Biopolymer Synthesis Catalyzed by Tailored Lipases

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Summary: Enzyme-catalyzed synthesis of biopolymers is a growing area of green chemistry because the exquisite selectivity and control of the polymer structure allowed by the enzymatic synthesis. In addition, the metal traces of chemical catalysts are avoided, being this feature particularly useful in biomedical applications. Lipases are remarkable catalysts and they are efficient in the synthesis of several kinds of biopolymers. However, one of the limitations of the industrial application of lipase-catalyzed polymerizations is the cost of commercial immobilized lipases. To overcome this drawback, in this work we present some cheap and simple strategies to produce and obtain immobilized lipases, as well as some examples of biopolymer synthesis using these tailored lipases.

Keywords: biopolymer; C*arica papaya*; immobilization; lipase; Y*arrowia lipolytica*

Introduction

Lipases are ubiquitous biocatalysts in organic syntheses due to its broad substrate acceptation and its natural ability to work in non-aqueous media.[1–2] As lipases act naturally on ester bonds, they have been successfully used in polyester synthesis by different routes.[3] Lipases can also degrade some chemically synthesized polymers and practically all the lipase-synthesized polymers.[4–5] In consequence, biodegradable polymer syntheses catalyzed by lipases are increasing, because the polymerization conditions are better controlled and the purification costs of the biopolymer are reduced.[6–7]

Lipases are interfacial enzymes which usually become active at hydrophilic/hydrophobic interfaces through a rearrangement of their structure. The structure rearranged is a short α-helix called ''lid'', which was originally covering the active site. In presence of interfaces, the lid moves away and the active site becomes accessible for the substrate. This phenomenon is called ''interfacial activation'' (Scheme 1). Interfacial activation is absent in a few lipases that do not posses a lid.

Although several lipases have been used in polymer synthesis,[3] the immobilized lipase B from *Candida antarctica* (Novozyme 435) seems to be the most widely used biocatalyst for polymer synthesis.[7,8–10] Looking at topologies of active sites of lipases,[11] it could be hypothesized that the absence of lid in lipase B from *Candida antarctica* avoids steric hindrances during polymerization, although *Rhizomucor sp.* and *Rhizopus sp.* lipases with large hydrophobic active sites near surface are also adequate for certain polymer synthesis.[3,6] Another explanation for the success of Novozyme 435 as biocatalyst in polymer synthesis is the fact that Novozyme 435 is a very stable biocatalysts due to its immobilization support, a strong hydrophobic acrylic resin. Indeed, it is well known that strong hydrophobic supports could activate and stabilize the lipases.[12] Immobilization offers of course other well-known advantages: adequate dispersion of the enzyme and the possibility of enzyme reuse.[13] However, in industrial-scale polymer

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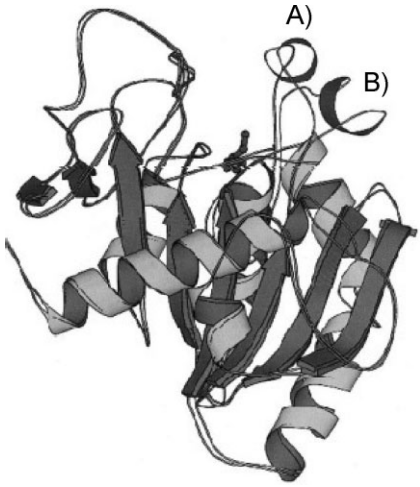

Scheme 1.
Interfacial activation of lipases. The example of *Thermomyces lanuginosa* lipase is shown. A) Closed (inactive) and B) open (active) forms superimposed. The short α-helix called "lid", which covers the active site (catalytic serine is shown in "ball & stick"), moves away in the presence of hydrophilic/hydrophobic interfaces.

synthesis the high cost of commercial immobilized lipases limits its applicability and the preferred lipase in polymer synthesis (Novozyme 435) is even more expensive than the average immobilized lipases. Therefore, alternative cheap immobilized lipases are still required for biopolymer synthesis.

In the light of these considerations, we propose on one hand the use of the extracellular lipase 2 from the yeast *Yarrowia lipolytica* immobilized on hydrophobic supports and on the other hand the auto-immobilized lipase from agro-wastes from *Carica papaya* as a new cheap and efficient biocatalyst for biopolymer synthesis.

Experimental Part

Polyester Synthesis

Adipic acid, glycerol, t-buthanol and Lewatit beads were purchased from Sigma-Aldrich and used as received. Accurel was a kind donation of Akzo Nobel. Papaya lipase was obtained from papaya agro-wastes (latex from unripe fruits), coming from an experimental papaya plantation in INIFAP Costa de Jalisco (Mexico). Partial purification of the latex was performed by washing off proteases using distilled water. ε-caprolactone (ε-CL) (Aldrich) was distilled at 97-98 °C over CaH_2 at 10 mmHg. In a typical run, 1.08 mmol of ε-CL and 12 mg of immobilized lipase were placed in a 10 mL vial previously dried. Vials were stoppered with a teflon/silicon septum and placed in a thermostated bath at predetermined temperatures and predetermined time. The enzyme was filtered off to stop the reaction. Products were purified by dissolving in chloroform (1:1 v/v), precipitating in methanol (10:1 v/v) and drying in a desiccator at room temperature.

