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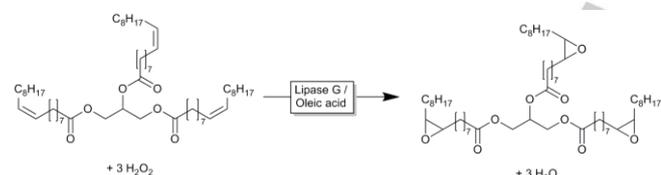
Deep eutectic solvents enable more robust chemoenzymatic epoxidation reactions

Pengfei Zhou,^[a] Xuping Wang,^[b] Chaoxi Zeng,^[b] Weifei Wang,^[b] Bo Yang,^[a] Frank Hollmann,^[c] and Yonghua Wang^{*[b]}

Abstract: A chemoenzymatic method for the production of epoxidized vegetable oils is presented. The unique combination of the commercial lipase G from *Penicillium camembertii* with certain deep eutectic solvents enables efficient production of epoxidised vegetable oils.

Epoxidized vegetable oils are promising substitutes for phthalates as plasticizers,^[1] lubricants^[2] and coatings.^[3] Traditional synthetic routes for the epoxidation of unsaturated triglycerides entail stoichiometric use of peracids such as peracetic acid under acidic reaction conditions. The challenges of these methods reside with the rather corrosive reaction conditions, their poor atom efficiency, the relatively harsh reaction conditions and the considerable amounts of waste generated.

In situ generation of the reactive peracid from catalytic amounts of carboxylic acids and hydrogen peroxide (H₂O₂) using hydrolases as catalysts represents a very promising solution to the aforementioned challenges (Scheme 1).^[4]



Scheme 1. Chemoenzymatic epoxidation of glyceryl trioleate.

First reported in 1990 by Björkling and coworkers,^[5] this perhydrolase activity has found widespread application in the chemoenzymatic synthesis of epoxides^[6] and lactones.^[7]

One challenge to be faced in the synthesis of epoxidized esters is the hydrolytic activity of the enzymes leading to epoxidized fatty acids in the presence of water. Therefore, if epoxidized esters such as epoxidized vegetable oils are desired, particular attention has to be

paid to the exclusion of trace amounts of water.

Very recently, we reported that lipase G from *Penicillium camembertii* exhibits no activity towards triglycerides making it a promising catalysts for the direct epoxidation of epoxidised triglycerides.^[8] Unfortunately, however, lipase G is not very robust under the reaction conditions limiting its practical applicability. Inspired by a very recent contribution by the previous publications,^[9] we decided to evaluate some natural deep eutectic solvents as stabilizers for lipase G. In a first set of experiments, we compared different DESs as reaction media for the chemoenzymatic epoxidation of glyceryl trioleate (GT) to epoxidized glyceryl trioleate (EGT) (Figure 1). It is worth mentioning here that the acid number of the GT used in this study was comparably high (1 mg(KOH) per gram of GT corresponding to an oleic acid concentration in GT of approx. 19 mM). The presence of free oleic acid in the starting material made any external addition of further (fatty) acids unnecessary.

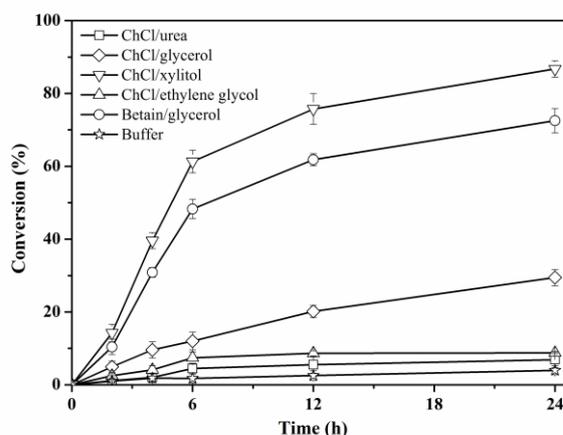


Figure 1. Time courses of the chemoenzymatic epoxidation of GT to EGT in different reaction media. General conditions: reactions were performed in a 10 mL Erlenmeyer flask at 40 °C for 24 hours (h) with magnetic stirring (500 rpm). The reaction mixture contained glyceryl trioleate (1.69 mmol), hydrogen peroxide (30%, 5.08 mmol), lipase G (150 mg) and DESs (1.5 g).

Pleasingly, DESs had a very beneficial effect on the overall activity. Thus, the EGT formation rate increased more than 22-fold from aqueous reaction conditions (7 $\mu\text{M}\cdot\text{h}^{-1}$) to 172 $\mu\text{M}\cdot\text{h}^{-1}$ using ChCl/xylitol as reaction phase. Furthermore, the reaction in buffer ceased after 6 hours while in the presence of ChCl/xylitol it smoothly proceeded to almost full conversion. We attribute this to a stabilizing effect of ChCl/xylitol on lipase G. It is worth mentioning here that reactions using the thermally inactivated biocatalyst under otherwise

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identical conditions yielded no significant product formation.

