarly, C4 and C3 diols were not copolymerized with sebacic acid by HiC-AO. Figure 3b displays results of N435-catalyzed polycondensation reactions with the same set of diol monomer substrates. In contrast to HiC-AO, N435 is active on diols of all chain lengths. Diol monomers with C5, C6, and C8 chain lengths are polymerized at similar rates, hence N435 does not differentiate between these substrates. Copolymerizations of C3 and C4 diols proceed relatively slower. By 1 h, M_n values for copolymerizations of sebacic acid with C3, C4, C5, C6, and C8 diols are 2.0, 3.0, 3.8, 4.0, and 4.2 kg/mol, respectively, with PDI values of 1.9, 1.7, 1.6, 1.7, and 1.8, respectively. Thus, for HiC-AO, its activity for diol substrates was as follows: C8 > C6, where C3, C4, and C5 diols were not polymerized. In contrast, N435 showed greater substrate promiscuity such that all diol chain lengths studied were converted to polyesters. N435's relative activity for diol substrates was as follows: C8 = C6 = C5 > C4 > C3. In a set of control experiments where no catalyst was used, M_n values of under 1 kg/mol were attained for each of the substrates.

Copolymerization of 1,8-Octanediol with Diacids of Varying Chain Lengths. Reactions were performed without solvent, at 70 °C, under vacuum as above. 1,8-Octanediol and diacids

with chain lengths of 6, 8, 9, 10, and 13 carbons were used as monomers for HiC-AO- and N435-catalyzed copolymerization reactions (see Figure 4). Figure 4a shows HiC-AO has similar activity for C10 and C13 diacids. For 2 h, M_n values for poly(octylsebacate) and poly(octylbrassyate) are 6.6 and 5.1 kg/mol, respectively, while at 8 h, M_n values are 10.2 and 11.1 kg/mol, respectively (PDI 2.9 and 1.8, respectively). In agreement with the above results, HiC-AO activity for polyester synthesis is extraordinarily sensitive to diacid chain-length. HiC-AO showed little activity for conversion of C9-diacid to polyester, whereas, for C10-diacid, polyester synthesis was rapid. In accord with the low activity found for C9-diacid, a further decrease in diacid chain length to C8 and C6 resulted in similarly low activity for conversion to polyester. Figure 4b displays the results of N435-catalyzed polycondensation reactions with the same set of diacid monomer substrates. In agreement with studies of diols with differing chain lengths, N435 shows very different selectivity than HiC-AO as it is active on diacids of all chain lengths studied herein. By 1 h, M_n values for N435-catalyzed copolymerizations of 1,8-octanediol with C6, C8, C9, C10, and C13 diacids are 4, 4, 5, 5, and 6 kg/mol, respectively. Therefore, N435 does not differentiate between these substrates even though they differ in chain length by up to seven carbons. Thus, for HiC-AO, its activity for diacid

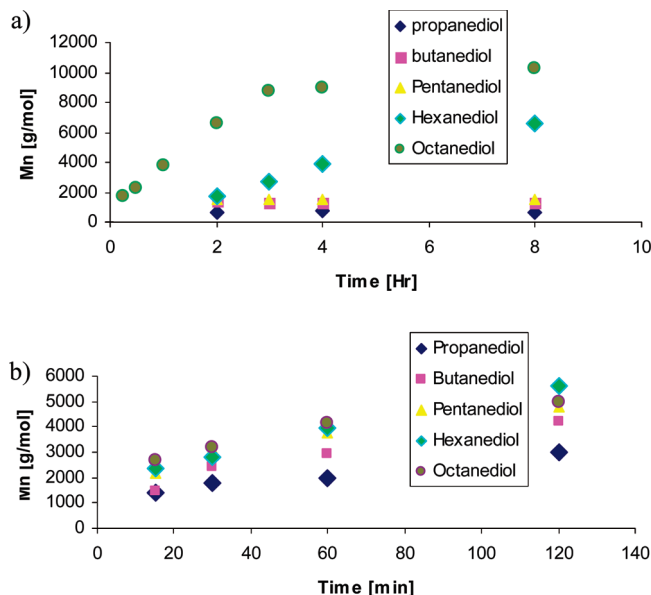


Figure 3. Enzyme-catalyzed polyesterification of linear diols with sebacic acid (70 °C, in bulk, diol diacid 1:1 mol/mol, 1% w/w of enzyme-to monomer, application of vacuum after 2 h): (a) HiC-AO catalysis, M_n as a function of time; (b) N435 catalysis (immobilized CALB), M_n as a function of time.