Analysis

Solution ^{1}H -NMR spectra was recorded at room temperature on a Varian Gemini 2000. Chloroform-d ($CDCl_3$) was used as solvent. Molecular weight was determined from ^{1}H –NMR data as number-average weight.

Biopolymer Synthesis by Immobilized Lipase 2 from *Y. lipolytica* (YLL2)

Y. lipolytica is a dimorphic yeast that secretes an extracellular lipase (lipase 2) in high quantities.[14] The availability of modified lipase-overproducing strains[15] and already optimized fermentation processes for the production of *Y. lipolytica* lipase 2 (YLL2),[16–17] ensures high production and activity at reasonable costs. Among other applications, low molecular weight polycaprolactones (PCLs) have been successfully synthesized by ring-opening polymerization using non-immobilized YLL2 as biocatalysts.[18] Polylactones have applications in tissue engineering and as drugs vectors.[19] In these biomedical applications it is important to avoid metal traces and for this reason lipase-catalyzed synthesis of polylactones is preferred over

metal-catalyzed synthesis.[19] Immobilization of YLL2 on hydrophobic supports was performed according to the procedure of Al Duri *et al*. (2002).[20] Two strong hydrophobic resins were chosen: Lewatit, which is similar to the support of Novozyme 435, and Accurel, which has been reported as an efficient support for immobilization of lipases.[21] The main characteristics of these supports are given in Table 1.

The resulting biocatalysts were applied in the synthesis of PCL. As it can be observed in Table 2, their performances are comparables to those of Novozyme. The best support in this case seems to be Lewatit. Higher conversion and molecular weight were obtained using this support, probably it is able to retain more enzyme in an active form.

Immobilized YLL2 derivatives were also applied to a condensation polymerization of adipic acid and glycerol to obtain dendritic polymers. Indeed, there is a growing interest in these type of biopolymers (dendrimeric and hyperbanched, collectively called dendritic polymers), which are being used in biomedicine as bioabsorbable nano-carriers of drugs and genes and as vectors of colorants in molecular imaging.[22–23] YLL2-Lewatit was again the best biocatalyst for this reaction at the conditions tested. The ^{1}H-NMR spectrum of the dendrimer of adipic acid/glycerol obtained using YLL2-Lewatit is shown in Figure 1.

Chemical synthesis of these type of polymers usually requires several steps including protection and deprotection of functional groups[24,25] while in lipase catalysis this drawback is avoided. The selectivity of lipases for primary hydroxyls produces an incompletely ramified polymer (Figure 1-E), thus leaving some internal unreacted groups that could be used for coupling the targeted molecule to be carried or for further polymerization. In contrast, the chemically synthesized polymer (Figure 1-C) is fully branched. Additional advantages of the YLL2-catalyzed synthesis are that solvent and catalyst used are non toxic and that the synthesis is realized in one pot.

Table 1.
Characteristics of the immobilization supports.

FEATURE/SUPPORT	ACCUREL	LEWATIT
Material	Polypropylene	Polymethacrylate
Particle size (μm)	400–1000	100–400
Pore size (nm)	microporous*	27

*the fabricant does not give the values

Table 2.
Polycaprolactones obtained by lipase-catalyzed ring-opening polymerization in bulk during 6 h at 150 °C, 1.08 mmol of ε-CL and 10% (w/w) of biocatalyst.

IMMOBILIZED LIPASE	CONVERSION (%)*	Molecular weight (Da)*
Novozyme 435	6.3	601
YLL2-Lewatit	74	1358
YLL2-Accurel	3	653

*Number-average weight determined by ^{1}H-NMR.

Further advantages of YLL2-catalyzed synthesis of biopolymer could arise from the facts that some function-structure relationships of the *Y. lipolytica* lipase 2 have been elucidated[16,26] and that high-throughput methodologies to generate[27] and screen[28] mutants of this lipase have been implemented. The complete biotechnological pack to generate customizable YLL2 mutants for biopolymer synthesis is therefore available.

PCL Synthesis by Auto-Immobilized Lipase from Agrowastes of *Carica papaya* (CPL)

Carica papaya is an unbranched tree native to the Central America which is easily adapted to tropical and subtropical climates. Brasil and Mexico are the biggest producers of papaya fruit, followed by Nigeria and India.[29] As the tree grows up, fruit recollection becomes expensive and the entire plantation is removed every 2–3 years. Moreover, papaya trees are exposed to several viral and bacterial infections and sick plantations must be also removed.[30] For these reasons several tonnes of papaya agrowastes are available.

