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Effects of Protein Content on Pickering-assisted Interfacial Enzyme Catalysis

Christoph Plikat,[a] Anja Drews,[b] and Marion B. Ansorge-Schumacher*[a]

In recent years, water-in-oil Pickering emulsions have been introduced as promising reaction systems for multiphase enzyme catalysis, in particular lipase-catalyzed esterification and transesterification. Here, we for the first time gained insight into the effects that the presence of the proteins exert on the fineness and stability of the emulsion system and thus, the catalytic performance. We demonstrated a distinct, concentration- and enzyme-dependent decrease of droplet sizes in the dispersed phase, accompanied by decreasing stability against coalescence. This was due to a probably quantitative adsorption of lipases at the interphase intercalating the solid particles. Destabilization was reduced slightly at increased particle content and increased volume portion of the dispersed phase, respectively. However, the low tolerable lipase concentrations in the reaction system considerably limited its productivity. Thus, our study points at the enzyme content, or rather enzyme location, in Pickering emulsions being the crucial parameter for optimizing catalytic performance.

Introduction

In recent years, Pickering emulsions (PE) have been recognized as promising reaction systems for both chemo- and biocatalysis.[1] Comprising solvent droplets dispersed in a second immiscible solvent and stabilized by micro- or nanoparticles, PE provide conditions decidedly favorable for multiphase reactions. They enable broad contact between phases and thus fast exchange of reactants. At the same time, they efficiently keep away vulnerable compounds from detrimental solvents. Distinct from emulsifying surfactants, the solid particles do not spontaneously detach from the interface due to an exceedingly large energy barrier[2] and thus can obviate product spoiling as a major drawback of reactions performed in surfactant-induced emulsions.

PE for catalytic uses may involve various solvents and types of particles, with the catalysts dissolved in the solvent phase or bound to the particles at the interface, yielding so-called Pickering-assisted or Pickering interfacial catalysis, respectively.[1a] Biocatalysis most commonly uses dissolved isolated enzymes in water-in-oil (w/o)-type PE, i.e. salt-buffered aqueous solution dispersed in a water-immiscible organic solvent, stabilized with surface-modified silica-based particles. Presently, the choice of enzymes focuses on lipases as versatile and robust catalysts of esterification and transesterification reactions,[1] although our group also demonstrated compatibility with more sophisticated enzymes, when we proposed the use of PE for biocatalysis for the first time.[3]

Employment of PE for multiphase catalysis often improves the activity of catalysts compared to standard, i.e. mixed biphasic systems considerably. The actual performance predominantly depends on the (average) size of the droplets building the dispersed phase, as indicated by the relevant literature.[1] A small diameter ensures a large interface and thus appropriate mass exchange between phases, and a high reaction efficiency. Consequently, a constantly small droplet size over time ensures a constantly high reaction efficiency and thus high product yields. The droplet diameter itself depends on the hydrodynamic conditions during PE formation, and on the PE composition, in particular the solvent ratios and the particle size, type and concentration.[4] Small diameters generally arise from high dispersion energy,[5] a small volume fraction of the dispersed phase in the whole system,[5a] and a high concentration of small stabilizing particles with rough surfaces.[6] The features strongly interact. PE stability, in terms of droplet size constancy over time, mainly depends on the solvent combination and the properties of the particles involved, in particular their surface hydrophilicity/hydrophobicity.[7]

Considering the importance of droplet sizes for the performance of catalytic PE and their multiple and complex dependencies on the system composition, it is quite astonishing that the effects of enzyme presence in catalytic active PE have hardly been investigated so far. In a previous study, we observed a distinct impact of protein addition on the drop size distribution in a PE as well as on the filterability of the emulsion over a membrane.[8] We attributed these effects to the adsorption of the proteins between particles at the interface. It

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is well known that proteins in general and lipases in particular tend to adsorb to liquid aqueous-organic interfaces.[9] Lipases even require this contact for optimal catalytic activity.[10] Some other proteins, most prominently whey proteins like κ-casein or β-lactoglobulin, stabilize emulsions of the oil-in-water (o/w) -type on their own.[11] It is therefore reasonable to expect that in biocatalytically active PE (BioPE) dissolved enzymes settle at the interface and thus support a Pickering-assisted interfacial catalysis, where the enzyme is not bound to the stabilizing particles, but nevertheless located at the interface, rather than real Pickering-assisted catalysis, where the enzyme acts within the dispersed phase.

Here, we systematically assessed the impact of the protein content on droplet formation and stability in a typical w/o BioPE. Different concentrations of the molecular distinct lipases A (CALA) and B (CALB) from *Pseudozyma aphidis* (formerly *Candida antarctica*) and the lipase from *Thermomyces (Humicola) lanuginosa* (TLL) were introduced to a PE stabilized with silanized and fluorescence labelled silica particles. The resulting mean droplet sizes were determined upon PE formation and after 24 hours stirring. Likewise, we investigated possible cross-effects of lipase presence on the impact of particle content, solvent ratio and dispersion speed on the droplet sizes. The actual relation of the droplet sizes and the emulsification process to catalytic activity was monitored for CALA using transesterification of 1-phenylethanol with vinyl butyrate as model reaction.

