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Relative Effectiveness of Pretreatments on Performance of *Rhizomucor miehei* Lipase in Nonpolar Reaction Media

Rafael A. Garcia* and Mark R. Riley

Department of Agricultural and Biosystems Engineering, Shantz Building, The University of Arizona, Tucson, AZ 85721, E-mail: rgarcia@errc.ars.usda.gov

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Abstract

Enzymes can be used in nonpolar reaction media to modify water-insoluble substrates. A variety of pretreatments, applied to the enzyme prior to introduction to the nonpolar media, can improve enzyme activity. However, the various pretreatments have not been studied using directly comparable conditions, nor have they been applied simultaneously to test for interactive effects. This work evaluates pretreatment of lipase with various classes of additives. The pretreated lipase is used to catalyze esterification between citronellol and acetic acid in a medium of *n*-hexane. The effectiveness of a particular pretreatment is presented in terms of relative performance (RP), which is equal to the number of times faster the pretreated lipase catalyzes the reaction relative to untreated lipase. The individual and interactive effects of the pretreatment factors were studied and compared. Buffer salts had a much stronger performance-enhancing effect than nonbuffer salts; pretreatment with 90% (w/w) sodium phosphate yielded lipase with an RP of approx 64. A strong interaction was found between the treatments with sodium phosphate and pH adjustment. These treatments may mitigate the inhibitory effect of acetic acid. Activating effects of phase interfaces and active-site protectants are shown to be complementary to other treatments, demonstrating that they likely act by distinct mechanisms.

Index Entries: Nonaqueous enzymology; lipase; pretreatment; lyophilization; fragrance.

*Author to whom all correspondence and reprint requests should be addressed. Current address: USDA-ARS, 600 E. Mermaid Lane, Wyndmoor, PA 19038.

Introduction

Use of a nonpolar reaction medium for enzymatic conversions can provide a number of advantages *(1–3)*, but enzymes typically retain only a small portion of their original activity in nonpolar environments. This low activity is the primary obstacle to industrial adoption of nonaqueous enzymatic processes.

Several factors prevent enzymes in nonpolar environments (ENPEs) from achieving their full catalytic potential *(4)*. In a nonpolar environment an enzyme's active site is typically somewhat distorted, placing active-site functional groups in nonideal positions and reducing the "fit" of the active site to the substrate *(5)*. ENPEs are often clumped together, limiting the mass transfer rate of substrates and products in and out of the active site.

In synthetic reactions involving short-chain organic acids as substrates, ENPEs may be further inhibited by the substrate itself *(6–8)*. Although many different types of ENPE-catalyzed reactions have been reported in the literature, reactions with short-chain organic acids as substrates have consistently reacted slowly and given low product yields.

An additional mechanism can contribute to low activity when lipase is used as an ENPE. Lipase has a "lid" covering its active site *(9)*; in aqueous solution, the lid is in the closed position unless the lipase is on a water-lipid phase interface. When used as an ENPE, lipase's structure is rigid, including the lid, which is locked in the closed position, reducing access to the active site *(10–12)*.

The catalytic performance of ENPEs can be greatly improved by applying a pretreatment to the enzyme before its introduction to the reaction *(4,12–17)*. Pretreatment involves dissolving commercial enzyme in aqueous solutions or emulsions containing various additives that may include buffers, salts, acid, base, substrate analogs, or nonpolar materials. The enzyme solution is then frozen and lyophilized to remove the water. It has been hypothesized that these different pretreatment additives and variables improve the performance of ENPEs by relieving one or more of the inhibitory factors that we have described *(3)*.

Typically, each type of pretreatment variable has been investigated individually, with each study using different experimental methods. Studying the different variables individually neglects possible interactions between two or more variables. Owing to varying experimental methods employed by prior researchers, the relative effectiveness of different pretreatment conditions cannot be adequately compared. Finally, pretreatments have rarely been studied using a reaction system that includes short-chain organic acids, which are required for the production of a number of industrially relevant products. It is not clear from the literature whether pretreatment can be used to counteract short-chain organic acid inhibition of ENPEs. The present work uses a consistent experimental method to investigate the effect of several different pretreatment variables,

alone and in combination. The model reaction is the lipase-catalyzed esterification between citronellol and acetic acid (a short-chain organic acid) to produce citronellol acetate (CA), a higher-value material. The results of this work demonstrate that the various pretreatments produce a wide range of rate enhancements, and that important interactions among pretreatment variables exist.

