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Candida antarctica Lipase B Catalyzed Copolymerizations of Non-proteinogenic Amino Acids and Poly(Ethylene Glycol) to Generate Novel Functionalized Polyesters

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ABSTRACT

The condensation copolymerization of non-proteinogenic amino acid derivatives **1–9** with polyethylene glycol (PEG, Mw 600) (**10**), catalyzed by lipases in bulk is reported. Five different lipases (porcine pancreatic lipase, *Candida rugosa* lipase, *Candida antarctica* lipase, *Pseudomonas AY* lipase, and *Pseudomonas cepacia* lipase) were tested to catalyze the copolymerization of diethyl-α-acetamido, α-(3-trifluromethyl-benzyl)-malonate (**7**) with poly(ethylene glycol 600) (**10**) in bulk at 65°C. All other enzymes except *C. antarctica* lipase B (Novozyme-435) failed to produce the polymer. Therefore, *C. antarctica* lipase B was used for the synthesis of copolymers starting with non-proteinogenic amino acid derivatives **1–9** and PEG (Mw 600) (**10**) to give to

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polymers **11–19**, which were characterized by ^{1}H (1D and 2D) and ^{13}C NMR spectroscopic data. Light scattering photometry, as well as gel permeation chromatography were used to evaluate the particle size and molecular weights of the polymers. The effects of reaction parameters in chain extension have been systemically investigated. In principle, the method developed is flexible so that it can be used to generate a wide array of functionalized polyesters. In the absence of biocatalytic transformation, such structural control would be extremely difficult or currently impossible to obtain.

Key Words: Candida antarctica lipase B; Non-proteinogenic amino acids; Functionalized polyesters; Polyethylene glycols; Diethyl-α-acetamido; α-Alkylmalonates; Condensation polymerization; Transesterification; Novozyme-435; Lipases; Chain extension.

INTRODUCTION

Amino acids are well known for their wide natural occurrence and are one of the five major classes of natural products.[1–3] Among them, dicarboxylic amino acids have recently drawn attention because of their specific functions in biological systems. Aminomalonic acid (Ama) is the first representative example of the homologous α-amino dicarboxylic acid series, including important proteinogenic acids such as glutamic and aspartic acids. Recently peptide derivatives of Ama have invoked significant interest because of their possible physiological activities as enzyme inhibitors, i.e., of rennin and HIV-1 protease.[4] The Ama molecule has a prochiral center and all the derivatives, having different substituents at the two carboxyl groups, should be chiral. Obviously, the chiral moiety of the Ama derivatives might become an important stereocontrolling element if the Ama residue was incorporated in the peptide chain.

The importance of polymers in medical applications has been well recognized, one of the most widely researched area being their use as pharmaceutical carriers in drug delivery. A considerable amount of research has been directed towards the use of natural and synthetic polymers as polymeric drugs and drug delivery systems. Studies have largely been confined to two different areas of research. One utilizes the insoluble polymers for the development of controlled release systems in which the drug is physically adsorbed or entrapped within an insoluble polymeric matrix, while the other area of development includes soluble prodrugs or drug carriers where the drug is either chemically bound to the polymer backbone or is part of the backbone itself.[5,6]

Due to the exponential increase in interest in the area of enzyme-catalyzed organic reactions, we tried to carry out the synthesis of functionalized polymers using enzymes. More so, because many families of enzymes can be utilized for transformation of not only their natural substrates, but a wide range of unnatural substrates to yield a variety of useful compounds.[7–10] The use of enzymes in organic synthesis has many other advantages also, such as superior catalytic power and high selectivity under mild conditions with regard to temperature, pressure, and pH; promising substrate conversion efficiency; high diastereo-, enantio-, regio-, and chemoselectivities, as well as regulating stereochemistry, to provide development of new reactions.[11,12] The naturally occurring polymers are produced in vivo by enzymatic catalysis. The use of enzymes for the synthesis of useful polymers has

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been well exploited,[13–18] most of which are otherwise very difficult to produce by conventional chemical catalysts, as they require undesirable protection–deprotection steps. These features allow for the generation of functional compounds for pharmaceutical and agro-chemical sectors employing non-toxic natural catalysts with "green appeal." Additional advantages include catalyst recyclability and use in bulk reaction media avoiding organic solvents.[19–23]

As a part of our ongoing research program to develop highly biocompatible compounds/polymers[24–27] which can be utilized for such purposes, we have studied the copolymerization of Ama derivatives with poly(ethylene glycol) (PEG) using *Candida antarctica* lipase B. We used PEGs, because they are known to be biocompatible, non-toxic, and water soluble. All the polymers have been reported for the first time and have been characterized by different physical techniques.

