Biocatalysis

DOI: 10.1002/anie.201410633

An Enzyme Cascade Synthesis of ε-Caprolactone and its Oligomers**

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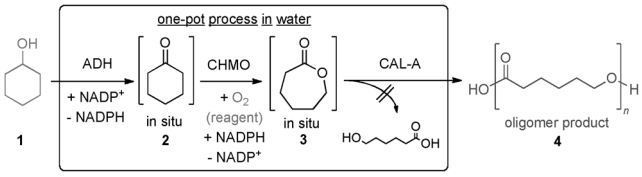
Abstract: *Poly-ε-caprolactone (PCL) is chemically produced on an industrial scale in spite of the need for hazardous peracetic acid as an oxidation reagent. Although Baeyer–Villiger monooxygenases (BVMO) in principle enable the enzymatic synthesis of ε-caprolactone (ε-CL) directly from cyclohexanone with molecular oxygen, current systems suffer from low productivity and are subject to substrate and product inhibition. The major limitations for such a biocatalytic route to produce this bulk chemical were overcome by combining an alcohol dehydrogenase with a BVMO to enable the efficient oxidation of cyclohexanol to ε-CL. Key to success was a subsequent direct ring-opening oligomerization of in situ formed ε-CL in the aqueous phase by using lipase A from Candida antarctica, thus efficiently solving the product inhibition problem and leading to the formation of oligo-ε-CL at more than 20 g L^{-1} when starting from 200 mM cyclohexanol. This oligomer is easily chemically polymerized to PCL.*

Biocatalytic processes are well established for the synthesis of high-value fine chemicals, especially for chiral pharmaceutical intermediates, by using natural or engineered enzymes.[1] In contrast, examples for the enzymatic synthesis of bulk chemicals are still rare.[2]

ε-caprolactone (ε-CL, **3**) is an important industrial chemical that is currently produced at a multi-10000 ton scale per year by the UCC process to serve as a precursor for polymer synthesis.[3] In this process, cyclohexanone is oxidized by using stoichiometric amounts of peracetic acid. Besides only modest selectivity (85–90%), further drawbacks arise from the perspective of toxicity, ecology, and safety.

One obvious enzymatic alternative for the production of ε-CL is the use of Baeyer–Villiger monooxygenases (BVMO).[4] These flavin-dependent enzymes only require molecular oxygen as an oxidation reagent and the cofactor NADPH. Within this enzyme class, the cyclohexanone monooxygenase (CHMO) from *Acinetobacter calcoaceticus*, which was already described almost 40 years ago,[5] is the preferred candidate. Furthermore, this enzyme can be produced recombinantly in yeast[6] and *E. coli*.[7] To date, however, biocatalytic large-scale production of ε-CL has not been achieved for a number of reasons. These include stability of the enzyme, the issue of cofactor regeneration, and the need for a stoichiometric amount of a co-substrate. The major challenge is to overcome substrate and product inhibition of the CHMO. To enable an economic process, cheap and efficient recycling of the cofactor NADPH without the need for an external co-substrate is necessary. We recently reported such a system, in which this CHMO is combined with an alcohol dehydrogenase (ADH from *Lactobacillus kefir* or a designed polyol dehydrogenase from *Rhodobacter sphaeroides*, left part of Scheme 1) to create self-sufficient cofactor



Scheme 1. Synthesis of oligo-ε-caprolactone (oligo-ε-CL) **4** through an enzyme cascade. First, cyclohexanol **1** is oxidized to cyclohexanone **2** by an alcohol dehydrogenase (ADH), followed by Baeyer–Villiger oxidation by a cyclohexanone monooxygenase (CHMO) to produce ε-CL (**3**), with concurrent recycling of the cofactor NADPH. Severe product inhibition is completely avoided and significantly higher productivity is achieved through the use of lipase CAL-A as a result of its acyltransferase activity in an aqueous system. The result is the formation of oligo-ε-CL only, without the formation of 6-hydroxycaproic acid.

recycling when starting from the readily available bulk chemical cyclohexanol.[10] These studies revealed that even at concentrations of 60 mM, severe product inhibition by ε-CL takes place in addition to modest inhibition by cyclohexanol **1** and cyclohexanone **2**.[8]

Substrate inhibition can easily be addressed through appropriate feeding of cyclohexanol. When both ADH and CHMO are expressed at sufficiently high levels, the concentration of cyclohexanone as an in situ formed intermediate also remains at low levels. By contrast, product inhibition and

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[**] We thank the "Deutsche Bundesstiftung Umwelt" for financial support (AZ 13268-32).

