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Polymer Chemistry

ARTICLE

Mixed systems to assist enzymatic ring opening polymerization of lactide stereoisomers

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Abstract.

Both D- and L- lactide enantiomers have been selectively enzymatically polymerized by Novozyme 435 and Lipase from *Burkholderia cepacia*, respectively. An optimal temperature was determined for each enzyme (90 and 70 °C, for lipase from *Burkholderia cepacia* and Novozyme 435, respectively). Various polymerization conditions were tested to improve reaction kinetics and modify the macromolecular architecture. Main results show that enzymes activation by triethylamine, an aprotic amino base, leads to a great improvement in the kinetics (five to six times faster reaction) but also induces slight molar mass variation. Variation of the molar mass is observed for longer reaction times. In a first step, there is an increase possibly due to an activation of coupling chain reaction, then in a second step a decrease is shown likely due to chain transfer reactions.

Introduction

The synthesis and the properties of polylactide (PLA) have been widely studied for 40 years [1], and more extensively during the last decade. PLA is now one of the most promising biobased and biodegradable polymers [2], with a large worldwide production, higher than 200 kTons/year. PLA is suitable for a large range of applications, from short-term packaging to biomedical purposes.

The most wide-spread production process of PLA is a combination of (i) fermentation for producing lactic acid [3-4], and (ii) chemical synthesis [5]. At first, lactide is obtained from lactic acid produced by sugar fermentation followed by the lactide chemical ring opening polymerization (ROP).

Nowadays, metallic-based catalysis is the main way to obtain well-controlled PLA [5]. However these catalysts may induce some toxicity (which could be detrimental for e.g., implants or tissue engineering) [6], environmental pollution (in the case of e.g., compostability) and also increase the polymer degradation kinetics [7]. Some common organocatalyst for ROP (like DMAP or triflic acid or N-heterocyclic carbene) [8] are efficient but present also some toxicity [6]. Currently, enzymatic catalysis shows a great potential to substitute metal-based catalysts or toxic organocatalysts to limit final toxicity, environmental impacts and preserve abiotic degradation of the polymer, in perfect agreement with a sustainable development and concepts of green chemistry [9].

Lipases (E.C. 3.1.1.3) seem to be the most efficient enzymes for ROP[10]. They can be found in most organisms from microbial, plant and animal kingdom [11]. They are serine hydrolase that catalyse ester bond cleavage in aqueous medium (physiological action is cleavage of triglyceride) and they are also able to catalyse esters bond formation in organic medium (reverse reaction) [10]. By enzymatic catalysis, reactions can be performed under mild conditions (low temperature and pressure, neutral pH). High catalytic activity and very good reaction control of enantio-, chemo-, regio-, and stereo-selectivities can also be expected. Owing to these advantages, enzymatic processes could provide precise control of polymer architectures, allowing the synthesis of polymers with specific structures using a clean process, without by-products, and energy savings [12]. Enzymatic polymerization can thus be regarded as an environment-friendly synthetic process, providing one of the best examples on “green polymer chemistry”.

Enzymatic Ring Opening Polymerization (eROP) of lactones has been studied since 1993, [13] but most of the studies concern ε-caprolactone [14] and the few ones on lactide often show inconsistent results [9], [15]. It has been reported that lipases with a funnel-binding site, such as lipase B from *Candida antarctica*, are the most appropriate choice for lactone polymerization [16]. However, eROP of lactones still shows some strong limitations: kinetics is usually slow and only some monomers can be polymerized [9].

Some methods to improve the polymerization kinetics have been used, such as continuous flow reaction [17], or ionic liquid as solvent [18], with mixed results. However, the main investigations concerned the immobilization of proteins onto

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various supports (e.g. acrylic resins, silicones or nanoclays) to increase the enzyme stability and catalytic activity for polyester synthesis [19]. Additional methods to improve lipases activity have also been tested and developed. Esterification, interesterification or transesterification catalysed by lipases have been studied and optimizations of these syntheses have been performed [20]. Some authors studied the influence of the medium composition such as the solvent type [15] or the optimum water content [21-22].

Regarding the lactide enzymatic polymerization, among all existing enzymes, only a few of them have shown an activity, especially lipase B from *Candida Antarctica*, lipase from porcine pancreas, lipase from *Pseudomonas fluorescens* and from *Burkholderia cepacia* [23]. Results are usually quite heterogeneous, but most of the time eROP of lactide produces oligomers of low molar mass with poor yield and slow kinetic (reaction time from 1 day to 1 week) [24-25]. To overcome these weaknesses different approaches have been tested such as the copolymerization with better recognized monomers such as ε-caprolactone [26-28] or glycolide [29], the immobilization of enzymes onto nanoclays [26], or special reaction media like supercritical fluids [30] or ionic liquids [31]. However, most of these approches resulted in rather limited improvements.

Apart from enzymatic polymerization, some authors studied the influence of various organic bases (aromatic tertiary amine with pyridine and some pyridine derivatives, and aliphatic tertiary amine like triethylamine, *N*-methylpiperidine) on lipases activity towards the hydrolytic resolution of esters reporting significant lipase activation with some of the tested bases [32]. Interestingly, to the best of our knowledge, such activation of lipase by organic bases has not been tested and reported so far for lactide eROP.