EXPERIMENTAL

Materials

Candida antarctica lipase B (Novozyme-435), an immobilized enzyme, was a gift from Novozymes Inc., USA. *Candida rugosa* and porcine pancreatic lipase were purchased from Sigma Co. (USA). *Pseudomonas cepacia* and *Pseudomonas AY* lipases were purchased from Amano Co. (Japan). All other chemicals and solvents were of analytical grade and were used as received unless otherwise noted.

Instrumentation

Gel permeation chromatography (GPC) was used to determine the molecular weights, Mn (number average molecular weight), and molecular weight distributions, Mw/Mn, of polymer samples. Light scattering data were collected by a laser light scattering photometer (Wyatt Technology DAWN Model F) equipped with a 632 nm He–Ne laser as a light source. The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Instrument Inc. 250 MHz ARX spectrometer equipped with a Silicone Graphics station.

Preparation of Diethyl-α-alkanamidomalonates 1–4

The diethyl-α-alkanamidomalonates **1–4** (Sch. 1) were synthesized as per the literature procedure[28] by the reaction of diethyl malonate with sodium nitrite and acetic acid/propanoic acid/butanoic acid/heptanoic acid and their corresponding anhydrides, followed by the reduction and in situ N-acylation of the resulting diethyl isonitrosomalonates. These compounds were obtained as white crystalline solids in 80–85% yields. The compounds **1–3** are known in the literature[29] and were analyzed by comparing their spectral data with the reported data.

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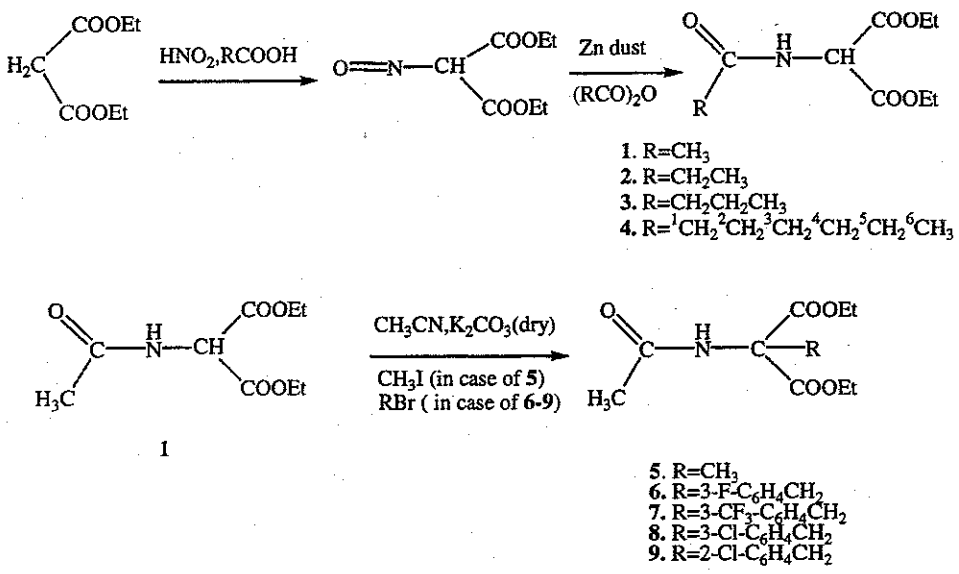

Scheme 1. Synthesis of diethylmalonate derivatives.

Diethyl-α-heptanamidomalonate (4)

^{1}H NMR Data ($CDCl_3$): δ 0.90 (t, 3H, C-6H), 1.20–1.40 (bm, 12H, C-3H, C-4H, C-5H, and 2 × OCH_2CH_3), 1.70 (m, 2H, C-2H), 2.30 (t, 2H, C-1H), 4.30 (q, 4H, 2 × OCH_2), 5.20 (d, 1H, -*C*HNH), 6.50 (d, 1H, NH).

