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CAL-B Catalyzed Regioselective Bulk Polymerization of L-Aspartic Acid Diethyl Ester to α-linked Polypeptides

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This paper reports that the bulk polymerization of L-aspartic acid diethyl ester catalyzed by immobilized CAL-B at 80 °C for 24 h gives primarily (~95%) α-linked poly(L-aspartate) in 70% yield with DP_{avg} = 50 and regioselectivity (α/β) = 94:6. Plots of log{$[M]_0/[M]_t$} vs time and DP_{avg} vs conversion indicate that this polymerization proceeds in a controlled manner by a chain-growth mechanism up to 90% conversion. Thereafter, a competition occurs between chain growth and step mechanisms.

Poly(aspartic acid) is a water-soluble poly(amino acid) that is biodegradable in various bioactive environments.[1] It has a number of unique properties that result in its utility in a broad range of applications such as drug delivery, corrosion inhibitors and superadsorbants.[2-5] Established synthetic routes to poly(α-aspartic acid) include ring-opening polymerization (ROP) of the corresponding N-carboxyanhydride (NCA) monomer, protease-catalysis,[6] and solid phase peptide synthesis.[7] While protease-catalyzed polymerizations in bulk[6a] or organic/aqueous media[6b] have several advantages including reduced use of protection-deprotection steps and toxic chemical reagents, it results in oligomer mixtures with degree of polymerizations (DP) from about 9-19. Solid phase peptide synthesis (SPPS) is impractical for the synthesis of high molecular weight homo-polypeptides since it procedes stepwise using multiple protection-deprotection steps, solvents and toxic reagents. However, for short chains (2-30 residues), SPPS provides a reliable route to uniform sequence and chain length peptides. For the synthesis of high molecular weight poly(α-amino acid) including poly(α-aspartic acid), NCA ROP is the preferred method.[8] Though, NCA ROP requires the use of toxic reagents such as phosgene, protection-deprotection and high purity monomers. An alternative route to polyaspartate is by thermal or acid-catalyzed polymerizations.[3,9] However, the harsh conditions involved result in racemization and chains consisting of a complex mixture of α- and β-linked repeat units. This work describes for the first time a simple and environmentally friendly route to enantiopure poly(α-aspartic acid).

Important advantages of enzyme vs chemically catalyzed reactions include that enzymes: *i*) are of natural origin, *ii*) provide high enantio- and regio-selectivity, *iii*) function under mild conditions (e.g. temperature) and *iv*) do not require strict exclusion of water and oxygen.[10,11] Immobilized Lipase B from *Candida antarctica* (CAL-B) is known to efficiently catalyze polyester synthesis by polycondensation and ring-opening polymerization mechanisms.[11-15] Immobilized CALB has also been reported to catalyze the synthesis of polyamides by reaction of aliphatic and aromatic diesters and diamines.[16-18] Of particular interest herein is the regioselective amidation of aspartic acid at either the α- or β-carboxyl group. Conde *et al*. conducted a model study on the amidation of N-protected (benzyloxycarbonyl, *N*-Cbz; *tert*-butyloxycarbonyl, *N*-Boc; and acetyl, N-Ac) aspartic acid diethyl esters.[19] Reactions were performed in anhydrous diisopropyl ether at 60 °C using immobilized CAL-B as catalyst. Generally, the L-aspartate substrate series yielded α-monoamides whereas the corresponding D-aspartate substrates showed β-selectivity. The degree of regioselectivity was a function of the N-protecting group. The smallest protecting group gave the lowest α/β-ratio (3.8). It is well known that regioselectivity may also vary due to changes in the reaction media (e.g. solvent vs. bulk reactions) as well as when the substrate is no longer a simple monomeric substrate.[20]