Results and Discussion

In accordance with our previous observations,[8] the addition of lipases to a PE with fixed phase ratio (10% dispersed phase per volume v_{dp}/v) and particle content (30 g per liter of dispersed phase, $g \cdot L^{-1}_{dp}$) had a distinct effect on the initial droplet sizes of the dispersed (aqueous) phase (Figure 1). Compared to the protein-free system the droplet diameter in the freshly prepared PE was by at least 10.9% lower. The extent of the decrease depended on both lipase concentration and type. It was highest upon addition of TLL and lowest with CALB. The higher the enzyme concentration was the smaller was the initial droplet size. At an enzyme concentration of 5 $g \cdot L^{-1}_{dp}$, we were not able to detect droplets with either TLL or CALB.

The presence of lipases also affected the emulsion stability in terms of droplet size constancy over time. In contrast to the protein-free PE, which was rather stable, the droplet diameters increased within 24 hours by at least 20%, 33,4% and 416%, respectively, when CALA, CALB or TLL were added to the system. Again, the effect intensified with increasing protein concentration. Large droplets even emerged with CALB at a concentration of 5 $g \cdot L^{-1}_{dp}$, while such a belated emulsion formation did not occur with the same concentration of TLL.

The observed response of droplet size and stability to the presence of lipases suggests that in PE these enzymes behave as emulsifiers. True, as stated before,[8] the lipases were not able to stabilize the here investigated w/o emulsions alone demonstrating that these proteins lack distinct features of typical

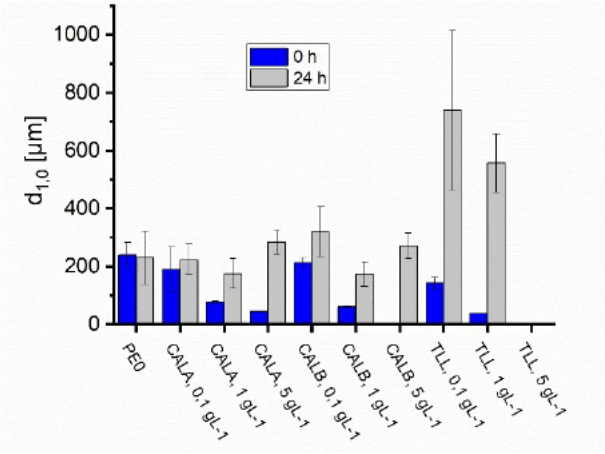

Figure 1. Mean diameters ($d_{1,0}$) of aqueous droplets in w/o PE at a phase ratio of 10% (v_{dp}/v) buffer in CPME and a particle content of 30 $g \cdot L^{-1}_{dp}$ without additional protein (PE_0) and in the presence of different concentrations of CALA, CALB and TLL (0.1, 1 and 5 $g \cdot L^{-1}_{dp}$) directly after emulsion formation and after 24 hours stirring at 440 rpm. Standard deviations refer to triplicate experiments.

emulsifiers like low-molecular mass surfactants, solid particles or β-lactoglobulin.[4] Amphiphilic surface arrangements like in whey proteins or typical surfactants in fact are missing. The globular structures of approximately $3 \cdot 4 \cdot 5\ nm^3$ (CALB and CALA) and $3.5 \cdot 4.5 \cdot 5\ nm^3$[12] are within the size limit for PE stabilization, but too small to form a PE with the here applied emulsification process.[13] Nevertheless, the enzymes can still interact with the interface and interfere with the stabilizing effect of the silica particles.

In a review on the mechanisms operative in surfactant-, particle- or protein-mediated emulsification, Tcholakowa and co-workers explain that, over a wide experimental range, the initial droplet size of the dispersed phase of an emulsion is mainly determined by the emulsifier content, at which an almost complete adsorption layer is formed on the droplet surface.[4] At constant volume fraction of the dispersed phase, the droplet size decreases with increasing emulsifier concentration, because the emulsifier suffices to cover a larger interfacial area. In PE, the droplet size also decreases with decreasing particle size, when the particle content remains constant, because smaller particles can achieve full interfacial coverage only at a denser packing.[7] On this background, our observation that the addition of increasing amounts of lipase to an otherwise unaltered PE gradually decreases the droplet diameter confirms the enzymes acting as additional emulsifiers. Despite their low concentration compared to the employed particles, they exert a strong effect, which might be due to their by two orders of magnitude smaller particle sizes.