In the present study, we thus implemented a comprehensive mechanistic approach to improve enzymatic ring opening polymerization of lactide, starting from proposed mechanism and current knowledge to the development of a new approach of base-assisted activation of the monomers and/or the polymer growing chains. The first part was focused on the selection of active enzymes for the eROP of lactide. We selected two lipases with a funnel-like binding site (i) lipase B from *Candida antarctica* immobilized on acrylic resin, which has been extensively studied for this reaction and which is known for being specific of D-lactide [33-35] and (ii) lipase from *Burkholderia cepacia*, which is known to be specific of L-lactide [35]. The influence of the medium water content and drying method on the reaction kinetics and final molar masses were then investigated. Finally, the addition of an amino base was precisely studied to investigate if such a co-solvent could activate the lactide eROP with the aim to further enhance the polymerization kinetics and to reach higher molar masses.

Results and discussions

In a preliminary step, the optimal activity temperature was determined for each lipase, by performing a simple esterification reaction (octanol and oleic acid) in toluene, determining the initial reaction rate at different temperatures. Figure 1 shows the activity profiles with optimal temperatures of 90 and 70 °C for lipases from *Burkholderia cepacia* (LBC) and for N435, respectively. These temperatures were thus set as the optimal temperatures for the polymerization reaction.

The high temperature optimum observed for LBC and the absence of activity at low temperature can seem to be surprising, but such behavior, even if not fully explained and understood so far, has already been reported for reverse reaction with LBC working only at high temperature [15],[29].

Two other lipases (PPL and LCR) were also tested since they were previously reported to be efficient for eROP of lactone [36-37]. However in these esterification conditions, PPL and LCR were not active. Because of their poor activity in our conditions, these lipases were not further considered in the next experiments.

Table 1 (experiments 1 to 6) shows that lactides eROP showed mixed results. Only low molar mass oligomers were produced (about 700 to 3500 g.mol^{-1} equivalent PS). These values are significantly lower than those usually obtained by conventional chemical ROP, equivalent for CALB and slightly lower for LBC, than those reported in the literature for eROP [12], [23].

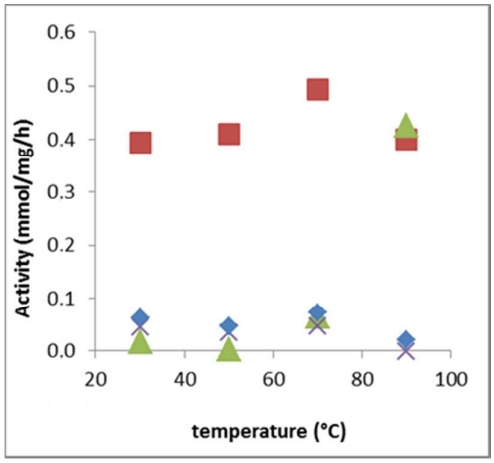

Figure 1. Enzymatic activity for olecic acid esterification by octanol (expressed in mmol of ester formed per mg of enzyme and per hour) at various temperatures; ■ N435; ▲ LBC; × LCR; ◆ PPL.

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Table 1. Enzymatic ring opening polymerization of lactide isomers catalyzed by lipase from *Burkholderia cepacia* (LBC) and immobilized lipase B from *Candida antarctica* (N435)

entry	enzyme	monomers (1g)	E/M (w)[a]	Solvents (ratio)	T °C	t(h)	Final ξ (%)[c]	η (%)[d]	Mn (g.mol^{-1})[b]	Đ[b]
1	N435	L-Lactide	0,1	Toluene	70	48	23	12	-	-
2	N435	DL-Lactide	0,1	Toluene	70	48	5	-	-	-
3	N435	D-Lactide	0,1	Toluene	70	48	98	90	3500	1,3
4	LBC	D-Lactide	0,05	Toluene	90	48	14	5	-	-
5	LBC	DL-Lactide	0,05	Toluene	90	48	20	10	500	1,1
6	LBC	L-Lactide	0,05	Toluene	90	48	80	65	700	1,1
7	N435	D-Lactide	0,1	Toluene	70	36	98	90	4200	1,2
8	LBC	L-Lactide	0,05	Toluene	90	48	80	65	1000	1,1
9	N435	D-Lactide	0.1	Toluene: TEA (2:1)	70	8	>95%	89%	4300	1.4
10	N435	D-Lactide	0.1	Toluene: TEA (2:1)	70	24	>95%	89%	4900	1.9
11	N435	D-Lactide	0.1	Toluene: TEA (2:1)	70	48	>95%	89%	3800	2.4
12	LBC	L-Lactide	0.05	Toluene: TEA (2:1)	90	8	92%	78%	1500	1.6
13	LBC	L-Lactide	0.05	Toluene: TEA (2:1)	90	24	94%	80%	1800	2.0
14	LBC	L-Lactide	0.05	Toluene: TEA (2:1)	90	48	>95%	85%	1500	2.4

[a] Enzyme/Monomer weight ratio;
[b] Determined by SEC, values given in PS equivalent.
[c] Final monomer conversion determined by NMR
[d] Recovery yield

The first experiments (Table 1, experiments 1 to 6) have shown that each enzyme is specific to one lactide stereoisomer. N435 is specific of D-lactide and LBC is specific of L-lactide. These results are in agreement with current knowledge [33-35]. Although the reaction time has been fixed at a maximum of 48h, if conversion was almost complete and not evolving anymore, the reaction was stopped before. Quantitative reaction was obtained for N435 (conversion greater than 95% after 1.5 days) and quasi-quantitative (about 80% monomer conversion after 2 days) with LBC as catalyst. The recovered PLA yields were of 94 and 81% of converted monomers for N435 and LBC, respectively. The small amounts of converted monomer that are not recovered likely correspond to the shortest chains, which could be eliminated with unreacted monomer during the precipitation step.