To gain further insights into the effect of ChCl/xylitol we determined the stability of lipase G under the reaction conditions (Figure 2).

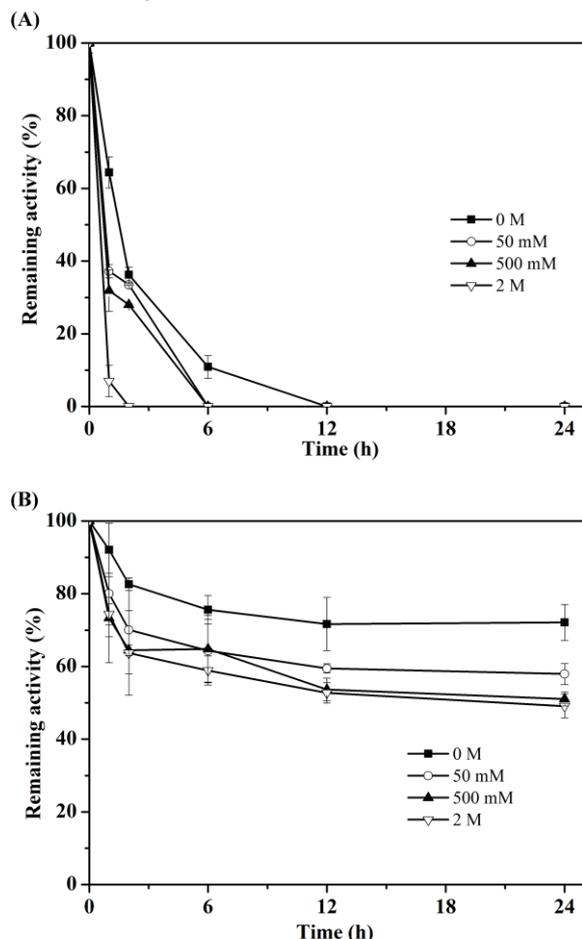


Figure 2. Effects of hydrogen peroxide on perhydrolysis activity of lipase G in the presence of phosphate buffer (a) and ChCl/xylitol (b). General conditions: Lipase G was soaked in 50 mM phosphate buffer pH 6.0 or ChCl/xylitol containing H₂O₂: 0 mM, 50 mM, 500 mM and 2 M.

Lipase G exhibited a comparably poor stability in buffer only (half-life time $t_{1/2} \approx 2$ hours). In the presence of H₂O₂, this effect was even more pronounced showing a H₂O₂-concentration dependent acceleration of lipase G inactivation. ChCl/xylitol, however, significantly alleviated both, the intrinsic instability of lipase G and the H₂O₂-dependent inactivation. Hence, even in the presence of 2 M H₂O₂ more than 50% of the original activity was retained after 24 hours. This result further gave evidence that DES effectively enhance the stability of lipase G.

The stabilization of lipase G in DES was further studied by circular dichroism spectroscopy (Table 1). Lipase G in buffer with or without H₂O₂ folds in α -helix and β -strand secondary structures as well as a rise random coil compared to native lipase (Table 1), which agree with a partially unfolded state of protein.^[10] It is noticeable how the secondary structure of the enzyme in

DESS is very similar to that of the native structure (Table 1). Interestingly, a marked enhancement in the β -strand content was observed in DESS with or without H₂O₂. We attribute this to a decrease in the amount of water molecules around the protein surface similar to the effect of lyophilization on the structure of lipase.^[11] In this way, DESS could be involved in the preservation of the essential water in the microenvironment of the enzyme to stabilize it.

Table 1. Secondary structure percentages of lipase G in different media and incubation at 30°C for 24 hours. The secondary structure results were analyzed using the CDNN tool.

Medium*	α -helix (%)	β -strand (%)	Turn (%)	Random coil (%)
Native lipase G	21.2	7.8	30.3	40.7
KP ₁ ^[a]	7.7	7.5	32.3	51.5
KP ₁ /H ₂ O ₂ ^[b]	5.5	7.4	32.5	52.6
ChCl/xylitol ^[c]	7.8	16	31.2	45
ChCl/xylitol/H ₂ O ₂ ^[d]	7.9	24.4	28.7	39.4

*a: lipase G incubated in 50 mM pH 6.0 phosphate buffer; b: lipase G incubated in 50 mM pH 6.0 phosphate buffer contained 1 M H₂O₂; c: lipase G incubated in ChCl/xylitol DESS; d: lipase G incubated in ChCl/xylitol DESS contained 1 M H₂O₂.

Next, we set off to characterize the reaction conditions. Therefore, we systematically varied the reaction temperature, the H₂O₂-concentration and the amount of ChCl/xylitol (Figure S1). 40°C proved to be the optimal reaction temperature. We attribute the rather counterintuitive lower productivity at elevated temperatures to inactivation of the biocatalyst.