Intercalation of the lipases as emulsifiers between the stabilizing silica particles at the droplet interfaces also explains their observed devastating effects on the long-term stability of our PE. Emulsion stability, in terms of increasing droplet sizes over time with an ultimate emulsion collapse, generally depends on the ability of an emulsifier to maintain the liquid

films between neighboring dispersed droplets preventing coalescence.[4] Emulsifying particles achieve this via capillary forces between the particles on both sides of the film.[14] The film ruptures when the two opposite film surfaces approach each other at any point below a critical thickness, upon which the droplets coalesce. The fluid interfaces in the film acquire a distinct shape so that the film thickness varies at different points. The shape depends on various factors such as capillary pressure across the fluid interface, particle radius and hydrophobicity, inter-particle distance, and oil-water interfacial tension.[4] Introduction of protein molecules into a layer of solid particles or substitution of particles within the layer by proteins must have a strong effect on several of these factors. The resulting film shape might facilitate falling below the critical thickness at some point and thus decrease the emulsion stability. An increasing protein concentration must pronounce the effect, which is in accordance with the observations in our study.

Eventually, Tcholakova and co-workers noted that globular proteins could behave similar to solid particles in emulsions.[15] Thus, individual particulate features of the three lipases might explain the observed mutual differences in their impact on the initial droplet size and emulsion stability. Binks and Lumsdon reported a dependence of the droplet size in PE on the wettability of the particle surface, which they directly related to the ratio of hydrophilic and hydrophobic functionalities on the material.[7] In w/o PE, the droplet size increased with increasing surface hydrophobicity. Hydrophilicity plots according to Kyte and Doolittle[16] on the available monomeric crystal structures of CALA, CALB and TLL, respectively, reveal obvious differences in the overall surface hydrophilicity/hydrophobicity also for these enzymes (Figure 2). The overall hydrophilicity on the enzyme surfaces, marked as blue residues, seems to be highest with TLL (Figure 2C) and lowest with CALB (Figure 2B), which agrees with our observation that addition of TLL to the emulsion system reduces the droplet diameter most, while CALB has the least impact. Thus, these differences in the mean surface hydrophilicity of the enzymes might contribute to their different impact on the initial droplet size at a fixed enzyme concentration.

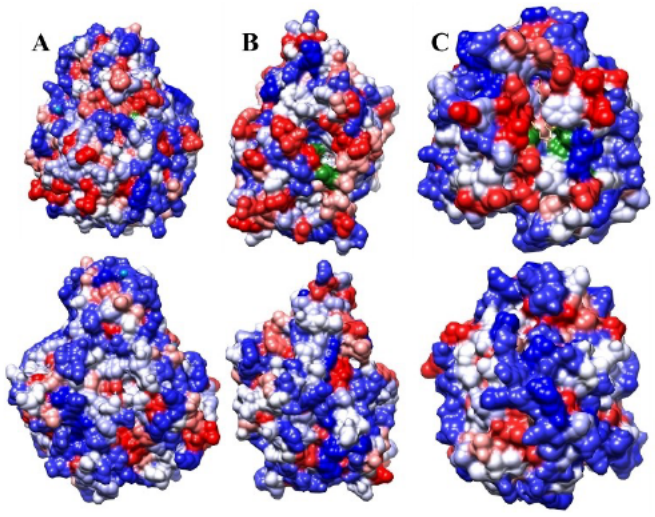

Figure 2. Lipase hydrophilicity plots of the lipase surfaces in Kyte-Doolittle scale. blue: hydrophilic amino acids, red: hydrophobic amino acid, white: neutral amino acids, green: amino acid residues comprising the catalytic triad; color intensity denotes the scale; for illustration of the Janus-like surface characteristic, front sides (including the active site) are shown in the upper row, back sides in the lower row. A: CALA based on RCSB Ref. 3GUU; B: CALB based on RCSB Ref. 4K6G; C: TLL based on RCSB Ref. 1GT6. Structures were obtained with software Chimera 1.12.

Binks and Lumsdon also related the stability of PE to the particle wettability postulating a sharp maximum for intermediate hydrophobicity and a shallower destabilizing effect towards increasing hydrophobicity than towards increasing hydrophilicity.[7] This again nicely agrees with our finding that CALA, the enzyme with a hydrophobicity intermediate between TLL and CALB, causes the least coalescence, while the most hydrophilic of the enzymes, TLL, has the most destabilizing effect. The observation also implies that, for the investigated conditions, the optimum mean hydrophilicity for the protein surfaces lies somewhere between that of TLL and CALA.