Supporting information for this article (including experimental details) is available on the WWW under http://dx.doi.org/10.1002/anie.201410633.

enzyme deactivation by the product ε-CL, particularly at higher concentrations, represents a major hurdle to overcome. Several routes to remove this fully water-miscible lactone by in situ extraction with an organic solvent or the use of adsorbents suffer substantially from low productivity and additional costs and environmental disadvantages.

Herein, we report an elegant solution in which the ε-CL produced by the one-pot two-step enzymatic method is directly subjected to in situ ring-opening oligomerization by using lipase CAL-A from *Candida antarctica*[9] (Scheme 1). Such a process solves the problem of enzyme inhibition and deactivation by ε-CL (**3**) at higher concentrations. Conveniently, the formed oligo-ε-CL is hydrophobic and can be isolated by extraction or precipitation. Although the enzymatic synthesis of polymers, especially enzymatic ring-opening polymerization, is well documented,[10] the formation of polyesters always required bulk organic solvents and notably the absence of water to prevent undesired hydrolysis.[11] Interestingly, lipase CAL-A has unique acyltransferase activity that enables efficient formation of the ester product despite the presence of bulk amounts of water.[12] Indeed, when we determined the hydrolytic activity of CAL-A towards 1 M of **3** in aqueous solution, we unexpectedly did not detect any hydrolysis of this ester. After a few hours of incubation, we observed the formation of a white precipitate in the aqueous phase (Figures S1, S2 in the Supporting Information), which after isolation and examination by gel permeation chromatography revealed that CAL-A catalysis led to oligomers of compound **3** with a maximum molecular weight of 1200 g mol^{-1} (Table 1, peak retention volume 16.4 mL; Figure 1). Both end groups can be specified from the expected enzymatic reaction mechanism, which is identical to the ring-opening polymerization performed in organic solvents.[10h] These end groups are thus a carboxyl moiety and an alcohol moiety.

Table 1: Characterization of oligo- and poly-ε-caprolactone obtained through different routes and analyzed by GPC.

Product[a]	Peak retention volume [mL]	M_n [Da]	M_w [Da]	M_w/M_n
PCL_{chem}	12.0	35.426	48.248	1.4
Oligo-ε-$CL_{Cascade}$	18.4	160	375	2.3
Oligo-ε-$CL_{CAL\text{-}A}$	16.4	615	1154	1.8
Oligo-ε-$CL_{Cond.}$	12.2	21.662	37.773	1.7

[a] PCL_{chem}: chemically synthesized; Oligo-ε-$CL_{Cascade}$: obtained by using all three enzymes starting from **1**; Oligo-ε-$CL_{CAL\text{-}A}$: obtained by using lipase A starting from 1 M **3**; Oligo-ε-CL_{Cond}: chemically polymerized Oligo-ε-$CL_{CAL\text{-}A}$.

This oligomer (oligo-ε-$CL_{CAL\text{-}A}$, Table 1) could be easily converted chemically into high-molecular-weight polymer (oligo-ε-$CL_{Cond.}$), which showed properties identical to standard chemically synthesized poly-ε-caprolactone (PCL_{chem}). ^{1}H NMR spectroscopy was used to confirm the identity of the two polymers (Figure S3–S5).

Next, we used ADH and CHMO as a mixture of two *E. coli* cell suspensions containing these recombinant enzymes and studied the effect of CAL-A in one-pot biocatalysis reactions at different cyclohexanol concentrations. The concentration of **3** strongly decreased in the presence of CAL-A compared to the biotransformation without the addition of CAL-A (Figure 2). The lower concentration of ε-CL presumably results from the CAL-A-catalyzed formation of oligo-ε-CL. With 1 g L^{-1} (per batch) of lyophilized CAL-A during the biotransformation, much lower concentrations of ε-CL were observed compared to reactions without CAL-A, in which only ε-CL was formed. This result strongly indicates the conversion of ε-CL into oligo-ε-CL, and the conversion was confirmed by ^{1}H NMR spectroscopy analysis (Figure S5). Furthermore, these experiments demonstrated that whole-cell biotransformation could be performed with all three enzymes (ADH, CHMO and CAL-A) in a single one-pot cascade reaction.