Materials and Methods

Chemicals

DL-Citronellol (95%), CA (83.6%), dodecane (≥99%), glacial acetic acid (ACS reagent), *n*-hexane (≥99%), all salts, and lipase from *Mucor miehei* (minimum of 4000 U/mg) were purchased from Sigma (St. Louis, MO).

Pretreatment of Lipase

An aqueous pretreatment solution was prepared by adding the various salts to 18 mL of ultrapure water. The water-insoluble citronellol and hexane were added to the solution and homogenized using a Cole-Parmer 8890 Ultrasonic bath. Specifically, citronellol or hexane was introduced to the solution, stirred vigorously for 15 s, placed in the bath for 15 s, and then cycled between the stirring and the bath twice more. This produces an emulsion that is stable for several hours. The solution pH was then adjusted to the specified pH ± 0.03 by the addition of small aliquots of 0.1–1.0 *M* NaOH or HCl and measured using an electrode specially designed for unbuffered and/or low-ionic-strength solutions (Star electrode; Beckman Coulter, Fullerton, CA). Lipase (25 mg) was added and the pH checked and readjusted if necessary.

The solution was placed in a lyophilization flask and allowed to freeze quiescently in a bath of liquid nitrogen for 5 min. The flasks of frozen solution were stored in a –20°C freezer for a minimum of 1 h and then lyophilized for 17 h on a Labconco 4.5 L freeze-dryer (Labconco, Kansas City, MO). The resulting dry material had a volume roughly equal to the original liquid volume (18 or 20 mL). Eighty percent of the dry material (containing 20 mg of enzyme) was compacted with a metal spatula, weighed, and transferred to a reaction vial. To prevent degradation and absorption of moisture from the atmosphere, the enzyme preparation was stored tightly sealed in a freezer until use.

Reaction

The reaction was a lipase-catalyzed esterification between citronellol and acetic acid in hexane to produce CA. The reaction mixture (0.250 *M* DL-citronellol, 0.200 *M* dodecane in *n*-hexane) was stored at approx 5°C over a mixture of anhydrous sodium acetate and sodium acetate trihydrate (5 g of each salt). The sodium acetate salt hydrate pair acts as a water activity buffer, holding the water activity of the reaction mixture at 0.28, assuming

that the mixture is at equilibrium and the temperature is 25°C *(18)*. Just before use, acetic acid was added to the reaction mixture.

Reactions were carried out in 5.5-mL glass vials fit with gastight Mininert (Supelco, Bellefonte, PA) sampling valves. Three milliliters of the reaction mixture was added to the vial containing the pretreated enzyme. The vial was placed in a 37°C incubator and shaken at 325 rpm using a rotary shaker, to suspend the enzyme. Samples (1–1.5 µL) were periodically removed for gas chromatography (GC) analysis.

GC Analysis

A Varian model 3700 gas chromatograph (Varian, Walnut Creek, CA) equipped with a packed glass column (6 ft × 2 mm id) filled with 10% SP-1000 (Supelco) and a flame ionization detector was used. The carrier gas was N_2 at a flow rate of 5 mL/min. The temperature program consisted of an initial temperature of 115°C held for 1 min after sample injection, followed by a 21°C/min temperature ramp up to a final temperature of 165°C held for 1 min. This method completely resolves all the reaction mixture components and the reaction product. Dodecane, which does not participate in the reaction, was used as an internal standard.

The measure of enzyme performance is based on the rate of generation of the desired product, CA. The concentration of CA in each reaction was measured at the initial time point, and at three additional time points during the course of the reaction. The sampling times were selected so that at the final sampling, the CA concentration would be within the range of 0.03–0.06 *M*. This requires that the spacing of the sampling times vary according to the velocity of the reaction.

Measure of Performance

A plot of the concentration of CA vs time was examined to determine whether a linear relationship was appropriate. If so, a simple linear regression was used to fit the data. The slope taken from this regression is the rate of the reaction. This rate divided by the concentration of enzyme in the reaction is defined here as "performance":

$$\text{Performance} = \frac{\text{Rate of CA production}}{\text{Lipase concentration}} = \frac{\left(\frac{\Delta[\text{CA}]}{\text{Time}}\right)}{\left(\frac{\text{Mass lipase}}{\text{Reaction volume}}\right)} \quad (1)$$

Results in terms of performance are difficult to interpret (the units are moles of CA/[grams of lipase·second]), so results are presented in terms of relative performance (RP), a dimensionless parameter, defined as follows:

$$RP = \frac{\text{Performance}}{\text{Average performance of untreated lipase}} \quad (2)$$

The interpretation of RP is straightforward; lipase with a relative performance of 8 catalyzes a reaction eight times faster than untreated lipase (lipase used as received from a commercial supplier without any pretreatment). The number of replications of each experiment varied, as indicated in the figure captions. A regression equation was fitted to each series of results, and in each case, a significant relationship between the variable and relative performance was found.