However, Binks and Lumsdon also remarked that relatively small changes in the composition of particle surfaces bring about a large change in emulsion stability.[7] We therefore expect that surface effects on emulsion stability by lipases cannot simply be described by the overall surface hydrophilicity/hydrophobicity alone. For one thing, the distribution of hydrophilic and hydrophobic functionalities on the enzyme surfaces is much more inhomogeneous than on typical PE stabilizing particles such as silica. Apart from a Janus-like arrangement, i.e. an overload of hydrophilic or hydrophobic amino acid residues on the front side (upper part in Figure 2) and backside (lower part in Figure 2) of the molecules, respectively, the contributing amino acid residues form differently sized enzyme-specific hydrophilic and hydrophobic clusters, which might have locally different impact. In addition, lipases in particular display a distinctly hydrophobic active site, which in an aqueous-organic system preferably orients towards the organic phase. Many lipases, such as TLL and CALA, possess a lid structure near the active site that specifically adsorbs to the interface and thus exposes the catalytic center.[17] Consequently, the ratio of surface hydrophilicity/hydrophobicity on the "lid side" of the enzyme molecule might have a stronger influence on its effects on droplet size and stability in PE than the overall mean hydrophilicity. In comparison with lipases without a lid structure such as CALB,[18] the effects would be more distinct. This is in agreement with our observations here and in our previous study that CALB affected PE more like a non-catalytic protein than like the other lipases.[8] Finally, structural refolding, which lipases usually undergo upon contact with an interface[19] might change the effective distribution of hydrophilic and hydrophobic residues on the lipase surface and consequently their particulate properties as emulsifiers. During aging of the PE, even further spreading of the molecule and formation of intra- and intermolecular cysteine-bridges might be possible,[9] which could further change droplet sizes and emulsion stability over time.

Overall, proteins possess a strong affinity to any kind of hydrophobic interfaces, which physical and chemical molecular

properties of the proteins such as size, shape, charges, location and distribution of charges as well as flexibility determine.[11] In principle, they might affect PE properties by adsorption either to the particle surface or to the liquid interface. However, the here observed devastating effects of 5 $g \cdot L^{-1}_{dp}$ TLL and CALB, respectively, on PE formation indicate rather a direct interaction with the liquid interface, which at high concentrations prevents particles from adsorption. Kinetics of particle adsorption to interfaces are considerably slower than that of protein adsorption.[4] Thus, it can be expected, that in a competition situation for interfacial space, proteins bind more than particles. In case of proteins, which do not stabilize PE of their own such as the lipases in our experiments, formation of a stable emulsion is probably impossible when the particle concentration at the interface falls below a critical concentration. Obviously, this occurred at a concentration of 5 $g \cdot L^{-1}_{dp}$ TLL and CALB. The observation corresponds to the previously discussed stronger destabilizing effects of these two enzymes compared to CALA. Assuming particle substitution at the interface by lipase molecules also agrees with our previous finding free silica particles at a high protein content.[8]

In a subsequent experiment, we investigated how strongly lipase addition changed the typical effects of solid particles on droplet sizes and PE-stability by varying the particle content to half (15 $g \cdot L^{-1}_{dp}$) and double (60 $g \cdot L^{-1}_{dp}$) the amount, respectively, at fixed lipase concentration and phase ratio. To ensure a distinct enzyme effect as well as PE formation with all three investigated enzymes, the lipase concentration was set to 1 $g \cdot L_{dp}^{-1}$.

As illustrated in Figure 3, at a particle content of 30 $g \cdot L^{-1}_{dp}$ the initial droplet sizes of the dispersed phase in the PE were in good agreement with the values obtained in the previous experiments at the set lipase concentration. At half as well as double the particle content related to the dispersed phase, hardly any differences in the droplet sizes arose. Outliers were found only for CALB at the lowest and for TLL at the highest particle content, where the obtained droplet sizes were considerably larger (about 227 μm) or smaller (about 36 μm), respectively. In contrast, PE-stability increased with increasing particle content, albeit to a very different extent. At the same time, the polydispersity of the droplets increased, as indicated by the increasing standard deviation in the triplicate experiments.

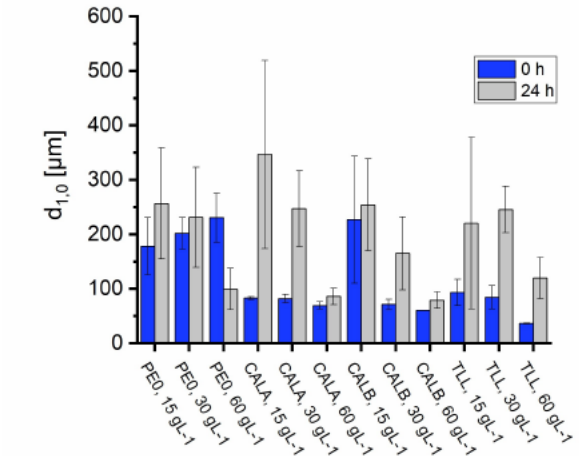

Figure 3. Mean diameters ($d_{1,0}$) of aqueous droplets in w/o PE at a phase ratio of 10% (v/v) buffer in CPME and a particle content of 15, 30 and 60 $g \cdot L^{-1}_{dp}$ without additional protein (PE_0) and in the presence of 1 $g \cdot L^{-1}_{dp}$ CALA, CALB and TLL, respectively, directly after emulsion formation and after 24 hours stirring at 440 rpm. Standard deviations refer to triplicate experiments.