Furthermore one can notice that the obtained PLA show very narrow molar mass distribution, with dispersity ranging from 1.1 to 1.3, which means that chain coupling or other side reactions such as cyclisation or chain scission are negligible (Charts 1). Significant occurrence of these reactions would induce a larger or even a polymodal molar masses distribution. However, cyclisation or limited chain scission reactions cannot be totally excluded since the corresponding products could be eliminated during the precipitation step as in the case of short oligomers. But this monomodality also indicates that there are no chain-splitting side reactions or, at least, that such reactions are negligible. However, transesterification likely occurs as side reaction, as it will be demonstrated hereafter, but this reaction does not lead to increased dispersity.

These results were partially confirmed by MALDI-ToF mass spectroscopy (Figure 2), that shows a bimodal oligomers distribution respectively centered on 545 g.mol^{-1} (Dp=7) and on 1193 g.mol^{-1}, corresponding to Dp=16 initiated by water molecules (M= 72Dp + $M_{endchain}$ + M_{Na+}, for water initiated reaction end-chain are H and OH). This bimodal distribution cannot correspond to cyclic oligomers and seems to be too narrow for chain splitting reaction but it could mean that two distinct water populations are involved. Besides, the presence of peaks every 72 m/z, corresponding to one lactic acid unit, clearly attests for to the occurrence of numerous and efficient transesterification reactions.

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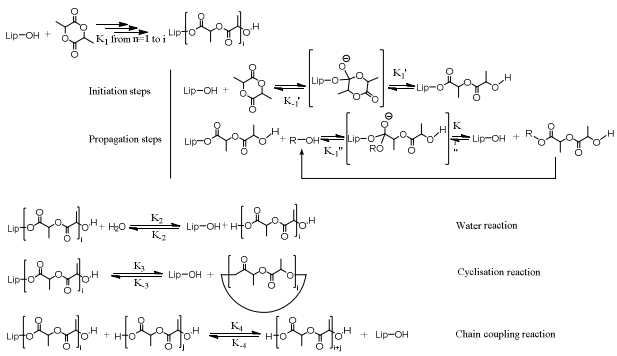

Charts 1. Mechanism and reaction pathways for enzymatic ring opening polymerization of lactide, adapted from PM Johnson and coworkers [38]

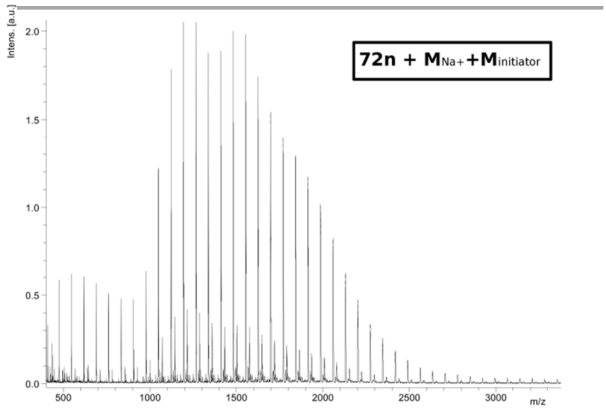

Figure 2. MALDI-ToF MS spectrum of PDLA oligomers synthesized with non-dried N345 as catalyst.

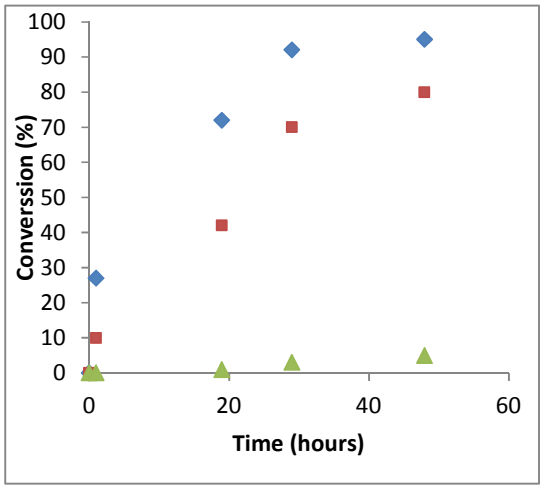

Figure 3. Monomer conversion ▲ L-lactide without enzymes, ◆ with N435 –on D-lactide and ■ with LBC on L-lactide

As shown in Figure 3, reactions kinetics are quite slow compared to metal-based catalysts or organocatalysts. To reach 80% monomer conversion, the time required is at least 24 and 48h, for N435 and LBC, respectively. Such slow kinetics are usual for enzymatic reactions on lactide substrate, especially for the enzyme reverse reaction such as lactide eROP [15]. For the eROP of lactide, N435 shows higher results than LBC, with a reaction two times faster, a higher yield (90 instead of 65% for LBC) and a higher PLA average molar mass (five times greater). Such quantitative conversion (80% to total conversion), significant yields, slow kinetics and low molar mass (less than 4000 $g.mol^{-1}$) are in accordance with literature. Some authors explain this low reactivity by a weak affinity between lipases binding site and lactide [38].