substrate copolymerization with 1,8-octanediol was as follows: C13 = C10, where C6, C8, and C9 diacids showed little activity for copolymerization. N435 showed greater substrate promiscuity such that all diacid chain lengths studied were converted to polyesters. Furthermore, within experimental error, N435 copolymerizations in Figure 4b proceeded with equivalent rates of M_n increase as a function of reaction time. In the set of control experiments where no catalyst was used, M_n values of under 1 kg/mol were once again attained for each of the substrates.

Small Molecule Model System: Esterification of Lauric Acid with *n*-Alkanols of Varying Chain Lengths. This work compares the chain length selectivity of HiC-AO and N435 for a series of small molecule esterification reactions and determines the extent that this selectivity correlates with that observed above for polycondensation polymerizations. Reactions were performed in diphenylether (1:1 ratio) in capped tubes at 70 °C. Lauric acid and *n*-alkanols with chain lengths of 3, 4, 5, 6, 8, and 12 carbons were used as substrates for HiC-AO- and N435-catalyzed esterification reactions.

Results of reactions conducted for 30 min are displayed in Figure 5a. HiC-AO shows a preference for the C6 alcohol relative to alcohols of longer and shorter chain length. Percent conversion for HiC-AO-catalyzed esterifications between lauric acid and 3-, 4-, 5-, 6-, 8-, and 12-carbon *n*-alkanols are 18, 37, 36, 72, 38, and 6, respectively. Comparison to N435-catalyzed esterifications between lauric acid and *n*-alkanols of differing chain length shows similar trends. Hence, hexanol is the preferred substrate and alcohols of shorter and longer chain lengths reach lower conversion values. Percent conversion for N435-catalyzed esterifications between lauric acid and 3-, 4-, 5-, 6-, 8-, and 12-carbon *n*-alkanols are 41, 63, 58, 84, 73, and 46, respectively. For all *n*-alkanol chain lengths, N435 catalysis results in higher ester %-conversion values. Thus, N435 has higher reactivity than HiC-AO for this series of substrates. Furthermore, consistent with results discussed above for polycondensation reactions, N435 shows a broader specificity as a function of *n*-alkanol chain length. That is, using N435, the maximum difference in %-conversion between any two sub-

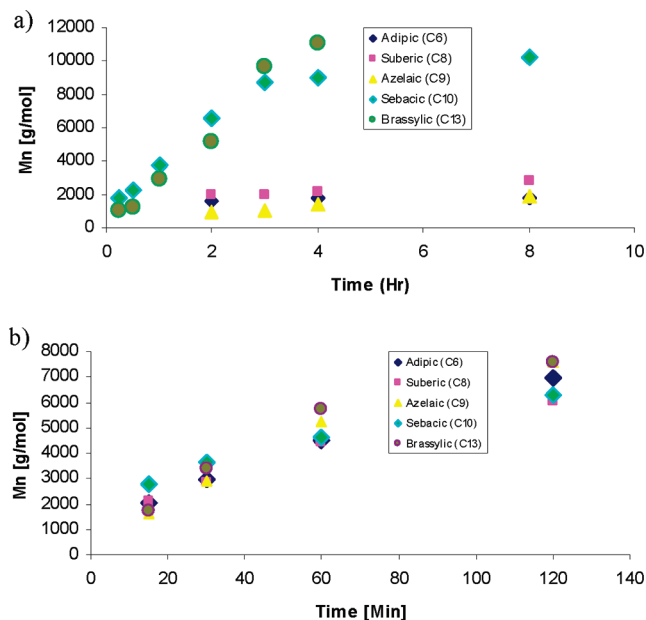


Figure 4. Enzyme-catalyzed polyesterification of 1,8-octanediol and linear diacids (70 °C, in bulk, diol diacid 1:1 mol/mol, 1% w/w of enzyme-to monomer, application of vacuum after 2 h): (a) HiC-AO catalysis, extent of M_n as a function of time; (b) N435 catalysis (immobilized CALB), M_n as a function of time.