^{13}C NMR Data ($CDCl_3$): δ 14.39 (C-6, 2 × OCH_2CH_3), 22.87 (C-5), 25.46 (C-4), 29.22 (C-3), 31.90 (C-2), 36.49 (C-1), 56.76 (NHCH), 62.98 (2 × OCH_2), 166.89 (CONH), 173.25 (2 × COO).

IR (cm^{-1}): 1723 (COO), 1672 (CONH, amide I), 1450 (CONH, amide II).

Preparation of Diethyl-α-acetamido, α-Alkylmalonates 5–9

The compounds **5–9** were obtained by the alkylation of diethyl acetamidomalonate (**1**) with alkyl halide (methyl iodide, 3-flurobenzyl bromide, 3-trifluromethyl benzyl bromide, 3-chlorobenzyl bromide, and 2-chlorobenzyl bromide) and ignited potassium carbonate in dry acetonitrile as crystalline solids in 55–59% yields and characterized by comparing their spectral data with those of the earlier reported compounds.[29]

General Method of Polymerization

To a mixture of the Ama derivatives **1–9** (1.0 mmol) and PEG (**10**, 1.0 mmol, Mw 600) taken in a round-bottom flask (25 mL), enzyme (10% by weight of monomers) was added and the reaction flask placed in a constant temperature oil bath maintained at a

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predetermined temperature under vacuum. The reaction was allowed to proceed for 48 h, after this time, it was quenched by adding chloroform and filtering off the enzyme under vacuum. The organic solvent was then evaporated under vacuum and the residue was re-dissolved in water and dialyzed using membrane (MWCO 6000). After the completion of dialysis, the product polymers **11–19** were obtained by freeze-drying. The structures of the polymers were characterized using NMR (^{1}H and ^{13}C) spectroscopy (Bruker 250 MHz) and the molecular weights of the polymer products were determined by GPC/light scattering techniques.

RESULTS AND DISCUSSION

Monomer Synthesis and Characterization

Nine diethyl-α-acetamidomalonate derivatives **1–9** were synthesized in three steps starting with isonitrosoylation reaction of diethylmalonates as shown in Sch. 1. The diethyl α-heptanamidomalonate (**4**) has been synthesized for the first time and characterized on the basis of its ^{1}H NMR, ^{13}C NMR, and IR spectral data.

Enzyme Screening

Screening was performed to select an enzyme that could successfully catalyze the copolymerization of non-proteinogenic amino acid derivatives **1–9** and PEG (Mw 600). Five different lipases (porcine pancreatic lipase, *C. rugosa* lipase, *C. antarctica* lipase, *P. AY* lipase, and *P. cepacia* lipase) were tested to catalyze the copolymerization of diethyl-α-acetamido, α-(trifluromethylbenzyl)-malonate (**7**), with PEG (Mw 600) (**10**) in bulk at 65°C. All other enzymes, except *C. antarctica* lipase B (Novozyme-435) failed to produce any polymer.

Polymerization Reaction

Scheme 2 represents the general strategy that was used for the synthesis of copolymers starting with non-proteinogenic amino acid derivatives **1–9** and PEG (Mw 600) (**10**). These monomers, which contain both the amide and ester functionalities, have been used for the first time in enzymatic polymerizations. *Candida antarctica* lipase was chosen to catalyze these copolymerization reactions, due to its high catalytic activity for ester synthesis as seen in enzyme screening, high thermal stability and the immobilization on the large surface area material.

The *C. antarctica* lipase B catalyzed condensation of **1–9** and PEG (**10**, Mn 600) under solvent-less conditions gave the polymers **11–19**. In the attempted polymerization without enzyme (control experiment), the monomers were recovered unchanged. Furthermore, no polymer formation was observed by using the deactivated *C. antarctica* lipase B. These data imply that these polymerization reactions proceeded through lipase catalysis.