Influence of the enzymes water content.

As it can be seen from Charts 1, the main parameters to be controlled in order to maximize the PLA chains length are the relative rate of the chain coupling (K2) reaction with regard to the water reaction rate (K4) [37] and the water content, in the medium. As described in the experimental section, the monomers, solvent and reactor atmosphere are thoroughly dried. However, enzymes also bring water molecules. This last parameter is quite sensitive to control since enzymes drying may cause their denaturation. Enzymes water can by divided in two types, namely (i) the hydration water, and (ii) the structural water [38-39]. Thus, the freeze-drying method was tested to remove hydration water while keeping the structural water in order to preserve enzymes efficiency. The enzymes final water contents were determined by thermogravimetric analysis coupled with infrared spectroscopy (hydration water leaves between 100 and 120°C whereas structural water is lost just before the main degradation) and the results are shown on Figure 4.

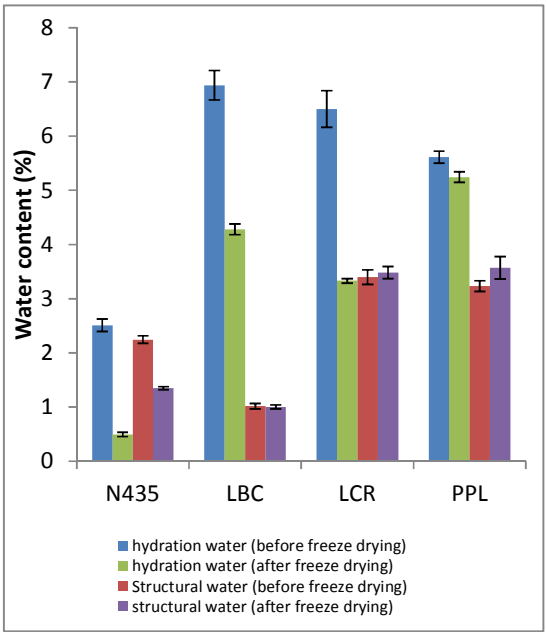

Figure 4. Hydration and structural water contents of the lipases, before and after freeze-drying.

The freeze-drying method allowed a significant reduction of hydration water level, without having a negative impact on the structural water content. This has been confirmed by testing the efficiency of the freeze-dried enzymes for lactide eROP.

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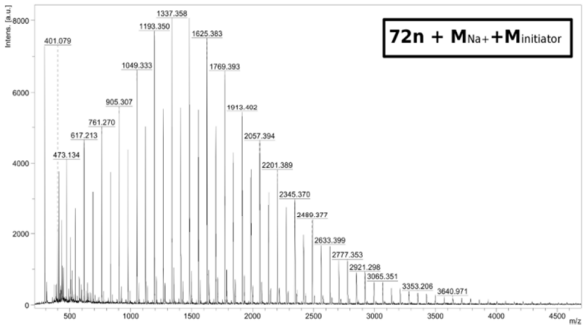


Figure 5. MALDI-ToF spectrum of PDLA oligomer synthesized with dry N435

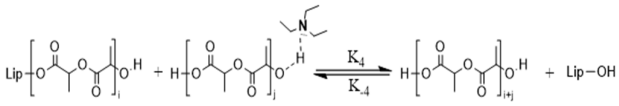
As expected this reduction in the hydration water content of the enzyme allowed increasing significantly the final PLA average chain length from 3500 to 4200 g.mol^{-1} for N435 and from 700 to 1000 g.mol^{-1} for LBC, respectively, with a corresponding short increase in the reaction time. 80% of conversion was reached after 28h (instead of 24h) and 50h (instead of 48h) for N435 and LBC, respectively. These reactions gave very similar final conversion (>98% for N435 and 80% for LBC) and yield (about 90% and 65% for N435 and LBC, respectively) than the previous experiments without drying. As previously explained, the difference between conversion and yield could be explained by the loss of the short oligomers during the precipitation step. One can also notice that, for N435 the control of these key parameters to minimize the water content allows to obtain PLA chains with narrower molar mass distribution (Đ =1.3 in the first experiments compared to 1.1 in this case). As far as freeze-dried LBC is concerned, Đ does not significantly change (1.1 to 1.2) but remains very low. As previously mentioned, such low Đ likely attests for the absence of chain coupling or cyclisation as side reactions.

The MALDI-ToF results (Figure 5) show a monomodal distribution centered on 1481 g.mol^{-1} (Dp=20, water initiated) and show the disappearance of the first oligomer population (Figure 2) which confirms that such a population is likely due to the hydration water of our enzymes.

In all the cases, the molar mass values reach a kind of plateau at 4500 g.mol^{-1}. This could be due to the clear differences in the chemical structures and shapes between the conventional substrates of the lipase (i.e., fatty acids and triglycerides) and the lactide monomers and oligomers, which are much more polar and more rigid molecules. This may result in a strain for the PLA growing chains to enter, and being sufficiently stable, into the lipase active/binding site and thus to incorporate additional monomer units.