At first glance, our results imply an overruling effect of lipase addition over the previously stated effects of particle content on the initial droplet size. However, the effect was not only absent in the presence of lipases, but also was not detectable in our protein-free PE. This missing response of the protein-free PE to the changes in particle content indicates that in our experimental set-up the particle content always remained within the emulsifier-rich regime of emulsification, despite a particle content of only 15 $g \cdot L^{-1}_{dp}$.

In stable emulsions, two qualitatively different regimes of emulsification, the emulsifier-poor and the emulsifier-rich regime, are generally distinguished.[4] In the emulsifier-poor regime, i.e. at low emulsifier content, the droplet size of the dispersed phase rapidly increases with decreasing amount of emulsifier because of droplet coalescence under these conditions. In the emulsifier-rich regime, i.e. at high emulsifier concentrations, the droplet size is practically independent of the emulsifier amount and due to the closed layer of emulsifier adsorbed to the interface mostly determined by the emulsification process. The higher the dispersion speed, the lower are the droplet sizes.[5b,22] The absolute concentrations governing these regimes vastly vary for different types of emulsifiers. In a w/o PE stabilized with latex particles, Golemanov and co-workers observed the emulsifier-poor regime at a particle content up to 30 $g \cdot L^{-1}_{dp}$ and the emulsifier-rich regime at particle contents above that concentration.[20] In an o/w PE stabilized with silica particles, Frelichowska and coworkers found the emulsifier-poor regime at particle contents between 15 $g \cdot L^{-1}_{dp}$ and 55 $g \cdot L^{-1}_{dp}$.[21] From these values, we assumed that in our study a particle content of 15 to 60 $g \cdot L^{-1}_{dp}$ would cover both regimes, which interestingly was not the case. The explanation probably lies in the fundamentally different composition of our emulsion system in terms of particle features, solvent composition and phase ratios.[4] We cannot explain the particular opposite effects of CALB and TLL at the lowest and highest particle contents, respectively, yet, but the observation corresponds to the previously discussed adverse effects of the two enzymes on initial droplet sizes in the emulsion and might therefore be related to structural features such as hydrophilicity/hydrophobicity. Since lower particle contents yielded only very instable PE and thus are unattractive for practical catalysis, we did not look further into the effect of the particle content on the initial droplet sizes in the emulsifier-poor regime of our BioPE. The observed overall increase of the emulsion stability with the particle content was in full agreement with reports from literature.[13] Considering the theory of PE stability as described above, the decreasing effect of a fixed lipase concentration with increasing particle content is easily explained with the decreasing portion of lipase at the interface

and thus decreasing interference with the stabilizing forces between particles.

In our emulsion, we did not observe significant changes in the initial droplet sizes when we varied the portion of the dispersed phase at a fixed particle content and lipase concentration (Figure 4).

This is in accordance with our deduction that our investigation system operated in the emulsifier-rich regime of emulsification, since in this regime the droplet size is independent of the volume fraction of the dispersed phase.[4] Emulsion stability was affected more again. Optimum stability in the enzyme-containing PE corresponded with the highest volume ratio of 33% (v_{dp}/v), at which we also observed high viscosity of the emulsion. This agrees with a stronger bridging of the surfaces of neighboring droplets in the PE due to a denser packing of the droplets at a higher portion of the dispersed phase,[14] and with the perception that thickening generally slows destabilization in emulsions.[5] On this background, the low stability of the protein-free PE at 33% (v_{dp}/v) is hardy explicable and might best be laid down to experimental problems as indicated by the large error in the observed droplet sizes. The intercalating enzymes interfered with the emulsion stability to an individual extent. We observed the lowest destabilizing effects with CALA and the highest with TLL, which is in accordance with our previous results on these enzymes' impact on PE stability.

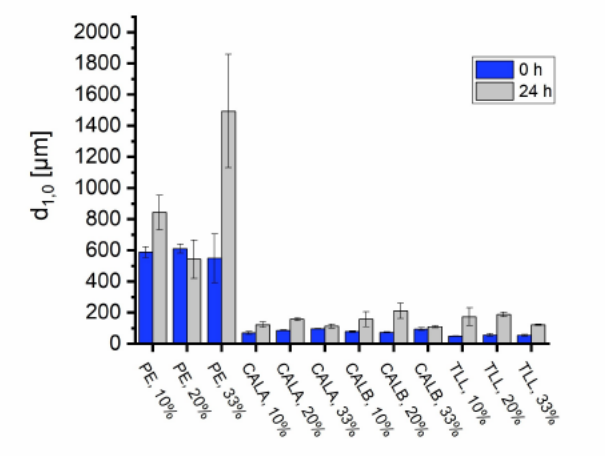

Figure 4. Mean diameters ($d_{1,0}$) of aqueous droplets in w/o PE at a phase ratio of 10%, 20% and 33% (v_{dp}/v) buffer in CPME and a particle concentration of 30 g·L $^{1}_{dp}$ without additional protein (PE_0) and in the presence of 1 g·L $^{1}_{dp}$ CALA, CALB and TLL, respectively, directly after emulsion formation and after 24 hours stirring at 440 rpm. Standard deviations refer to triplicate experiments.