Results and Discussion

Minimal Pretreatment

The performance-enhancing effect of a minimal pretreatment, simply dissolving lipase in water and freezing, followed by a 30-h lyophilization cycle, is useful in comparing the effectiveness of more complex pretreatments. This treatment yielded lipase with an RP of 11.34 (±0.28; n = 2), corresponding to a reaction rate approx 11 times that of untreated lipase. The untreated commercial enzyme is in dense chunks; after dissolution and lyophilization, the lipase is a large, fluffy, porous mass. This change in texture may alleviate mass transfer restrictions; the increase in surface area allows better access for the substrates to the active site. The manufacturer of the lipase likely lyophilizes the enzyme from a much more concentrated aqueous solution, producing material with a dense texture, compared with the pretreated material.

Lyophilization Time

Lyophilization removes water from the frozen lipase solution. In the initial phase of drying, the bulk water sublimates relatively rapidly; once the bulk water is removed, microscopic amounts of remaining water sublimate relatively slowly. The effect of lyophilization time on RP was evaluated using lipase from a pretreatment solution containing either 80% KCl (w/w) or 80% sodium phosphate (NaPhos). The KCl preparations completed initial drying within about 16 h, whereas the NaPhos preparations took at least 20 h, as determined by visual inspection. The difference in drying rates is presumably the result of the different water-binding strengths of the two salts. If preparations were removed from the lyophilizer before the end of initial drying, the remaining ice quickly melted and absorbed into the dry material. Preparations that had remaining macroscopic water were not used.

When lyophilized 18 h or more, the RP of the KCl preparations was roughly constant with increasing lyophilization time (*see* Fig. 1); there was a gradual decrease in performance with lyophilization times greater than 50 h. The NaPhos preparations were much more sensitive to lyophilization

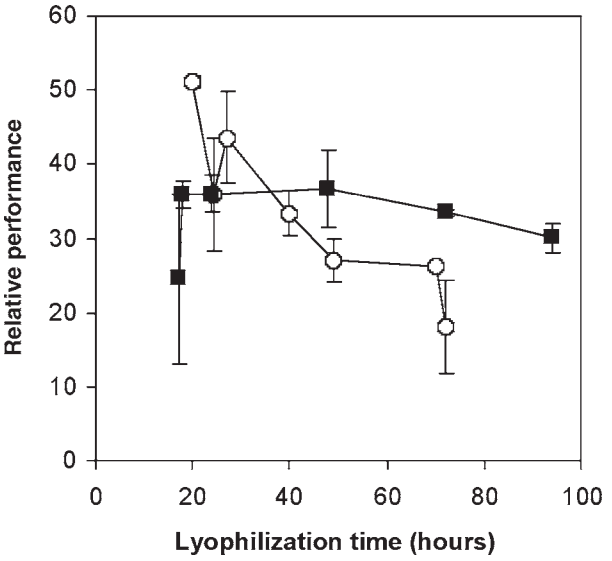

Fig. 1. Effect of lyophilization time on RP: (■) 80% (w/w) KCl, pH 7.5, originally 20 mL of water; (○) 80% (w/w) NaPhos, pH 8.5, originally 18 mL of water (n = 2–5). Error bars represent ±1 SD.

time compared with KCl preparations. RP was highest for short lyophilization times and dropped rapidly with increasing time.

Ru et al. *(15)* studied the effect of lyophilization time on enzyme activity and found somewhat different trends. They used lipase from *Mucor javanicus* pretreated with 98% (w/w) KCl. Their data show a sharp peak in activity at about 58 h. One major difference between their system and ours is the source of lipase used. Valivety et al. *(19)* report that lipases from different organisms vary widely in their sensitivity to water activity. Some of the lipases displayed flat activity profiles over a wide range of water activity values, whereas others were very sensitive to changes in water activity. Water activity of a preparation will drop with increasing lyophilization times, and it is possible that the lipase Ru et al. *(15)* used was simply more sensitive to water activity than the lipase studied here.