Study of the activation of lactide eROP with TEA.

Our second approach to increase the PLA molar mass was based on the increase of the relative rate constant of coupling chain reaction (K4/K2). For that, an amino base, TEA was added as a co-solvent with the initial aim to enhance the lactide solubility but also to potentially activate chain-ends of preformed oligomers (Charts 2). Besides, since TEA is slightly basic and nucleophilic, it could also activate the monomer for its ring opening and thus increase the reaction rate [24] (Charts 3). However, this co-solvent can also activate chain-splitting reactions.



Charts 2. Oligomers chain-end activation

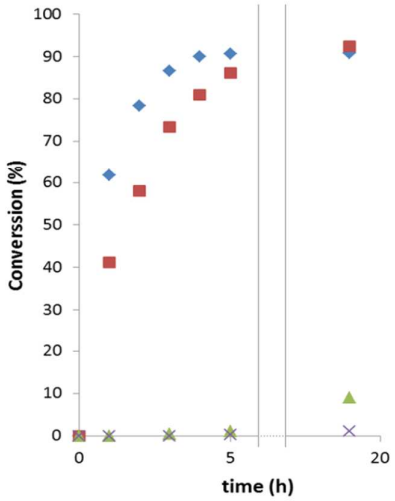


Figure 6. Influence of TEA addition on kinetics of eROP catalyzed by LBC (with L-lactide) at 90 °C (◆), N435 (D-lactide) at 70 °C(■), without catalyst (L-lactide) at 90 °C(▲), with neither catalyst nor TEA (L-lactide) 90 °C (x)

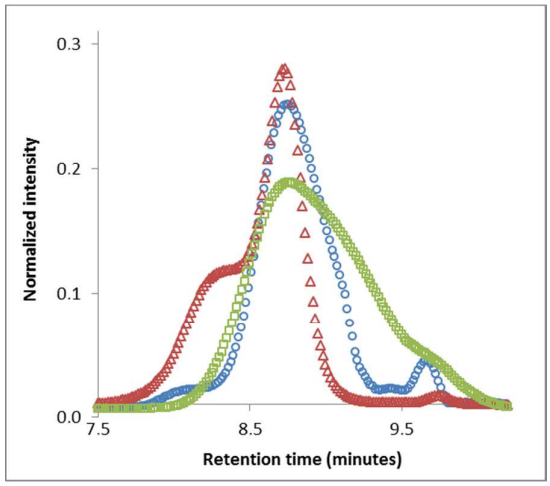


Figure 7. SEC elution profiles of PLA produced by eROP catalyzed by N435 in toluene:triethylamine solvent mixture. After 8h (○), 24h (△) and 48h (□) of reaction. All curves were normalized on flow marker intensity.

Figure 6 shows the main results of the addition of TEA with a great impact on the reaction kinetics. Reaction time was reduced about 5 to 6 times for both selected lipases (80% conversion reached after 4 hours for N435, and after 2.5 hours for lipase from *Burkholderia cepacia*). As expected, TEA alone shows some catalytic activity but does not lead to a significant extent of lactide ROP.

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Table 2. Specific rotation of some synthesized PLA n function of reaction time

Entry[a]	Solvents (ratio)	t(h)	$[\alpha]^{20}_D$	o.p (%)[b]
7	Toluene	36	114.4	76%
9	Toluene: TEA (2:1)	8	88.5	59%
10	Toluene: TEA (2:1)	24	49.8	33%
11	Toluene: TEA (2:1)	48	24.5	6%

[a] see Table 1 for details of each entry;
[b] optical purity;

This increase in the reaction rate is probably not only the result of the relative increase in the chain coupling reaction rate but some other side reactions may also have occurred.

Tertiary amines like TEA can also promote the epimerization of lactide especially when present in such a large amount like in our case. This could affect the polymerization since it is well known that meso lactide is much more favorable for enzymatic polymerization by CALB [39].

To verify the occurrence and the importance of epimerization, polarimetric measurements were performed on some of the PLA synthesized (Table 2). It has been found in the literature that specific rotation of enantiopure PLA is about $[\alpha]^{20}_D$ = +/- 150° (+ for the D form and – for L form) [40]. One can note that the eROP of lactide in toluene already caused some epimerization (o.p. 75%). The addition of TEA induces an important reduction in o.p. (decreased to 6% after 48h) but this decrease is too slow (only 17% reduction after 8h) to explain the increase in reactivity. Furthermore, the important reduction in the o.p. after 48h matches with an increase in the dispersity and a lowering of the Mn value. These results confirmed the epimerization of lactide, but the decrease of $[\alpha]^{20}_D$ after the total consummation of lactide indicates that TEA also promotes the racemization of the formed oligomers.