Recently, Zhang et al investigated the solvent-free lipase-catalyzed synthesis of D- and L-polyaspartates.[21] At 80 °C for 72 hours using 20%-by-wt immobilized CAL-B, D- and L-polyaspartates were obtained with DP_{avg} of about 60 in 82% and 72% yield, respectively. Due to the presence of two different acyl moieties in aspartic acid diethyl ester, the repeat units may be linked between the α-amino and either the α- or β-carboxyl group (e.g. α-links, β-links or a mixture of both) (**Figure 1**). Based on 1D-^{1}H NMR, Zhang et al concluded that the polyaspartates obtained were predominantly β-linked (β/α = 96:4) although the chemical shifts

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and CD signs in chloroform were inconsistent with those of poly(β-aspartates) reported in the same solvent.[22] .

This paper reports a reinvestigatation of the regioselectivity of this interesting lipase-catalyzed synthesis. This includes a full structural characterization of the resulting poly(Et-L-aspartate) by 1D and 2D-NMR along with further studies to interrogate the mechanism of chain growth, reactions were performed at times from 2 to 24 h and plots of DP_{avg} versus monomer conversion and $\log[M]_0/[M]_t$ vs reaction time were constructed. Further characterizations of poly(Et-L-aspartate) synthesized herein was conducted by trifluoroacetic acid (TFA)-induced conformational changes monitored by NMR and CD. Also, the thermal properties of poly(Et-L-aspartate) prepared by lipase catalysis is reported.

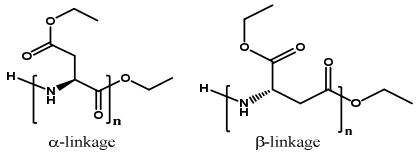

Figure 1. Chemical structures of poly(β-Et-α-aspartate) and poly(α-Et-β-aspartate) with repeat units linked through the α- (left) and β- (right) carboxylate moieties, respectively.

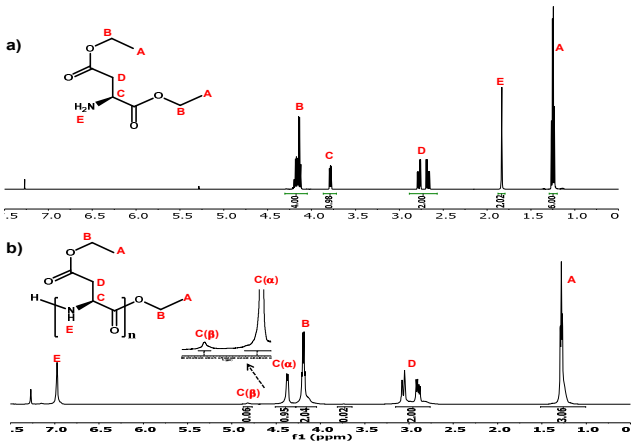

Figure 2. 1D ^{1}H NMR (600 MHz) in $CDCl_3$ of: (a) L-aspartic acid diethyl ester (L-Asp[OEt]$_2$) and (b) poly(Et-L-aspartate) obtained after 24 h reaction catalyzed by 20% immobilized CAL-B at 80 °C. [Can you improve the clarity of the x-axis ppm numbers]

The polymerization of L-aspartic acid diethyl ester (L-Asp(OEt)$_2$), using immobilized CAL-B (20%-by-wt), was carried out at 80°C for 24 hours. One-dimensional proton (^{1}H) NMR as well as 2D ^{1}H-^{13}C HMBC were used to assign the relative content of α- and β-links in the product. All signals were assigned based on 2D ^{1}H-^{1}H COSY and ^{1}H-^{13}C HSQC (**Figures SI-3** and **SI-4**). The 1D ^{1}H NMR spectrum of poly(Et-L-aspartate) obtained from the 24 hour reaction (**Figure 2b**) showed several signal shifts relative to the corresponding signals of the monomer (**Figure 2a**). Prominent examples include: *i*) the α-H (**C**) at 3.79 ppm of L-Asp(Et)$_2$ shifted downfield to 4.38 ppm (C_α) and 4.82 ppm (C_β), respectively (see below for discussion on assignments); ii) the free amine proton signal (**E**) of L-Asp(OEt)$_2$ at 1.83 ppm shifted downfield by ~ 7 ppm (8.18 ppm in DMSO-d_6, see