strates is a factor of 2× whereas, for HiC-AO, a difference in %-conversion of 12x is observed between *n*-hexanol and *n*-dodecanol. Comparison between HiC-AO catalysis as a function of alcohol chain length for the small molecule model system and polymerization results in Figure 2a shows interesting contrasts. Whereas *n*-propanol and *n*-butanol show significant extents of esterification over 30 min reactions with lauric acid, 1,3-propane diol and 1,4-butanediol show no significant chain growth over 8 h. Furthermore, whereas *n*-hexanol reacts more rapidly than *n*-octanol with lauric acid, chain growth occurs much more rapidly for copolymerizations between sebacic acid and 1,8-octanediol relative to 1,6-hexanediol. In contrast, there is good agreement found when comparing N435 catalysis as a function of alcohol chain length for the small molecule model system and polymerization results (Figure 2b). For polycondensations between sebacic acid and *n*-alkanediols, as the chain length increases from C3 to C4 and C6, there is an increase in the rate at which chain growth occurs. Furthermore, C6 and C8 *n*-alkanediols form polyester with sebacic acid at identical rates. Similarly as *n*-alkanol chain length increases from C3 to C4 and C6, esterification with lauric acid occurs to higher extents. Moreover, C6 and C8 *n*-alkanols form ester with lauric acid at similar rates.

Small Molecule Model System: Esterification of Hexanol with *n*-Alkanoic Acids of Varying Chain Lengths. In addition to the above model, studies between *n*-alkanols and lauric acid, a series of esterification reactions catalyzed by HiC-AO and N435 were performed where hexanol was reacted with *n*-alkanoic acids having chain lengths of 6, 8, 10, 12, 14, and 16 carbons. Results of reactions conducted for 30 min are displayed in Figure 5b. HiC-AO shows similarly high reactivity with *n*-alkanoic acids having 8, 10, 12, and 14 carbons. However, a small decrease and increase in *n*-alkanoic acid chain length to C6 and C16, respectively, results in a large decrease in ester formation. Percent conversion for HiC-AO-catalyzed esterifications between *n*-hexanol and 6-, 8-, 10-, 12-, 14-, and 16-carbon *n*-alkanoic acids are 14, 70, 73, 70, 65, and 13%, respectively. Comparison to N435-catalyzed esterifications