Another major difference between the methods of the present study and those of Ru et al. *(15)* is the amount of KCl in the pretreatment (98 vs 80%). This difference is greater than it may appear at first glance: the KCl concentration in the study by Ru et al. *(15)* was more than 12 times greater than that used in the present study. Higher salt concentrations will tend to slow the drying process and retain more "tightly bound" water.

The results of our studies and the earlier work both show that lipase performance drops off with very long lyophilization time. Potentially two different phenomena may be responsible for this drop off in performance. Simple thermal denaturation may lead to some loss in performance; the lipase is being held relatively warm over a long period of time. Long lyophilization also slowly removes small amounts of tightly bound water.

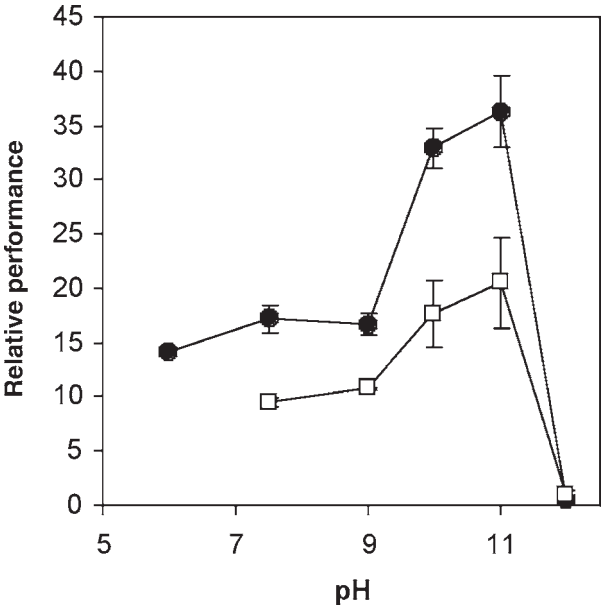

Fig. 2. RP of lipase treated with (□) 55% (w/w) KCl and (●) 80% (w/w) KCl, with both series lyophilized for 17 h (n = 2–5). Lipase in the two series came from separate lots. Error bars represent ±1 SD.

The literature suggests that these tightly bound water molecules are critical for the maintenance of native structure and structural flexibility *(20,21)*. Unfortunately, we were unable to measure directly the water content in our samples; the amount of enzyme needed for a Karl Fischer titration was cost prohibitive.

In further experiments described here, the lyophilization time is fixed. The chosen amount of time is a compromise among three goals: high performance, short time, and low sensitivity to minor variations in time (robustness). For KCl preparations, the choice of lyophilization time is straightforward—17 h meets the goals best. For NaPhos the choice is more difficult and arbitrary. RP was fairly sensitive to lyophilization time at all times tested. Thirty hours was chosen primarily because early results suggested that the slope of the lyophilization time vs RP was shallow in the region of 30 h; the process would be robust to small changes in lyophilization time. Later results did not support the choice of a 30-h lyophilization time, but this lyophilization time was retained for consistency.

pH

Zaks and Klibanov *(1)* observed that, in general, an enzyme's optimum pH in an aqueous environment is also the optimum pH to use in the pretreatment of that enzyme. Laboret and Perraud *(8)* reported that lipase performance in nonaqueous reactions is strongly inhibited by acetic acid substrate in the reaction mixture. We studied the effect of pretreatment pH with both these observations in mind. Figure 2 shows the RP of lipase

pretreated with either 55 or 80% (w/w) KCl over a wide range of pretreatment pH values. Profiles for both series closely parallel each other, except for the magnitude of response. The RP profile is flat in the pH range of 6.0–9.0, increases from 9.0 to 11.0, and drops sharply above 11.0. Both series have optima at about pH 11.0. The magnitude of the 80% series is consistently higher than that of the 55% series, and the difference in magnitudes is larger near the optima. Note that the lipase used in the two series came from different manufacturer's lots; the manufacturer claims similar activity for each lot; however, lot-to-lot variation probably accounts for a small portion of the difference between the series.

The observation of optimum RP at pH 11.0 is unexpected. In aqueous solution, most enzymes, including *M. miehei* lipase, have pH optima close to neutral. According to Zaks and Klibanov *(1)*, one should expect an optimum pretreatment pH not far from neutral. We believe that our unusually high optimum pH results from the use of acetic acid as a substrate. The lyophilized enzyme is surrounded by a very thin layer of water (the microaqueous layer) and has a surface that is both polar and charged. Acetic acid is miscible in both hexane and water but will tend to partition into the microaqueous layer and onto the surface of the enzyme. Although the initial acetic acid concentration in the reaction mixture is low, the local concentration in the small volume of the microaqueous layer can be very high. Consequently, an unbuffered microaqueous layer will have a very low pH, which will depress the enzyme's performance. We believe that this explains the inhibitory effect observed by Laboret and Perraud *(8)*.