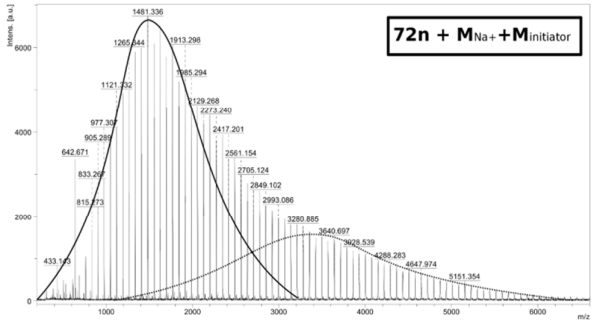


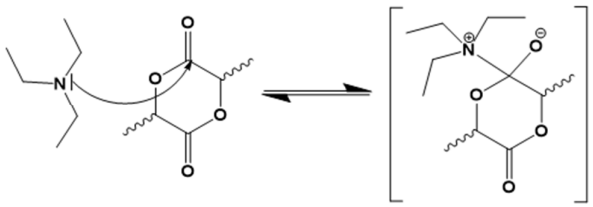
Figure 8. MALDI-ToF spectrum of PDLA oligomer synthesized with dry N435 in toluene:TEA mix

The SEC results (Table 1, experiment 10 and 13) show a significant improvement in the average molar masses, with the addition of TEA, with a 17% increase for N435 catalyst and over 80% increase for LBC. However, one can notice an important increase in dispersity (Đ =1.9 and 2.1, instead of 1.15 and 1.1 in previous experiments, respectively), which may indicate the occurrence of, at least, another type of reaction. The SEC chromatograms show a bimodal profile (Figure 7) with a marked shoulder corresponding to an average molar mass, twice the one of the main peak.

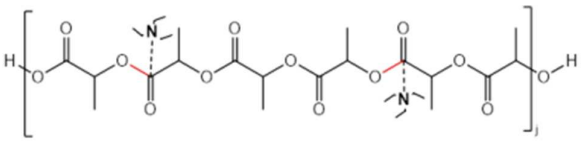
Such a doubled molar mass value seems to confirm that chain coupling reaction has been promoted by TEA addition as a result of the chain-end activation of the oligomers.

However, chain scission and chain transfer reactions have also been confirmed by additional experiments. As shown in Table 1 (experiments 9 to 14), average molar masses increase until 24 hours of reaction, until a probable progressive inactivation of the catalytic system. Then, for long reaction times, chain scission reactions and chain transfer are likely responsible of the gradual reduction of the average molar mass. Such a decrease in Mn values with transesterification reactions for long reaction times is in perfect agreement with previously reported results [41]. One can also notice a small peak or shoulder at about 9.7 minutes retention times, which could be due to cyclic oligomers, as it has already been reported in literature [29].

The activation of coupling chain reaction could also explain the



Charts 3. Nucleophilic activation of lactide



Charts 4. Nucleophile activation of oligomers esters bond.

obtained MALDI-ToF spectrum (Figure 8) which seems to be the superposition of 2 distributions, one centred on 1481 $g.mol^{-1}$ (Dp=20, water initiated) and the other centred on 3496 $g.mol^{-1}$ (Dp=48, water initiated), which is 2.4 times the first one.

The chain-end activation of the oligomers cannot totally explain our results, especially the kinetic improvement of eROP, and is probably not the only activation way. The influence of TEA is more likely much more complex. One can consider several additional activation mechanisms. Three simple possible activation mechanisms can be identified:

I. Similarly to the nucleophilic activation of the oligomers chain-end, one can consider that TEA could activate the lactide monomer through a nucleophilic attack on its carbonyl carbon (Charts 3). This would induce an elongation of the O-C=O bond facilitating the ring opening of lactide and then the formation of an O-acyl reactive intermediate.

II. A second possible activation way could consist in the basic activation of the serine amino acid of the binding site. As proposed and shown in Figure 9, this would facilitate the serine (Ser) deprotonation and

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thus increase the reactivity of lipases. In this case TEA would play a role very similar to that of histidine (His) in the enzyme binding site.

III. The third possible activation mechanism could also come from the nucleophilic character of TEA which could have an impact on the chain backbone through nucleophilic attack on the carbonyl of the ester bonds (Charts 4).

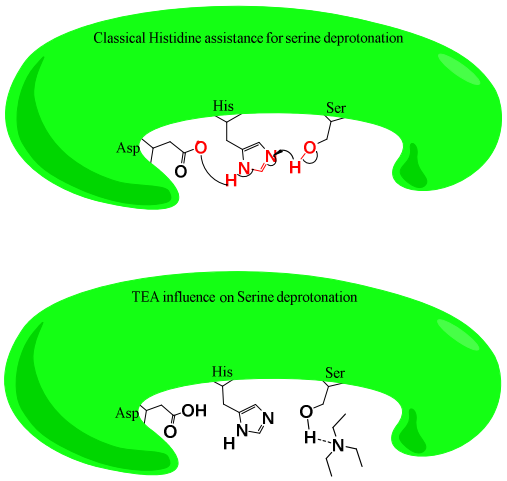

Figure 9. Lipase activation by facilitating serine deprotonation.

This last effect could result in increased occurrence of chain-transfer and chain scission reactions that could explain the molar mass decrease we observed (Figure 7). Besides, we confirmed the chain-splitting by a simple experiment: some previously formed oligomers (3500 $g.mol^{-1}$, Đ=1.3) were added to a mix of toluene and TEA and after 48h at 70 °C, we observed a significant lowering of initial molar mass and a significant increase in dispersity (2100 $g.mol^{-1}$, Đ=2.5). Whatever the involved mechanisms (these three activation mechanisms, the epimerization and the chain coupling) and whatever the enzyme we used, TEA has a clear and positive impact on eROP of lactide on both aspects, kinetic and final molar mass, with a controlled dispersity.