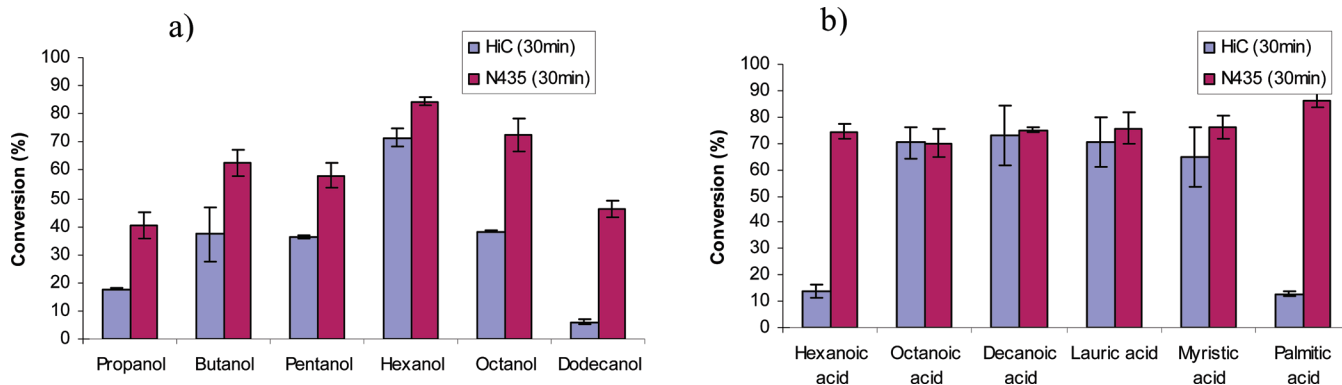


Figure 5. HiC-AO- and N435-catalyzed esterification reactions (30 min, 70 °C, in diphenylether, molar ratio of alcohol-to-acid is 1:1, 1%-by-wt enzyme to total monomer substrates): (a) extent conversion of reactions between lauric acid and *n*-alkanols of varying chain length; (b) extent conversion of reactions between hexanol and *n*-alkanoic acids of varying chain length.

between *n*-hexanol and *n*-alkanoic acids of differing chain length shows that, unlike HiC-AO, N435 does not differentiate between any of the *n*-alkanoic acid chain lengths studied. Percent conversion for N435-catalyzed esterifications between hexanol and 6-, 8-, 10-, 12-, 14-, and 16-carbon *n*-alkanoic acids are 74, 70, 75, 76, 76, and 83%, respectively. Thus, consistent with results discussed above for polycondensation reactions, N435 shows a broader specificity as a function of *n*-alkanoic acid chain length. That is, using N435, there is little difference in %-conversion between any two substrates, whereas, for HiC-AO, a difference in %-conversion of 5× is observed between decanoic acid and both *n*-hexanoic and palmitic acids. Comparison between HiC-AO catalysis as a function of *n*-alkanoic acid chain length for the small molecule model system and polymerization results in Figure 4a shows interesting contrasts. Whereas octanoic acid showed significant extent of esterification over 30 min reactions with *n*-hexanol, suberic acid (C8)/1,8-octanediol copolymerization showed no significant chain growth over 8 h. However, there is excellent agreement for HiC-AO-catalyzed esterification of C6 and C10 *n*-alkanoic acids and polyester synthesis with corresponding C6 and C10 diacids. That is, the C6 *n*-alkanoic acid and diacid (adipic acid) were poor substrates, whereas the C10 *n*-alkanoic acid and diacid (sebacic acid) were very good substrates for ester and polyester synthesis, respectively. In contrast, when comparing N435 catalysis as a function of *n*-alkanoic acid and diacid chain lengths for the small molecule model system (Figure 5b) and polymerization reactions (Figure 4b), respectively, excellent agreement is found. That is, for polycondensations between 1,8-octanediol and diacids of chain lengths C6 (adipic) to C13 (brassylic acid), all diacids were good substrates with similar rates of molecular weight increase. Similarly, for *n*-alkanoic acids of chain lengths C6–C14, esterification reactions with *n*-hexanol occurred to similar extents (~70%). Again, these results for small molecule and polyester synthesis for HiC and N435 catalysis with *n*-alkanoic acid and diacids of different chain lengths supports the generally broader range of substrates accepted by N435.

It is not surprising to discover there are differences between chain length selectivity for small molecule model systems and polycondensation reactions. Such variations may originate due to inherent differences in model and polycondensation reactions. Small molecule condensation reactions have well-defined acyl donor and acceptor species. In contrast, after conversion of monomers to dimers and higher molecular weight species, polycondensation reactions may have an array of acyl acceptor and donor species differing in oligomer and polymer chain lengths. Furthermore, small molecule substrates are monofunc-

Table 2. M_n and PDI (M_w/M_n) Values for Homopolymerizations of C16- ω HA Catalyzed by HiC-AO and N435

reaction time	HiC-AO catalysis		N435 catalysis	
	M_n (g/mol)	PDI	M_n (g/mol)	PDI
15 min	1800	1.3	4800	1.5
30 min	2500	1.5	5900	1.5
1 h	4700	1.4	8600	1.5
2 h	9700	1.4	11700	1.7
4 h	26000	1.4	19100	2.0
8 h	40400	1.5	25500	2.0

tional *n*-alkanol and *n*-alkanoic acids, whereas polyester synthesis is performed with difunctional acid and hydroxyl monomers. Nevertheless, determining the extent of agreement between small molecule esterification and polycondensation reactions as a function of precursor chain length is important since similarities in substrate specificities can greatly simplify investigations by modeling to explain enzyme selectivity. For this study, what is undoubtedly true is that comparisons of N435 and HiC-AO first for polycondensation reactions and then for small molecule substrates differing in chain length provides insights into similarities and deviations in substrate selectivity.