The structures of the repeating units of the polymers were identified using ^{1}H and 2D (COSY) NMR data, Fig. 1 shows the ^{1}H and ^{13}C NMR spectra of PEG (Mw 600)-co-diethyl-α-acetamido, α-3-trifluromethylbenzylmalonate (**17**). In the ^{1}H NMR spectrum

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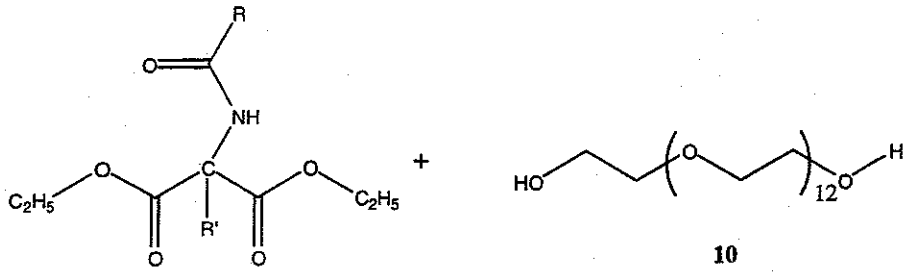
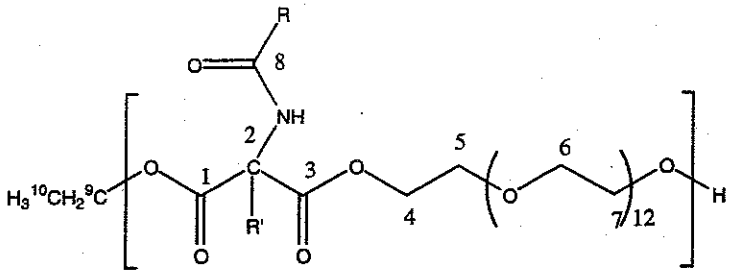


1. R=CH_3, R'=H
2. R=C_2H_5, R'=H
3. R=C_3H_7, R'=H
4. R=C_6H_{13}, R'=H
5. R= R'=CH_3
6. R=CH_3, R'=3-F-$C_6H_4CH_2$
7. R=CH_3, R'=3-CF_3-$C_6H_4CH_2$
8. R=CH_3, R'=3-Cl-$C_6H_4CH_2$
9. R=CH_3, R'=2-Cl-$C_6H_4CH_2$

Novozyme-435, Bulk
90°C, vacuum



11. R=CH_3, R'=H
12. R=CH_2CH_3, R'=H
13. R=$CH_2CH_2CH_3$, R'=H
14. R=$^{1'}CH_2{}^{2'}CH_2{}^{3'}CH_2{}^{4'}CH_2{}^{5'}CH_2{}^{6'}CH_3$, R'=H
15. R= R'=CH_3
16. R=CH_3, R'=3-F-$C_6H_4CH_2$
17. R=CH_3, R'=3-CF_3-$C_6H_4CH_2$
18. R=CH_3, R'=3-Cl-$C_6H_4CH_2$
19. R=CH_3, R'=2-Cl-$C_6H_4CH_2$

Scheme 2. Enzymatic synthesis of copolymers of diethylmalonate derivatives with PEG.

of **17**, the signal at δ 4.30–4.40 was assigned to C-4 protons and indicated the formation of ester linkages between the hydroxyl group of PEG (Mw 600) and ethoxy carbonyl moiety of **7**. The formation of ester was further confirmed by the disappearance of the signal at δ 4.20 (OCH_2) and δ 1.30 (OCH_2CH_3) as compared to that in the monomer **7**. The broad signal at δ 3.60–3.80 was assigned to the main chain protons of the PEG (Mw 600) units, i.e., C-6 and C-7. In the ^{13}C NMR spectrum, the signal at δ 167.44 was