Possibly also partial H₂O₂ decomposition may account for this observation. Similarly, a H₂O₂ surplus over the substrate of approx. 3 times molar excess appeared to be optimal for the chemoenzymatic epoxidation reaction. Possibly, this can be rationalized by the increasing peracid formation rate together with this increasing enzyme inactivation with increasing H₂O₂-concentrations. Finally, increasing the concentration of ChCl/xylitol above 1:1 (w/w) did not yield significant further stabilization of the enzyme.

Finally, we have compared the chemoenzymatic epoxidation of GT in the presence of ChCl/xylitol using CalB or lipase G as catalysts. Under otherwise identical conditions, lipase G gave significantly higher yields of the desired epoxide product (as estimated from the characteristic ¹H NMR signals for the C=C-double bond and the oxirane signals at 5.3 ppm and 2.9 ppm, respectively). Furthermore, the lipase G-catalyzed reactions yielded no hydrolysis products whereas the reaction mixtures with CalB showed an amount of the sn-1,2-glyceride product (5.1 ppm and 3.7 ppm in Figure 3).

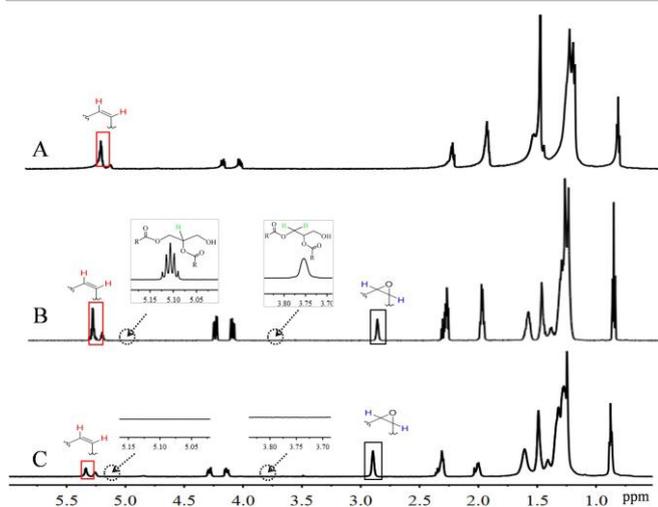


Figure 3. ^1H NMR spectra of fresh glyceryl trioleate (A) compared to the products of the chemoenzymatic epoxidation using Novozym 435 (B) and lipase G (C).

FT-IR spectroscopy qualitatively supports these findings as the characteristic C=C-double bond stretching and bending absorptions at 3007 cm^{-1} , respectively disappear whereas the characteristic oxirane band (831 cm^{-1}) appears (Figure 4).

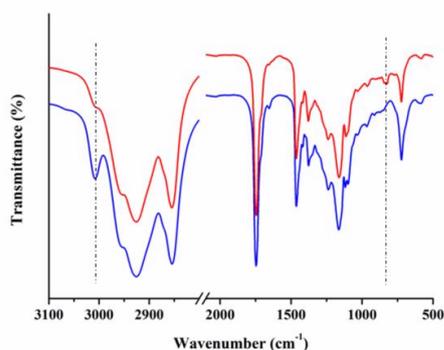


Figure 4. FT-IR spectra of glyceryl trioleate before (blue) and after the epoxidation (red). The characteristic peaks of the C=C-double bond (3007 cm^{-1}) and for the oxirane ring (831 cm^{-1}) are highlighted.

Overall, in the current contribution we have demonstrated the beneficial effect of using DESs as co-solvents to promote chemoenzymatic epoxidation reactions. The preliminary results demonstrated that highly catalysis efficient for epoxidized glyceryl trioleate was observed due to improving the stability of lipase G in DESs ChCl/xylitol. The molecular reason for the dramatic acceleration observed remains mysterious and further mechanistic studies are needed to fully understand (and even further optimize) the reaction system. Furthermore, we have demonstrated that lipase G is a promising biocatalyst to obtain high chemoselectivity for this reaction. Nevertheless, despite the very early stage of development of the reaction system proposed, already comparably high productivities of more than 8

$\text{g}_{\text{product}}/\text{g}_{\text{biocatalysts}}$ have been achieved pointing towards a possible economical applicability of an optimized reaction system.

Acknowledgements

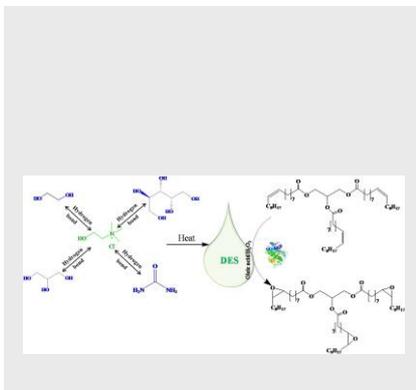
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COMMUNICATION

The combination of lipase G with deep eutectic solvents enables robust epoxidation of glyceryl trioleate without the undesired hydrolysis side-reaction.



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