Further Comments on Relative Activities of HiC and N435 for Polycondensation Reactions. Review of results above show that, for both N435 and HiC-AO, the preferred diacid, diol, and ω -hydroxyalkanoic acid substrates are brassylic acid (C13-diacid), 1,8-octanediol, and ω -hydroxyhexadecanoic acid (C16- ω HA), respectively. To facilitate careful comparison of N435 and HiC-AO activities using these preferred substrates, M_n and M_w/M_n (PDI) values are listed in Tables 2 and 3 for C16- ω HA homopolymerizations and copolymerizations of 1,8-octanediol with C13-diacid, respectively. For both polymerization reactions, N435 catalyzes more rapid chain growth from 0 to 1 h. Values of M_n at 1 h for N435- and HiC-AO-catalyzed C16-HA homopolymerizations are 8.6 and 4.7 kg/mol, respectively. Similarly, using these catalysts, M_n values at 1 h for 1,8-octanediol/C13-diacid copolymerizations are 5.7 and 3.0 kg/mol, respectively. For both the C16-HA and 1,8-octanediol/C13-diacid polymerizations, observation of Tables 2 and 3 shows that, from 2 to 8 h, chain growth occurs more rapidly using HiC-AO as catalyst. For example, for C16-HA polymerizations using HiC-AO and N435, the M_n increase from 2 to 8 h is 9.7 to 40.4 kg/mol and 11.7 to 25.5 kg/mol, respectively. PDI values for 1,8-octanediol/C13-diacid copolymerizations catalyzed by HiC-AO and N435 are similar and generally have values of about 1.7. However, PDI values for C16-HA polymerizations catalyzed by HiC-AO remain at ≤ 1.5 , whereas,

Table 3. M_n and PDI (M_w/M_n) Values for Copolymerizations of 1,8-Octanediol and Brassylc Acid Catalyzed by HiC-AO and N435

reaction time	HiC-AO catalysis		N435 catalysis	
	M_n (g/mol)	PDI	M_n (g/mol)	PDI
15 min	1000	1.6	1700	2.0
30 min	1200	1.7	3400	1.6
1 h	3000	1.3	5700	1.8
2 h	5000	1.7	6800	1.7
4 h	9600	1.8	8100	1.9
8 h	11000	1.8	9600	2.1

with N435 catalysis, PDI reaches 2.0 for products formed at 4 and 8 h, respectively. Narrow PDI values have previously been reported by our laboratory for both N435 and HiC catalysis^{7,21} and this phenomena was attributed to “chain selectivity” of these catalysts during propagation steps.

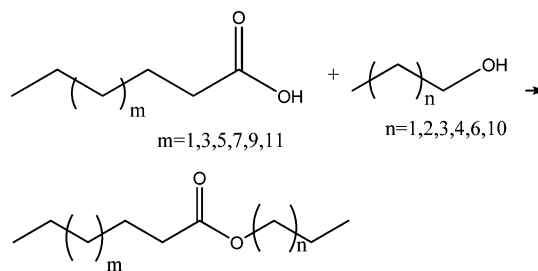
It is noteworthy that, during the first 2 h of reactions, vacuum is not applied. Hence, one explanation for more rapid chain growth at earlier and later reaction times with N435 and HiC-AO, respectively, may be differences in CALB and HiC activities as a function of reaction water content. HiC may have optimal activity at lower reaction water contents, whereas CALB may exhibit the opposite behavior. Indeed, previous work performed by our laboratory showed that, for N435-catalyzed reactions where no vacuum is applied, polymer chain growth is quite rapid at early reaction stages.²² To the best of our knowledge, there are no literature reports that describe the relationship between water concentration in organic media and its effect on HiC activity for ester formation.