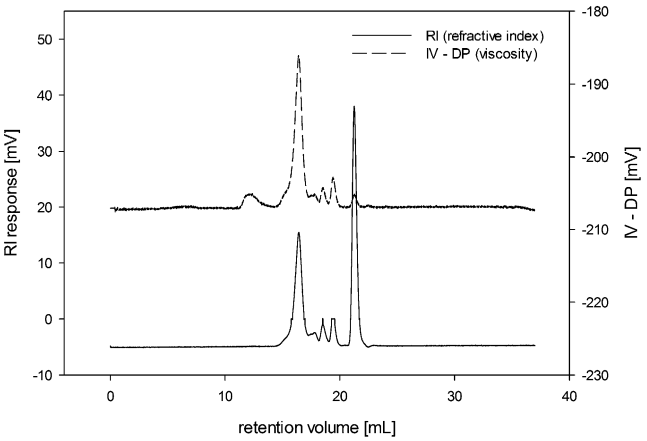

Figure 1. GPC chromatogram of oligo-ε-CL **4** synthesized directly by CAL-A (after extraction from the biocatalysis reaction). The single narrow peak represents the internal standard toluene; the various peaks between retention volumes 14.80 to 20.30 mL correspond to oligomer **4**.

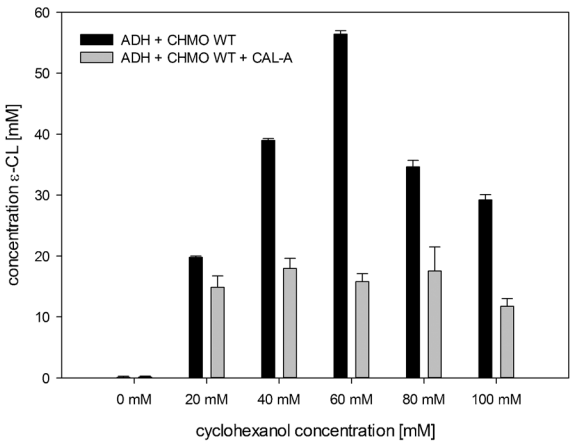

Figure 2. Comparison of the ε-CL concentration in biocatalysis by ADH and wild-type CHMO with (grey bars) and without (black bars) the addition of CAL-A. CAL-A leads to substantially lower levels of ε-CL as a result of the conversion of ε-CL into oligo-ε-CL.

In addition to inhibition of the CHMO by **3**, the instability of this enzyme is a further reason why large-scale enzymatic production of **3** has not so far been achieved. To overcome this problem, the double mutant C376L/M400I of CHMO was used since this double mutation has been reported[13] to confer higher oxidative and long-term stability on CHMO.

Preliminary experiments showed that expressing ADH and BVMO in separate *E. coli* cells is superior to expressing them in a single whole-cell system. Different ratios of whole-cell cultures containing recombinantly expressed ADH or the BVMO mutant C376L/M400I were thus used together for the biotransformation of cyclohexanol to ε-CL (Figure S7). The optimal ratio of ADH and CHMO is a very important factor because of differences in expression levels, specific activity, and stability between the two enzymes. At 60 mM **1**, the highest conversion was achieved at an ADH/CHMO ratio of 1:10.

Although the system is in principle self-sufficient with respect to cofactor recycling (Figure S6), we observed that the addition of acetone and glucose is useful for faster regeneration of NADPH at high substrate loading (Figure S8) since the two enzymes are expressed in separate *E. coli* cells (Figure S6). A stoichiometric amount of acetone gave a conversion of 95%.

Next, preparative-scale biocatalysis with substrate feeding (to avoid inhibition by **1**) was performed by using the combined system (ADH, CHMO double mutant C376L/M400I, and CAL-A) and after 48 h, complete conversion of 200 mM of **1** was achieved (Table 2). At 300 mM and 500 mM of substrate with the same amounts of the enzymes, 74% and 43% conversion, respectively, were observed. Analysis of the product from the 200 mM reaction by GC analysis and ^{1}H NMR spectroscopy confirmed that 75% oligomer was formed with 25% ε-CL present as co-product, which obviously can be easily co-extracted with the oligomer.

Table 2: Conversion of cyclohexanol (**1**) through the enzymatic cascade reaction (Scheme 1).