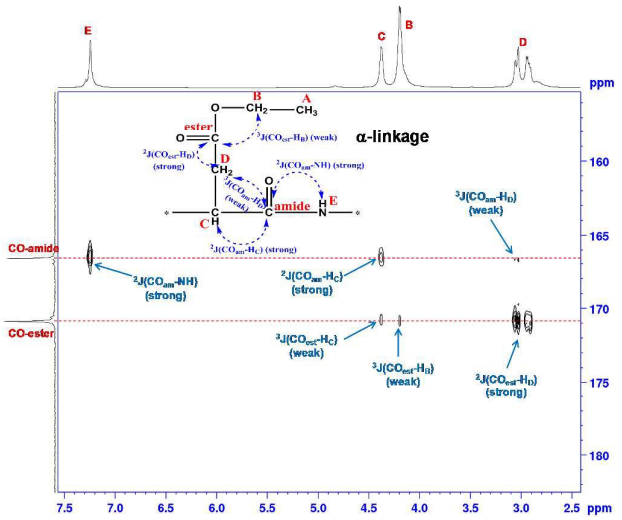

Figure 3. 2D 1H-Carbonyl region of HMBC spectrum (600 MHz) in $CDCl_3$ of poly(β-Et-α-Asp) obtained from 24 h reaction catalyzed by 20 wt.% CAL-B at 80 °C. A schematic representation of different correlations is also shown.

Figure SI-1) due to amide bond formation. The appearance of two carbonyl signals (amide and ester) in the ^{13}C NMR spectrum of poly(L-Et-Asp) (**Figure SI-2**) at 166.5 ppm and 170.8 ppm, respectively, confirms the formation of an amide bond. In addition, the integral ratio of the CH_3 ^{1}H NMR signal (**A**) of ethyl moieties to the β-CH_2 signal (**D**) decreased from 3 to ~ 1.5 (**Figure 2**). This corresponds to consumption of about 50% of ethyl ester moieties. Control reactions (without enzyme) were also carried out for 24 hours at 80 °C and no polymer/oligomer was detected by NMR (**Figure SI-5**).

L-Asp(OEt)$_2$ has ethyl ester groups linked to the α- and β-carbonyl carbons. Hence, enzymatic polymerization of this monomer could result in either poly(β-Et-α-Asp) with α-carboxylate moieties, poly(α-Et-β-Asp) with β-carboxylate moieties, or a mixture of both amide bond linkage chemistries (**Figure 1**). **Figure 2b** shows that there is a prominent peak at 4.38 ppm along with a less intense resonance at 4.82 ppm. Analysis by ^{1}H-^{1}H COSY and ^{1}H-^{13}C HSQC, as well previously reported data on poly(β-aspartate)s,[22] confirm that both of these signals correspond to protons (**C**) (see **Figures SI-3** and **SI-4)**. The assignment of NMR signals was further analyzed by conducting a 2D ^{1}H-^{13}C HMBC (Heteronuclear multiple-bond correlation spectroscopy) experiment. The ^{1}H-carbonyl correlation region from the 2D ^{1}H-^{13}C HMBC is displayed in **Figure 3**. Of the two carbonyls present, the up-field resonance at 166.5 ppm has a strong correlation with the –NH proton (**E**) and, therefore, is assigned to the –CO– next to the –NH amide (^{2}J(**C**O-N**H**), strong). Also, this carbonyl strongly correlates with the α-H (**C**) (^{2}J(CO_{amide}-$\mathbf{H_C}$), strong) at 4.38 ppm and displays a weaker correlation with β-CH_2 protons (**D**) (^{3}J(CO_{amide}-$\mathbf{H_D}$), weak) at 3.00 ppm. This reveals unambiguously that the amide bond is formed through the α-acyl group. The high preference by CAL-B for formation of α-linked repeat units agrees with work by *Conde et al* on CAL-B catalyzed regioselective amidation of Asp(OEt)$_2$[19] but contrasts with the report by Zhang et al.[21] Further confirmation is derived from that