Conclusions

In conclusion, this work reports enantioselective eROP of the two main enantiomers of lactide. Enantioselectivity of selected enzymes have been confirmed, with a selectivity towards D-lactide for N435 and towards L-lactide for LBC. The ability of freeze-drying to reduce enzymes hydration water while preserving their structural water has been demonstrated and polymerization tests confirmed that reducing the hydration water content results in increased oligo-PLA molar masses (Mn increase of 17 and 40%, for N435 and LBC, respectively).

It has also been demonstrated that eROP of lactide is activated by the addition of TEA as an aprotic amino base and that this kind of activator is efficient for both lipases we used. TEA addition permits a significant kinetic improvement (reaction was five time faster for N435) and also increased the molar mass (17% for N435). The kinetic improvement of eROP allows new opportunities for lipases reverse catalysis. In addition with others improvement methods, like continuous flow reaction and/or sonic activation [16-19], this biocatalyst could become competitive with classical organometallic catalysts for some application.

Five possible mechanisms have been proposed for the eROP activation by TEA: (i) a nucleophile activation of lactide, (ii) basic activation of lipases, (iii) basic activation of oligomers chain-end, (iv) the epimerization of lactide and (v) nucleophile activation of esters bond in the oligomers backbone. Our results seem to attest for the occurrence of at least three of these activation ways: the epimerization, the chain-end activation and the activation of ester bond in the chains backbone. However, one can say that a combination of these five mechanisms more likely occurs.

Our future works, by performing complementary experiments and numerical simulation, should allow us to describe and determine the contribution of each activation mechanism on the TEA activated eROP of lactide.

Experimental

Materials: Both D-lactide and L-lactide isomers were obtained from Purac (with the commercial name of Purasorb®) and were purified by sublimation and recrystallization prior use. Novozym®-435 (N435), acrylic resin-immobilized form of *Candida antarctica* lipase B (CALB), was purchased from Aldrich. Lipases from *Burkholderia cepacia* (LBC), from porcine pancreas (PPL) and from *Candida rugosa* (LCR) were purchased as lyophilized powders from Aldrich. Anhydrous toluene was freshly distilled over sodium under nitrogen atmosphere. Anhydrous triethylamine (TEA) was freshly distilled over molecular sieve under vacuum. Other solvents (GC grade) were purchased from Acros and used without further purification.

Enzymatic ring opening polymerization setup:

All reactions were carried out in selected dry solvent or in chosen mixture of dry solvents at 70 and 90 °C, for N435 and LBC, respectively. For that, 6.9 mmol of lactide (1 g) and 4Å molecular sieve (when necessary) were introduced into previously dried Schlenck tube under an inert dry argon atmosphere. The tube was immediately capped with a rubber septum and then immersed in a heated oil bath at 100 °C, until the melting of lactide monomer. Toluene was transferred with a syringe through rubber septum caps. The reaction tube was cooled down to reaction temperature. Predetermined amounts of catalysts, N435 (100 mg) or LBC (50 mg) were quickly introduced in the tube under an inert dry argon atmosphere. The tube was immediately capped with a rubber septum. The enzyme addition marked the beginning of the polymerization kinetic study (t0). Then, an aliquot was withdrawn at specified time intervals to monitor the

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polymerization reaction progress. Reactions were terminated by dissolving the reaction mixture in chloroform and when necessary removing the catalyst by filtration. Part of the solvent in the filtrate was then stripped by rotary evaporation at 35 °C. The polymer in the resulting concentrated solution was precipitated in cold methanol (previously stored at −20 °C) to ensure also the recovery of the shortest chains. The PLA precipitate was separated by filtration and dried overnight at 30 °C under vacuum.

System drying procedures:

To minimize the water content in the reaction medium, the monomers were dried by sublimation, the reaction solution medium has been dried using a molecular sieve and the reactor atmosphere was dried by placing molecular sieve or anhydrous calcium chloride in the argon flux tubing. For some experiments, a further drying of the reaction medium was performed by freeze-drying the enzymes for 24 to 36 hours.

Lipases esterification activity measurement:

3.3 mmol of octanol (0.429 g), 3.3 mmol of oleic acid (0.932 g) and 1 mL of toluene were introduced into a reaction tube with a magnetic stirrer under argon atmosphere. A determined amount of enzyme (about 0.1 g) was then added to the reaction medium. The tube was immediately capped with a rubber septum and immersed in a heated oil bath at the selected reaction temperature. After 30 minutes, the reaction was quenched by cooling in an ice bath and the enzyme was removed by filtration. Reaction extent (ξ) was determined according Equation 1 from ^{1}H NMR spectroscopy by relative integration of characteristic octyl oleate peak (O=C-O-CH_2- t 4.13ppm) and characteristic octanol peak (HO-CH_2- t 3.63ppm), $I_{octyl\ acetate}$ and $I_{octanol}$, respectively

$$\xi = 1 - \left(\frac{I_{octanol}}{I_{octanol}+I_{octyl\ oleate}}\right) \quad (1)$$

Size Exclusion Chromatography (SEC) analysis:

The number-average molar mass (Mn), the mass-average molar mass (Mw) and the dispersity (Đ) of the resulting samples were determined by SEC, using an Agilent PL 50 instrument. This device was equipped with a guard column 50 mm (5 µm) and a 300 mm column (PL Mixed C, 5 µm). Refractive index (RI) detector was used. Tetrahydrofuran (THF) was used as the eluent at a flow rate of 1 mL min^{-1}. The apparatus was calibrated with linear polystyrene standards from 900 to 1,000,000 g mol^{-1}.