1 (mM)	ADH [mU mg_{cells}^{-1}]	CHMO [mU mg_{cells}^{-1}]	CAL-A [kU][a]	Conversion [%][b]
200	1.78 ± 0.20	3.32 ± 0.18	30	99.0 ± 0.7
300	1.89 ± 0.17	3.56 ± 0.10	30	74.0 ± 1.3
500	1.93 ± 0.23	3.45 ± 0.20	30	43.0 ± 2.1

[a] Determined against the standard substrate tributyrin. [b] Based on consumption of **1** as determined by GC analysis.

In summary, we disclose a biocatalytic approach to the production of ε-caprolactone followed by direct in situ transformation into oligo-ε-caprolactone. This method leads to high product yields at substrate concentrations of more than 200 mM. This elegant setup enables the combination of monomer formation with direct in situ oligomer synthesis at substrate concentrations exceeding those of previous studies by at least six-fold. Future work will address further improvement of the overall productivity by using bioprocess engineering. Independently from our work, the Kroutil group very recently reported a related cascade process with ε-CL as an intermediate for the production of monomeric 6-aminohexanoic acid.[14] This further underlines the power of BVMOs for organic synthesis and especially the importance of enzyme cascade synthesis of ε-CL as a starting point to address different fields of application.

Received: October 31, 2014
Revised: December 3, 2014
Published online: ■■ ■■, ■■■■

Keywords: Baeyer–Villiger monooxygenases · cascade reactions · ε-caprolactone · enzyme catalysis · polymer synthesis

[1] a) B. M. Nestl, S. C. Hammer, B. A. Nebel, B. Hauer, *Angew. Chem. Int. Ed.* **2014**, *53*, 3070 – 3095; *Angew. Chem.* **2014**, *126*, 3132 – 3158; b) U. T. Bornscheuer, G. W. Huisman, R. J. Kazlauskas, S. Lutz, J. C. Moore, K. Robins, *Nature* **2012**, *485*, 185 – 194; c) M. Breuer, K. Ditrich, T. Habicher, B. Hauer, M. Keßeler, R. Stürmer, T. Zelinski, *Angew. Chem. Int. Ed.* **2004**, *43*, 788 – 824; *Angew. Chem.* **2004**, *116*, 806 – 843; d) H. E. Schoemaker, D. Mink, M. G. Wubbolts, *Science* **2003**, *299*, 1694 – 1697; e) A. Schmid, J. S. Dordick, B. Hauer, A. Kiener, M. Wubbolts, B. Witholt, *Nature* **2001**, *409*, 258 – 268.

[2] a) M. Kobayashi, T. Nagasawa, H. Yamada, *Trends Biotechnol.* **1992**, *10*, 402 – 408; b) C. E. Nakamura, G. M. Whited, *Curr. Opin. Biotechnol.* **2003**, *14*, 454 – 459; c) H. Yim, R. Haselbeck, W. Niu, C. Pujol-Baxley, A. Burgard, J. Boldt, J. Khandurina, J. D. Trawick, R. E. Osterhout, R. Stephen, J. Estadilla, S. Teisan, H. B. Schreyer, S. Andrae, T. H. Yang, S. Y. Lee, M. J. Burk, S. Van Dien, *Nat. Chem. Biol.* **2011**, *7*, 445 – 452.

[3] K. Weissermel, H. J. Arpe, *Industrial Organic Chemistry*, 4th ed., Wiley-VCH, Weinheim, **2003**.

[4] a) K. Balke, M. Kadow, H. Mallin, S. Sass, U. T. Bornscheuer, *Org. Biomol. Chem.* **2012**, *10*, 6249 – 6265; b) H. Leisch, K. Morley, P. C. K. Lau, *Chem. Rev.* **2011**, *111*, 4165 – 4222.

[5] N. A. Donoghue, D. B. Norris, P. W. Trudgill, *Eur. J. Biochem.* **1976**, *63*, 175 – 192.

[6] J. D. Stewart, K. W. Reed, M. M. Kayser, *J. Chem. Soc. Perkin Trans. 1* **1996**, 755 – 757.

[7] W.-H. Lee, Y.-C. Park, D.-H. Lee, K. Park, J.-H. Seo, *Appl. Biochem. Biotechnol.* **2005**, *123*, 827 – 836.