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the more downfield carbonyl at 170.8 ppm exhibits a strong correlation with β-CH_2 protons (**D**) ($^2J(CO_{ester}\text{-}\mathbf{H_D}$, strong) at 3.00 ppm and weakly correlates with both α-H (**C**) ($^3J(CO_{ester}\text{-}\mathbf{H_C}$), weak) at 4.38 ppm and the CH_2 ethyl ester (B) ($^3J(CO_{ester}\text{-}\mathbf{H_B}$), weak) protons at 4.19 ppm. The signal at 4.82 ppm is assigned to the α-H of β-linked repeat units. This assignment agrees with published data (4.8-5.0 ppm in $CDCl_3$) for poly(α-alkyl-β-aspartate)s prepared by ring-opening polymerization of optically pure (*S*)-4-alkoxycarbonyl-2-azetidinones.[22]

Table 1. Immobilized CAL-B catalyzed bulk polymerization of L-aspartate diethyl ester[a]

Entry	Time (h)	Conversion (%)[b]	Yield (%)[c]	DP_{avg}[d]	Regioselectivity (α/β)[d]
1	24	98	70	50	94:6
2	18	95	66	44	93:7
3	15	92	62	41	94:6
4	12	90	60	37	95:5
5	8	47	30	18	97:3
6	4	39	26	10	98:2
7	2	17	9	6	93:7

[a] reactions were conducted at 80 °C in bulk under vacuum by immobilized CAL-B (20 wt-% relative to monomer). [b] Estimated from ^{1}H NMR of crude products. [c] Isolated yields. [d] Calculated from ^{1}H NMR of pure products.

The effect of reaction time on the progress of immobilized CAL-B-catalyzed condensation polymerizations of L-Asp(Et)$_2$ at 80 °C was investigated. To assess the change in molecular weight with time, the DP_{avg} = n was calculated from 1D ^{1}H NMR spectra by measuring the integral ratio of methyl proton signals (**A**) of esters at 1.28 ppm to methylene β-CH_2 protons signals (**D**) at 2.75-3.16 ppm [3(n+1)/2n = A/D]. Alternatively, the DP_{avg} was calculated from the integral ratio of the internal methyne α-H protons (C_α) at 4.38 ppm to the N-terminal α-H ($C_{\alpha\text{-end}}$) at 3.74 ppm [n-1 = $C_\alpha/C_{\alpha\text{-end}}$]. Determinations of DP_{avg} for the 24 h reaction by both of the above integral ratios are in excellent agreement (50 and 48.5, respectively).

Both conversion and yield gradually increase from 17% and 9%, respectively, at 2 h to 98% and 70%, respectively, at 24 h (**Table 1**). Furthermore, from 2 to 24 h the DP_{avg} increases from 6 to 50. The semilogarithmic plot of conversion vs. time (**Figure SI-6**), when fit by linear regression analysis, has an R^2 of 0.9597. Hence, the polymerization does not proceed by a conventional step-growth mechanism but, instead, by chain growth. Furthermore, the DP_{avg} linearly increases with conversion up to 90% (**Figure SI-7**). Thereafter, a large linear increase in DP_{avg} from 37 to 50 occurs with a small increase in conversion (from 90% to 98%). This corresponds to a change in the dominant mechanism from chain to step-growth at high monomer conversions (>90%).[23]