NMR measurement:

^{1}H NMR spectra were recorded on a Bruker Ascend™ 400 spectrometer at 400 MHz, with, at least, 256 scans for kinetic measurements. $CDCl_3$ was used as solvent.

Monomer conversions were determined by NMR analyses on quenched aliquot, using Equation 2. This value was determined from the integrals of the lactide characteristic peak (-O-CH-CH_3 q 4.80-4.90 ppm, $I_{lactide}$), PLA characteristic peak (-O-CH-CH_3 m 5.00-5.15 ppm, I_{PLA}) and chain-end characteristic peak (HO-CH-CH_3 q 4.20-4.30 ppm, $I_{endchain}$).

$$Monomer\ conversion\ (\%) = 1 - \left(\frac{I_{lactide}}{I_{lactide}+I_{PLA}+I_{endchain}}\right) \times 100 \quad (2)$$

Thermogravimetric analysis (TGA) coupled Fourier transform infrared spectrometer:

Enzymes water contents were determined by TGA coupled with a FTIR for evolved gas analysis. TGA measurements were conducted under dry helium (at a flow rate of 25 mL min^{-1}) using a Hi-Res TGA Q5000 apparatus from TA Instruments. The samples (5-9 mg placed in a platinum pan) were heated up to 450 °C at 5 °C min^{-1}. FTIR spectra were recorded on a Nicolet 380 (Thermo Electron Corporation) by performing 16 scans with 4 cm^{-1} resolution. Hydration water content is determined from the weight loss between 100 and 120°C and the amount of structural water corresponds to weight loss in the 150-210°C temperature range.

MALDI-ToF Mass spectroscopy analysis:

For the sample preparation, matrix solutions were freshly prepared. Super DHB (9:1 mixture of 2,5-Dihydroxybenzoic acid and 2-hydroxy-5-methoxybenzoic acid, from Sigma Aldrich) was dissolved to saturation in a $H_2O/CH_3CN/HCOOH$ (50/50,1%) solution. Typically, a 1:1 mixture of the sample solution in CH_2Cl_2 was mixed with the matrix solution and 1 µL of the resulting mixture was deposited on the stainless steel plate.

Mass spectra were acquired on a time-of-flight mass spectrometer (MALDI-ToF-ToF Autoflex II ToF-ToF, Bruker Daltonics, Bremen, Germany) equipped with a nitrogen laser (λ = 337 nm).

An external multi-point calibration was carried out before each measurement using the singly charged peaks of a standard peptide mixture (0.4 µM, in water acidified with 1% HCOOH).

Scan accumulation and data processing were performed with FlexAnalysis 3.0 software.

Specific rotation measurement:

The $[\alpha]^{20}_D$ measurement was measured by a polarimeter MPC 200 thermostated at 20°C, in a 100 mm long cell (diameter = 0.3 mm). Measured solution was prepared at 0.9 to 1 mg.mL^{-1} in chloroform. The optical purity (o.p.) was calculated by the following equation:

$$\text{o.p.} = \frac{[\alpha_D^{20}]}{[\alpha_D^{20}]_0} \times 100 \quad (3)$$

$[\alpha_D^{20}]_0$ is fixed at +150° according to the literature [40].

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Notes and references

Abbreviations

LBC, lipase from Burkholderia *cepacia*; N435, Novozyme 435; LCR, lipase from *Candida rugosa*; PPL, Lipase from porcine pancreas; ROP, ring opening polymerization; eROP, enzymatic ring opening polymerization; PLA, polylactic acid; PS, polystyrene; TEA, triethylamine, SEC, size exclusion chromatography; NMR, nuclear magnetic resonance; TGA, thermogravimetric analysis; FTIR, Fourier transform infrared spectroscopy; MS, Mass spectroscopy; MALDI, Matrix-Assisted Laser Desorption/Ionization. ToF, Time of Flight.

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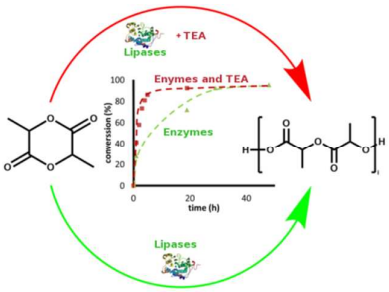

Mixed systems to assist enzymatic ring opening polymerization of lactide stereoisomers

S. W. Duchiron,[a] E. Pollet, [a] S. Givry,[b] and L. Avérous[a]

Enzymatic ring opening polymerization of both enantiomers of lactide was performed in toluene. The eROP was kinetically improved by solvent assisted method (by TEA) and gave 6 time faster reaction.

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