Conclusions

HiC was successfully immobilized on Amberzyme oxirane resin giving an active catalyst system (HiC-AO) to assess HiC activity during polycondensation reactions between diol/diacid copolymerizations and ω -hydroxy fatty acid homopolymerizations. The chain length of these substrate types was varied to assess chain length selectivity of HiC-AO and to compare this with that of N435 (CALB immobilized on Lewatit beads). Results of experiments proved that HiC-AO has higher chain length selectivity than N435. For the series of ω HA substrates studied, N435 catalyzed the homopolymerization of 3 out of the 4 substrate chain lengths, all except the shortest, while HiC-AO was active on only the longer ω HAs. HiC-AO was likewise highly selective in catalyzing polycondensation reactions with diols of varying chain lengths. In contrast, N435 was active in polymerizing all diol chain lengths with only small differences in activity between the different chain length substrates. The same experiment, but with varying diacid chain lengths, showed unequivocally the high selectivity of HiC for longer chain length substrates, as HiC was highly active with C13 and C10 substrates but was not active with substrates with chain length below C10. For this substrate series, N435 was not selective, showing almost the same activity for all diacids used. The list of substrates for which HiC-AO showed little or no activity included (i) C6 and C10 ω HAs, (ii) C3, C4, and C5 diols for copolymerizations with C10-diacid, and (iii) C6, C8, and C9 diacids for copolymerizations with 1,8-octanediol. However, N435 was active on all these substrates except C6 ω HA.

Activities of HiC-AO and N435 for step-condensation reactions using longer chain length substrates C16-HA and

Scheme 3. HiC-AO- and N435-Catalyzed Esterifications (70 °C, in Diphenyl Ether) between (i) Lauric Acid and n -Alkanols with Chain Lengths of 3, 4, 5, 6, 8, and 12 Carbons, and (ii) Hexanol was Reacted with n -Alkanoic Acids Having Chain Lengths of 6, 8, 10, 12, 14, and 16 Carbons



1,8-octanediol/C13-diacid were compared. During the first 2 h of reactions, N435 more rapidly builds chains giving higher M_n s. However, after 2 h, the relative activities of these two catalysts reverse and, ultimately, higher molecular weight polyesters were obtained by HiC-AO catalysis. It is noteworthy that, during the first 2 h of polymerizations, reactions must contain relatively high water contents because vacuum was not applied during this period. Hence, differences in CALB and HiC activities at earlier and later stages of polymerizations may be due to reaction water content. HiC might require lower reaction water contents to have optimal activity, whereas CALB may exhibit the opposite behavior.

To further study affects of substrate chain length on HiC-AO and N435 activity, two series of model small molecule esterification reactions was studied where either the n -alkanol or the n -alkanoic acid chain length was varied. Despite inherent differences between model esterification reactions and polycondensation reactions, N435 showed greater substrate promiscuity for a majority of reactions performed, and conversion results from substrate to ester were similar for most substrate chain lengths. This is in contrast to HiC-AO which showed large differences in percent conversion of preferred and nonpreferred substrates.

Current efforts are aimed at gaining an understanding of structural origins of HiC and CALB active sites that lead to these large differences in substrates selectivity. Experiments are underway to obtain high quality HiC crystals so that HiC crystal structure can be elucidated. Once this is completed, modeling work will seek to ascertain structure–activity relationships that provide rational explanations for experimental results obtained herein.

Acknowledgment. We thank the National Science Foundation Industry/University Cooperative Research Center (NSF-I/UCRC) for Biocatalysis and Bioprocessing of Macromolecules at Polytechnic Institute of NYU for their financial support, intellectual input, and encouragement during the course of this research. I also thank Minmin Cai and Chunxiao Han for their assistance with GC/MS analysis.

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BM901272R