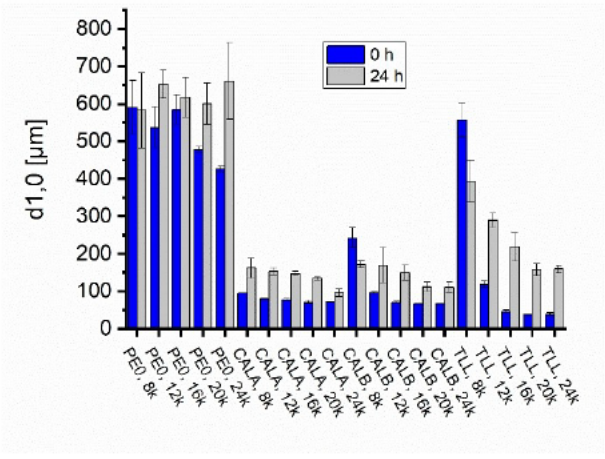

Figure 5. Mean diameters ($d_{1,0}$) of aqueous droplets in w/o PE at a dispersion speed of 8,000, 12,000, 16,000, 20,000 and 24,000 rpm directly after emulsion formation and after 24 hours stirring at 440 rpm. Dispersion was exerted with an Ultra Turrax T18 (IKA) for 2 min at a fixed phase ratio of 10% (v_{dp}/v) buffer in CPME, a particle concentration of 30 g·L $^{1}_{dp}$ without additional protein (PE_0) and in the presence of 1 g·L $^{1}_{dp}$ CALA, CALB and TLL, respectively. Standard deviations refer to triplicate experiments.

In the emulsifier-rich regime of emulsification, the initial droplet size exclusively depends on the emulsification process,[4] so we finally determined initial droplet sizes and stability in our emulsion at varying dispersion speed (Figure 5). Particle content, lipase concentration and phase ratio were fixed at 30 g·L^{-1}_{dp}, 1 g·L^{-1}_{dp} and 10% (v_{dp}/v) respectively.

In both the absence and presence of enzymes, the initial droplet sizes of the dispersed phase decreased with an increase of the dispersion speed, which was according to expectations.[5b,22] With the enzymes, an individual size plateau was reached at a dispersion speed of 20,000 rpm with a mean droplet diameter in PE with CALA and CALB around 70 µm and in PE with TLL of about 40 µm. Within 24 hours at gentle stirring, the droplet sizes increased again considerably showing the lowest values in the emulsions that were prepared with the highest dispersion speed of 24,000 rpm. The absolute size increase depended again on the participating lipase with CALA achieving the best and TLL the worst stabilization.

Surprisingly, when we finally examined the catalytic productivity of our system, we found it independent of the droplet size within the accessible size range as described in Figure 5. Using CALA as model catalyst because of the previously observed most favorable effect of this enzyme on PE stability, we achieved a space-time yield of 0.3 g·L^{-1}·h^{-1} and 4.9 $g_{product}$·$g^{-1}_{protein}$·h^{-1} for the transesterification of 1-phenylethanol with vinyl butyrate within a reaction time of 24 hours. This was identical for emulsions with an initial droplet size of either 94 µm in diameter (resulting from dispersion at 8,000 rpm) or 71 µm in diameter (resulting from dispersion at 24,000 rpm). As, at a fixed volume of the dispersed phase, the smaller droplet size entails a 1.3fold higher interfacial area than the larger droplet size ($a_2/a_1 = d_1/d_2$; a_1 and d_1 being surface area and diameter of the emulsion with the larger droplets, and a_2 and d_2 being surface area and diameter of the emulsion with the smaller droplets), we expected a higher catalytic activity of CALA with the smaller droplets. In a separate experiment, we observed that the stirring rate during emulsion formation hardly affected the specific activity of CALA, which excluded deactivation of the enzyme as an explanation for the missing impact of the interfacial area on catalytic performance. Thus, the results strongly indicate that the accessibility of CALA was identical in both PE, i.e. all enzymes contributed equally and independent of the volume of the dispersed phase to the

reaction at all times. This again indicates a quantitative adsorption of the enzyme at the interface under the here investigated conditions. Unfortunately, it was not possible to demonstrate this directly or indirectly. Direct detection of the location of the enzyme in the PE was not successful, while indirect proof would have required the considerable increase of the relative amount of the enzyme in the PE in order to saturate the interface. However, as demonstrated previously, such an increase has detrimental effects on emulsion formation and stability and therefore is not feasible.