[8] S. Staudt, U. T. Bornscheuer, U. Menyes, W. Hummel, H. Gröger, *Enzyme Microb. Technol.* **2013**, *53*, 288 – 292; b) H. Wulf, H. Mallin, U. T. Bornscheuer, *Enzyme Microb. Technol.* **2013**, *53*, 283 – 287.

[9] U. T. Bornscheuer, U. Menyes, S. Schmidt, R. Wardenga, S. Borchert, European Patent Application, EP14152848.9, **2014**.

[10] a) S. Kobayashi, K. Takeya, S. Suda, H. Uyama, *Macromol. Chem. Phys.* **1998**, *199*, 1729 – 1736; b) M. Labet, W. Thielemans, *Chem. Soc. Rev.* **2009**, *38*, 3484 – 3504; c) S. Kobayashi, *J. Polym. Sci. Part A* **1999**, *37*, 3041 – 3056; d) S. Kobayashi, H. Uyama, M. Ohmae, *Bull. Chem. Soc. Jpn.* **2001**, *74*, 613 – 635; e) R. T. MacDonald, S. K. Pulapura, Y. Y. Svirkin, R. A. Gross, D. L. Kaplan, J. Akkara, G. Swift, S. Wolk, *Macromolecules* **1995**, *28*, 73 – 78; f) G. A. R. Nobes, R. J. Kazlauskas, R. H. Marchessault, *Macromolecules* **1996**, *29*, 4829 – 4833; g) G. M. Sivalingam, G. Madras, *Biomacromolecules* **2004**, *5*, 603 – 609; h) H. Dong, S. G. Cao, Z. Q. Li, S. P. Han, D. L. You, J. C. Shen, *J. Polym. Sci. Part A* **1999**, *37*, 1265 – 1275; i) H. Uyama, S. Kobayashi, *Chem. Lett.* **1993**, *22*, 1149 – 1150.

[11] a) S. Hari Krishna, *Biotechnol. Adv.* **2002**, *20*, 239 – 267; b) I. K. Varma, A.-C. Albertsson, R. Rajkhowa, R. K. Srivastava, *Prog. Polym. Sci.* **2005**, *30*, 949 – 981; c) S. Namekawa, H. Uyama, S. Kobayashi, *Polym. J.* **1998**, *30*, 269 – 271.

[12] a) J. Müller, B. Fredrich, C. Kohlmann, L. Maksym, U. T. Bornscheuer, *Eur. J. Lipid Sci. Technol.* **2014**, *116*, 232 – 236; b) R. Brenneis, B. Baeck, *Biotechnol. Lett.* **2012**, *34*, 1459 – 1463; c) D. Briand, E. Dubreucq, P. Galzy, *J. Am. Oil Chem. Soc.* **1995**, *72*, 1367 – 1373; d) D. Briand, E. Dubreucq, P. Galzy, *Eur. J. Biochem.* **1995**, *228*, 169 – 175.

[13] D. J. Opperman, M. T. Reetz, *ChemBioChem* **2010**, *11*, 2589 – 2596.

[14] J. H. Sattler, M. Fuchs, F. G. Mutti, B. Grischek, P. Engel, J. Pfeffer, J. M. Woodley, W. Kroutil, *Angew. Chem. Int. Ed.* **2014**, DOI: 10.1002/anie.201409227; *Angew. Chem.* **2014**, DOI: 10.1002/ange.201409227.

Communications

Biocatalysis

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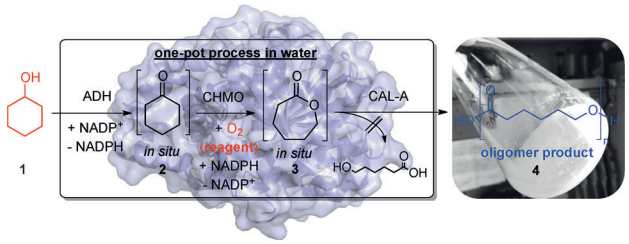
An Enzyme Cascade Synthesis of ε-Caprolactone and its Oligomers



Let's polymerize! Oligo-ε-caprolactone was produced in a one-pot enzymatic cascade synthesis starting from cyclohexanol. In the first step, cyclohexanol is oxidized by an alcohol dehydrogenase (ADH) in combination with the cyclohexanone monooxygenase (CHMO) from *Acinetobacter calcoaceticus*, followed by direct ring-opening oligomerization of ε-caprolactone in an exclusively aqueous phase by lipase A from *Candida antarctica* (CAL-A).