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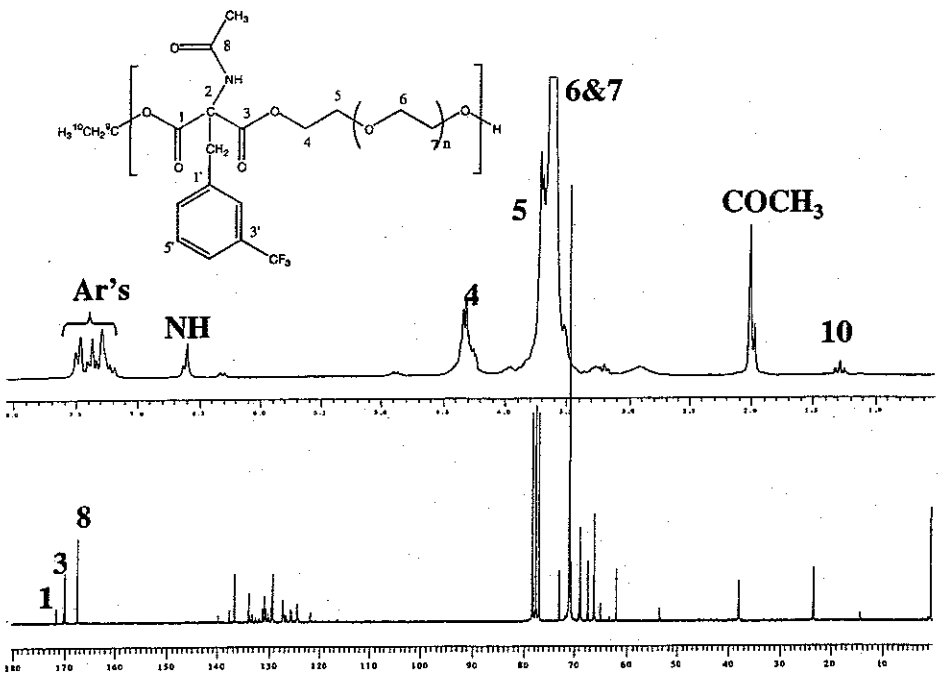

Figure 1. ^{1}H and ^{13}C NMR spectra of PEG600-co-diethyl-α-acetamido, α-3-trifluoromethylbenzyl malonate (**17**).

due to the amidic carbonyl, whereas the signals at δ 169.85 and 171.58 were assigned to ester carbonyls of the main chain ester and end group ester, respectively. The Mn of the polymers **11–19** were determined by GPC (Table 1).

Limiting Factors in Chain Extension

As shown during the enzyme screening procedure, *C. antarctica* lipase B was the most effective enzyme to carry out these copolymerization reactions. However, the Mn of the polymer from **7** and PEG (**10**) was much higher (34,000) as compared to that obtained (3000) by the reaction of **1** with PEG (**10**) performed in bulk with 10% (w/w) *C. antarctica* lipase B at 90°C. To further understand the limiting factors in chain extension, we evaluated the effects of various monomer structures on the enzymatic polymerization reaction.

It was observed that with the increase in chain length of the acyl group attached to the amine, the conversion and molecular weights of the polymers first increases, reaches a maximum value and then decreases (Table 1, entries **1–4**). In the case of **2** (having a propanoyl group), the highest molecular weight (Mn 7400) was obtained, whereas in the case of **4** (having a heptanoyl group), dimer and trimer formation was observed when reactions were performed under identical conditions. In the case of an acetyl group (**1**), a Mn of 3000 Da was obtained, indicating that in general the percentage conversion and the

Table 1. Percentage conversion and Mn of polymers **11–19**.