Conclusion

Our here described findings demonstrate that protein effects on the physical properties and stability of w/o PE are far from negligible. Small amounts of lipases added to the aqueous phase exert a strong decrease of the droplet sizes of the dispersed phase and severely reduce emulsion stability. Both effects result from the intercalation of the enzymes between the stabilizing particles at the interface and strongly depend on individual molecular, in particular surficial, properties of the proteins. Hydrophilicity and a distinct structure for interfacial adsorption increase emulsion destabilization. Destabilization can be counteracted slightly, but not eliminated, by increasing the content of solid particles or the volume portion of the dispersed phase in the emulsion. However, the concomitant establishment of excess particles in the continuous phase of the emulsion, which might also result from particle substitution by the enzymes at the interface, can reduce technical utility, in particular the filterability,[8] of the reaction system. It also could imply product spoiling as free particles might be difficult to separate. Overall, due to the destabilizing effect of the proteins in the BioPE, only limited catalytic productivity can be achieved. Thus, our study distinctly points at the enzyme content, or rather enzyme location, being the crucial parameter for optimization. Future research will have to identify methods to improve the interplay of PE and enzyme catalysts. As demonstrated, consideration of molecular and physical properties might pave the way towards this end, but probably hiding the enzymes within the stabilizing particles as introduced by some researchers[1b] will be the simplest and most effective approach.

Experimental Section

Chemicals and enzyme preparations. Tetraethoxysilane (TEOS, 99%, CAS 78-10-4), trimethoxy(octadecyl)silane (TMODS, 97%, CAS 3069-42-9), and 3-aminopropyltriethoxysilane (APTS, 97%, CAS 919-30-2) were purchased from abcr GmbH, Germany. Fluorescein isothiocyanate (FITC, 90%, CAS 3326-32-7) and cyclopentyl methyl ether (CPME, 99,9%, CAS 5614-37-9) were purchased from VWR International GmbH, Germany. 1-Phenylethanol (98%, CAS 98-85-1) was obtained from Sigma-Aldrich Chemie GmbH, Germany. Ammonium hydroxide (25%, CAS 1336-21-6), denatured ethanol (96%, CAS 64-17-5), dipotassium hydrogen phosphate (99%, CAS 7758-11-4) and potassium dihydrogen phosphate (99%, CAS 7778-77-0) were purchased from Carl Roth GmbH + Co. KG, Germany. Cinyl butyrate (min. 98%, CAS 123-20-6) was purchased from TCI GmbH, Germany. All chemicals were obtained with the highest available purity. CALA and CALB (lipases A and B, respectively, from *Candida antarctica* resp. *Pseudozyma aphidis*) were obtained from c-LEcta GmbH, Germany. Lipozyme® TL 100 L (lipase from *Thermomyces (Humicola) lanuginosa; TLL*) was purchased from Novozymes, Denmark. All enzyme preparations were dialyzed prior to use over a cellulose membrane (Sigma-Aldrich Chemie GmbH, Germany) with a cut-off of 14 kDa. SDS-PAGE confirmed enzyme purity (see Figure S1).

Nanoparticle synthesis. Hydrophobic silica nanoparticles were produced *via* modified Stoeber synthesis.[23] 20.7 mL APTS were solved in 265.7 mL denatured ethanol and mixed with one tip of a spatula FITC. To the labeled APTS solution, 531.3 mL denatured ethanol, 41.3 mL TEOS and 143 mL 25% ammonium solution were added and stirred at 100 rpm overnight. The obtained nanoparticles were washed three times with ethanol and dried. 30 gram of dry nanoparticles were crushed in a mortar and suspended in 930 mL of denatured ethanol, and 40 mL TMODS and 30 mL 25% (v/v) ammonium solution were added. The reaction mixture was stirred at 600 rpm and heated to 60 °C overnight. The nanoparticles were washed three times with denatured ethanol, dried and crushed in a mortar. The resulting mean size was 495 ± 166 nm in diameter (Figure S2).

Preparation of w/o Pickering emulsions. 1,5–6% (weight per volume dispersed phase) of nanoparticles were suspended in CPME with or without 81.25 mmol·L^{-1} 1-phenylethanol and 518.25 mmol·L^{-1} vinyl butyrate for 20 sec. with an UltraTurrax T18 (IKA) at 17,500 rpm. The mean diameter of the nanoparticles after this treatment was 345 ± 6 nm. 0.1, 1 or 5 g·L^{-1} of dialyzed CALA, CALB and TLL, respectively, were dissolved in potassium phosphate buffer (KPi, 50 mmol·L^{-1}, pH 7) and added to the particle suspension in CPME to a volume ratio of 10%, 20% and 33% (volume dispersed phase per total volume of the emulsion, v_{dp}/v), respectively. The phases were dispersed with the UltraTurrax at a speed between 8,000 and 24,000 rpm for 2 minutes.

Analysis of droplet size. Microscopic pictures of PE samples were taken with a 4- or 10-fold magnification (for example, see Figure S3) and manually analyzed using software AxioVision Rel. 4.8. An average of 355 droplets per PE set-up was included in the analysis. All droplets were assumed spherical. The arithmetic mean diameter ($d_{1,0}$) was calculated from the drop size distribution ($d_{1,0} = \sum_{i=1}^{n} \frac{d_{p,i}}{N_p}$).