CAL-B has been reported to catalyze a wide range of organic transformations with high stereo- and regioselectivities.[19] As the chemical environment in immobilized CAL-B bulk L-Asp(Et)$_2$ polymerizations changes with conversion, the regioselectivity during chain growth may also vary. Regioselectivity (ratio of α- to β-linked repeat units) was determined as described above from the integral ratio of signals at 4.38 ppm (**C(α)**, α-H in α-linkage) to that at 4.82 ppm (**C(β)**, α-H in β-linkage). The ratio of α/β-linked repeat units varies irregularly and to small extents with monomer conversion with an average value of about 95:5. Hence, high regioselectivity was retained throughout the polymerization. The effect of enzyme loading was also studied (**Figure SI-8**). Decrease in enzyme loading from 20 to 10 and 5 wt% resulted in decreases in conversion (98%, 81% and 68%, respectively) and DP_{avg} values (50, 25 and 18, respectively).

Poly(β-Et-α-L-Asp) is known to form a right-handed α-helix conformation.[24] Hence, circular dichroism (CD) and NMR measurements were performed to further confirm that repeat units are indeed α-linked. The CD spectrum of poly(β-Et-α-L-Asp) in methanol (**Figure 4B, inset**) displays a typical trough at 223 nm, consistent with the right-handed α-helix conformation reported in the literature for poly(β-Et-α-L-Asp) synthesized by ethanolysis of poly(β-benzyl-α-L-Asp).[24] In chloroform (**Figure 4B)**, the negative peak is slightly shifted to 231 nm, suggesting the presence of an additional side-chain ordered structure linked to the helix. This phenomenon was also observed by Tsujita et al when studying other poly(β-alkyl-α-Asp)s.[25] A loss of CD intensity occurred upon addition of TFA in the 1-5% range, suggesting a helix-coil transition. Furthermore, ^{1}H NMR spectra in $CDCl_3$ showed a down-field shift of α-H by about 0.25 ppm upon addition of 1-10% (v/v) TFA (**Figure 4A).** This is consistent with the down-field shift found in the helix-coil transition of poly(α-amino acid)s.[26] Furthermore, the down-field displacement observed for α-H is opposite to the upfield shift displayed by α-H in poly(α-alkyl-β-Asp)s.[22]

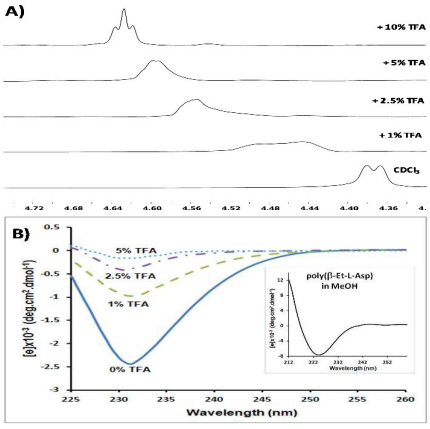

Figure 4. Trifluoroacetic acid (TFA)-induced conformational changes in poly(β-Et-α-Asp): (A) α-H signal shift in the presence of 1-10% TFA (v/v) in $CDCl_3$; (B) circular dichroism (CD) spectra in 0-5% (v/v) TFA in chloroform. The CD spectrum in methanol is also shown (inset).

Thermal analysis was performed on three poly(β-Et-α-Asp)s with Dp_{avg} of 18, 37 and 50 prepared at reaction times 8, 12 and 24 h, respectively. Thermogrametric analysis (TGA) (**Figure SI-9**) showed similar decomposition temperatures (T_d = 391, 393, 393 °C,

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respectively) for all three polyaspartates. Interestingly, the decomposition process occurs in a single step in contrast to the two-step process (formation of imides followed by chain cleavage) usually observed for β-polyaspartates [poly(α-Et-β-Asp): T_{d1} = 261 °C and T_{d2} = 368 °C].[27] The DSC traces of all three polymers (**Figure SI-10**) exhibited a clear endotherm in the range of 175-187 °C. Furthermore, the melting temperature (T_m) decreases as the DP_{avg} increases. This is likely due to increasing chain flexibility with increased DP_{avg}.

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