Entry	Polymer	^{1}H NMR ($CDCl_3$) ppm value	Mn (Dalton)	Yield
1	PEG600-co-diethyl-α-acetamidomalonate (**11**)	1.25 (t, C-10H), 2.05 (s, $COCH_3$), 3.00 (bs, NH), 3.50–3.70 (brs, C-6H & C-7H), 3.80 (brs, C-5H), 4.30 (q, C-9H), 4.35 (brs, C-4H), and 5.20 (d, C-2H)	3,000	10
2	PEG600-co-diethyl-α-propanamidomalonate (**12**)	1.20 (t, C-10H), 1.30 (t, CH_3), 2.30 (q, $COCH_2$), 3.50–3.70 (brs, C-6H & C-7H), 3.80 (brs, C-5H), 4.20 (q, C-9H), 4.30 (brs, C-4H), and 5.23 (d, C-2H)	7,400	22
3	PEG600-co-diethyl-α-butanamidomalonate (**13**)	0.95 (t, C-10H & CH_3), 1.79 (m, CH_2CH_3), 2.25 (q, $COCH_2$), 3.0 (bs, NH), 3.50–3.70 (brs, C-6H & C-7H), 3.80 (brs, C-5H), 4.0 (q, C-9H), 4.35 (brs, C-4H), and 5.25 (d, C-2H)	4,400	10
4	PEG600-co-diethyl-α-heptanamidomalonate (**14**)	0.90 (t, C-6′H), 1.15 (t, C-10H), 1.20–140 (bm, C-3′H, C-4′H & C-5′H), 1.60 (bm, C-2′H), 2.30 (m, C-1′H), 3.0 (bs, NH), 3.50–3.70 (brs, C-6H & C-7H), 3.80 (brs, C-5H), 4.20 (q, C-9H), 4.40 (brs, C-4H), and 5.25 (d, C-2H)	ND[a]	13
5	PEG600-co-diethyl-α-acetamido, α-methyl-malonate (**15**)	1.15 (t, C-10H), 1.70 (s, CH_3), 2.05 (s, $COCH_3$), 2.75 (bs, NH), 3.50–3.70 (brs, C-6H & C-7H), 3.80 (brs, C-5H), and 4.30 (q, C-9H & C-4H)	4,000	10
6	PEG600-co-diethyl-α-acetamido, α-3-fluorobenzyl-malonate (**16**)	1.30 (t, C-10H), 2.05 (s, $COCH_3$), 3.50–3.70 (brs, C-6H & C-7H), 3.75 (s, CH_2Ph), 3.80 (brs, C-5H), 4.25–4.45 (bm, C-9H & C-4H), 6.60 (bs, NH) and 6.75–7.20 (bm, Ar)	26,000	38
7	PEG600-co-diethyl-α-acetamido, α-3-trifluoromethylbenzylmalonate (**17**)	1.30 (t, C-10H), 2.00 (s, $COCH_3$), 3.50–3.70 (brs, C-6H & C-7H), 3.75 (s, CH_2Ph), 3.80 (brs, C-5H), 4.25–4.45 (bm, C-9H & C-4H), 6.60 (bs, NH), 7.15–7.50 (bm, Ar)	34,000	80
8	PEG600-co-diethyl-α-acetamido, α-3-chlorobenzyl-malonate (**18**)	1.25 (t, C-10H), 2.05 (s, $COCH_3$), 3.50–3.70 (brs, C-6H & C-7H), 3.75 (s, CH_2Ph), 3.80 (brs, C-5H), 4.20–4.40 (bm, C-9H & C-4H), 6.60 (bs, NH), 6.80–7.25 (bm, Ar)	4,400	75
9	PEG600-co-diethyl-α-acetamido, α-2-chlorobenzyl-malonate (**19**)	1.20 (t, C-10H), 2.10 (s, $COCH_3$), 3.50–3.70 (brs, C-6H & C-7H), 3.75 (s, CH_2Ph), 3.80 (brs, C-5H), 4.25–4.45 (bm, C-9H & C-4H), 6.60 (bs, NH), 6.85–7.7.20 (bm, Ar)	5,999	15

[a]Only dimer and trimers were formed.

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molecular weight of the product decreased with an increase in the chain length of the acyl group attached to the amine.

We have also studied the effect of alkyl/aryl groups directly attached to the prochiral center. It was observed that the presence of an alkyl group at the prochiral center results in an increased percentage conversion and Mn of the polymer formed. It was also observed that the monomers with aryl groups attached to the chiral center yielded polymers in higher percentage conversions compared to the alkyl groups, also the molecular weights in these cases are higher (Table 1, entries **1** and **5–9**). Among aryl groups, the one with fluoro substituents, gave very high molecular weights and percentage conversions (Table 1, entries 6 and 7) thus making them better substrates compared to those without fluoro substitutents (Table 1 entries **1, 8,** and **9**). The copolymerization with monomer **7** having a trifluoromethylphenyl group, resulted in the highest percentage conversion (80%) and Mn (34,000) among all the monomers studied.

Taking lead from monomers **1–9** and enzyme screening studies for the copolymerization reaction with PEG, temperature dependent studies were performed using the best substrate, viz. diethyl-α-acetamido, α-3-trifluoromethylbenzylmalonate (**7**) and *C. antarctica* lipase B; copolymers having higher molecular weights and low polydispersity values were obtained with an increase in reaction temperature from 90°C to 110°C.

CONCLUSION

Candida antarctica lipase efficiently catalyzed the polycondensation of non-proteinogenic amino acid derivatives **1–9** and PEG in a solvent-free system. The polymer molecular weight increased under reduced pressure and at higher temperature. The present reaction system affords a variety of biodegradable amphiphilic polymers via non-toxic enzymatic catalysis under mild reaction conditions without organic solvents, thus offering an environmentally benign "Green Technology."

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