Substrate & product analysis. Substrate and product concentrations were determined using a Shimadzu GC2010 gas chromatograph equipped with a flame ionization detector, an FS-EnantioSELECT® beta 1 column (CS - Chromatographie Service GmbH, Germany) and nitrogen as carrier gas at a flow of 0.52 mL·min^{-1}. Temperature program: 100 °C for 37 minutes, ramp of 10 °C min^{-1} up to 200 °C. Products were detected at 290 °C. Retention times: 14.4 min (*R*-1-phenylethanol), 15.25 min (*S*-1-phenylethanol), 34.35 min (*S*-1-phenylethyl butyrate), 35.07 min (*R*-1-phenylethyl butyrate).

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: multiphase catalysis · biocatalysis · lipase · Pickering emulsion · protein impact

[1] a) M. Pera-Titus, L. Leclercq, J.-M. Clacens, F. de Campo, V. Nardello-Rataj, *Angew. Chem. Int. Ed.* **2015**, *54*, 2006–2021; *Angew. Chem.* **2015**, *127*, 2028–2044; b) A. M. Bago Rodriguez, B. P. Binks, *Soft Matter* **2020**, *16*, 10221–10243.
[2] a) B. P. Binks, *Curr. Opin. Colloid Interface Sci.* **2002**, *7*, 21–41; b) R. Aveyard, B. P. Binks, J. H. Clint, *Adv. Colloid Interface Sci.* **2003**, *100–102*, 503–546.
[3] C. Wu, S. Bai, M. B. Ansorge-Schumacher, D. Wang, *Adv. Mater.* **2011**, *23*, 5694–5699.
[4] S. Tcholakova, N. D. Denkov, A. Lips, *Phys. Chem. Chem. Phys.* **2008**, *10*, 1608–1627.
[5] a) B. P. Binks, S. O. Lumsdon, *Langmuir* **2000**, *16*, 2539–2547; b) M. V. Kempin, M. Kraume, A. Drews, *J. Colloid Interface Sci.* **2020**, *573*, 135–149.
[6] Y. Li, G. Zhao, B. Hong, S. Zhao, X. Han, M. Pera-Titus, *Colloids Surf. A* **2020**, *599*, 124800.
[7] B. P. Binks, S. O. Lumsdon, *Langmuir* **2000**, *16*, 8622–8631.
[8] A. Heyse, C. Plikat, M. Grün, S. Delaval, M. B. Ansorge-Schumacher, A. Drews, *Process Biochem.* **2018**, *72*, 86–95.
[9] P. Reis, R. Miller, J. Krägel, M. Leser, V. B. Fainermann, H. Watzke, K. Holmberg, *Langmuir* **2008**, *24*, 6812–6819.
[10] L. Sarda, P. Desnuelle, *Biochim. Biophys. Acta* **1958**, *159*, 285–295.
[11] A. Schröder, C. Berton-Carabin, P. Venema, L. Cornacchia, *Food Hydrocolloids* **2017**, *73*, 129–140.
[12] M. Martinelle, M. Holmquist, K. Hult, *Biochim. Biophys. Acta Lipids Lipid Metab.* **1995**, *1258*, 272–276.
[13] Y. Chevalier, M.-A. Bolzinger, *Colloids Surf. A* **2013**, *439*, 23–34.
[14] a) N. D. Denkov, I. B. Ivanov, P. A. Kralchevsky, D. T. Wasan, *J. Colloid Interface Sci.* **1992**, *150*, 589–593; b) P. M. Kruglyakov, A. V. Nushtayeva, *Adv. Colloid Interface Sci.* **2004**, *108–109*, 151–158.
[15] S. Tcholakova, N. D. Denkov, I. B. Ivanov, B. Campbell, *Adv. Colloid Interface Sci.* **2006**, *123–126*, 259–293.
[16] J. Kyte, R. F. Doolittle, *J. Mol. Biol.* **1982**, *157*, 105–132.
[17] Z. Derewenda, A. Sharp, *Trends Biochem. Sci.* **1993**, *18*, 20–25.
[18] D. Ericsson, A. Kasrayan, P. Johansson, T. Bergfors, A. Sandström, J.-E. Bäckvall, *J. Mol. Biol.* **2008**, *376*, 109–119.
[19] F. K. Winkler, A. D'Arcy, W. Hunziger, *Nature* **1990**, *343*, 771–774.
[20] K. Golemanov, S. Tcholakova, P. A. Kralchevsky, K. P. Ananthapadmanabhan, A. Lips, *Langmuir* **2006**, *22*, 4968–4977.
[21] J. Frelichowska, M.-A. Bolzinger, Y. Chevalier, *J. Colloid Interface Sci.* **2010**, *351*, 348–356.
[22] N. Vankova, S. Tcholakova, N. D. Denkov, I. B. Ivanov, V. D. Vulchev, T. Danner, *J. Colloid Interface Sci.* **2007**, *312*, 363–380.
[23] W. Stöber, A. Fink, E. Bohn, *J. Colloid Interface Sci.* **1968**, *26*, 62–69.

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