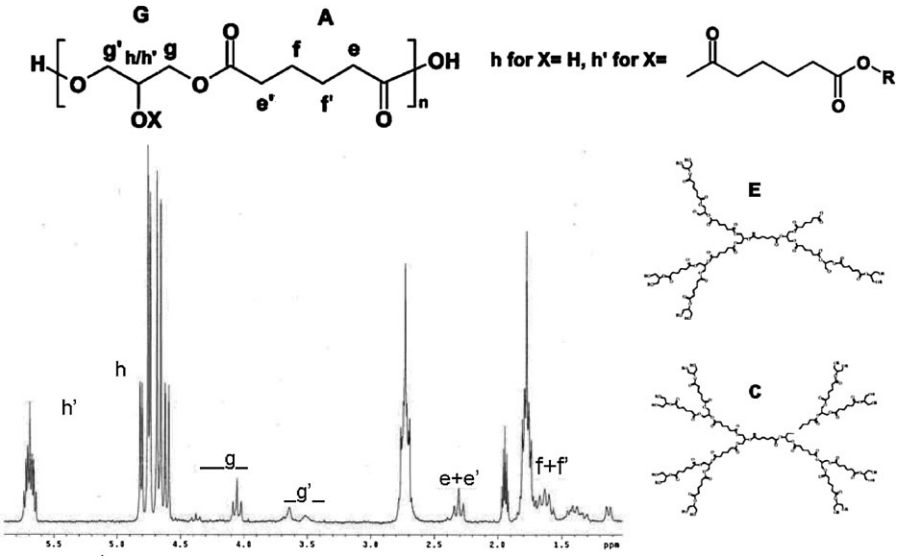

Figure 1.
^{1}H-NMR spectrum and possible structures of the adipic acid (A) - glycerol (G) dendrimers obtained using YLL2-Lewatit as biocatalyst. Reaction conditions: equimolar mixture of substrates, 40 g/l adipic acid, in 5 ml *t*-butanol, 50 °C, 100 mg of biocatalyst, 48 h.

These agrowastes and specially unripe or sick fruits are rich in hydrolases contained in its latex. The most studied and used is the endoprotease papain which is soluble in water. Conversely, the lipase is strongly attached to the insoluble fraction of the latex and is therefore considered as a naturally auto-immobilized enzyme.[31]

Partially purified papaya lipase (CPL), was used to catalyze the ring-opening

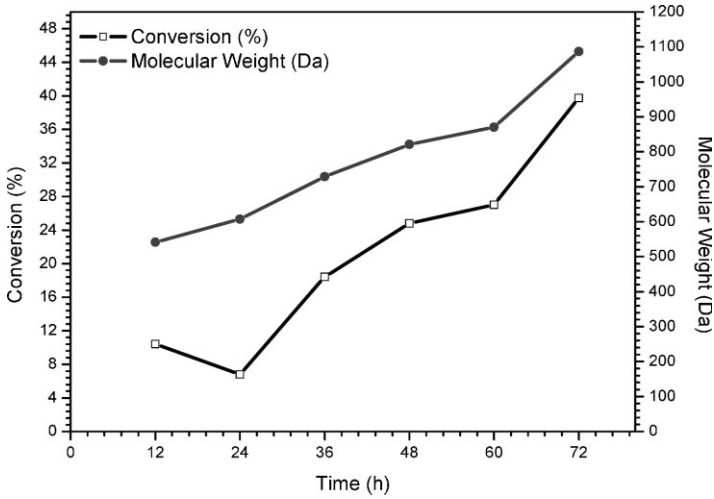

Figure 2.
Kinetics of the ring-opening polymerization of ε-caprolactone catalyzed by *C. papaya* in bulk at 70 °C, 1.08 mmol of ε-caprolactone and 10% (w/w) of biocatalyst. Conversion and number-avegarge molecular weight were determined by ^{1}H-NMR.

polymerization of ε-caprolactone. The kinetics of the reaction is depicted in Figure 2. A decrease in conversion is observed at 24 h because lipases could catalyze also the hydrolysis of polymers.[4-5] After equilibrium is established, the polymer continues to grow. This behavior was also observed with other lipases such as YLL2.[18] In this case, kinetics and molecular weight (number-average) of PCL obtained using CPL are comparable of those obtained by free YLL2 lipase.[18] Higher conversions and molecular weights could be achieved by reaction condition optimization (v.g. using more enzyme or increasing temperature). However, further biochemical characterization of the *C. papaya* lipase is needed in order to optimize the reaction conditions. As the lipase is insoluble, more advanced purification protocols are hard to implement, but the alternative strategy of cloning the lipase genes from *C. papaya* is ongoing in our group.

Conclusion

Lipases can be customized by immobilization and protein engineering to improve their performances and to produce cheap and custom-made biocatalyst for biopolymer synthesis. In this context *Y. lipolytica* lipase 2 is very promising and Lewatit was the best immobilization support for this application. In the same way, performances of auto-immobilized lipase from *C. papaya* agrowastes as biocatalyst in PCL synthesis merit further studies.

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