Raising the pH of the pretreatment solution can negate the effect of acetic acid. It is likely that the optimum pretreatment pH provides sufficient NaOH to balance the acetic acid at the surface of the enzyme, thus producing a microaqueous layer with a pH near neutral. Very high pretreatment pH (>11.0) may damage and inactivate the enzyme prior to lyophilization.

The results for lipase treated with NaPhos over a range of pH values are quite different from the KCl results (*see* Fig. 3). Note that the RP scales in Figs. 2 and 3 are different; the highest average RP in Fig. 2 is approx 39, whereas the highest in Fig. 3 is approx 62. In Fig. 3, the 90% (w/w) NaPhos series has an optimum in the neighborhood of pH 8.5; the optimum for the 80% (w/w) series is not well defined but has higher activity in the pH range of 10.0–11.5 compared with the 90% series.

Note that the two KCl series have similar shapes, shifted in magnitude, whereas the two NaPhos series have similar shapes and similar magnitude but shifted on the pH scale (Figs. 2 and 3). This shift is likely due to the interaction among pretreatment pH, NaPhos concentration, and acetic acid concentration described above. Higher NaPhos concentrations have more capacity to buffer the microaqueous layer, so the optimal pretreatment pH is closer to neutral. A shift in pH with changing KCl concentration would not be expected, because KCl has no buffering capacity (pK_a of HCl = –8.0).

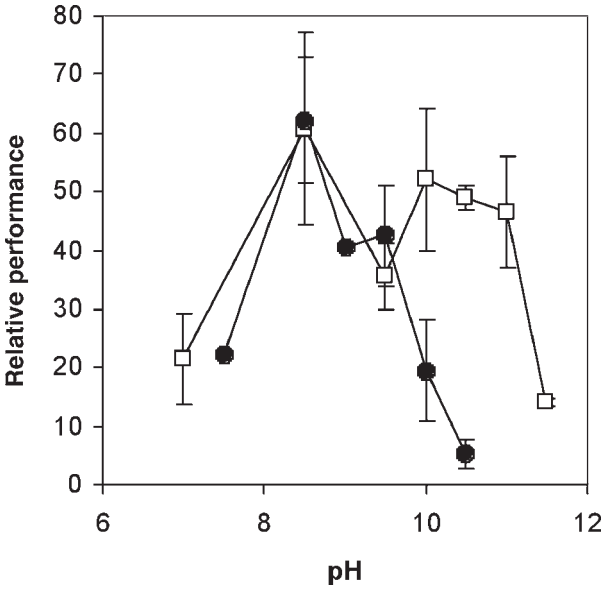

Fig. 3. RP of lipase treated with (□) 80% (w/w) NaPhos and (●) 90% (w/w) NaPhos, with both series lyophilized for 30 h (n = 2–5). Error bars represent ±1 SD.

Buffer and Nonbuffer Salt Additives

When the lipase was pretreated with buffer (NaPhos) or nonbuffer salts (KCl), RP improved compared to untreated controls for all salt concentrations tested (Fig. 4A). Increasing concentrations of KCl resulted in increasing RP, up to about 75 m*M*, which produced a 24-fold increase in reaction rate. There was no additional increase in RP for higher concentrations. Earlier research speculated that KCl and other nonbuffer salts may provide immobilization surfaces for enzymes, relieving mass-transport constraints created when large amounts of dry enzyme clump together, and may provide a local environment for the enzyme that promotes the native conformation *(17)*.

NaPhos is much more effective at increasing RP. Even very low concentrations produce large increases in RP. With increasing concentrations, RP rises rapidly. RP decreases when the concentration is higher than 88.7 m*M*.

NaPhos likely increases RP by the same mechanisms as nonbuffer salts, in addition to phosphate's inherent buffering activity. This hypothesis is supported by the comparison of our results with those of Triantafyllou et al. *(17)*. Using *Candida antarctica* lipase, they found an optimum pretreatment [NaPhos] of about 85% (w/w), above which activity dropped. The magnitude of the peak enhancement with NaPhos was much greater in the present study compared with the earlier work (64- vs 5-fold). This may have to do with the different reactions being catalyzed in each case. One substrate in the present study was acetic acid, which has the inhibi-

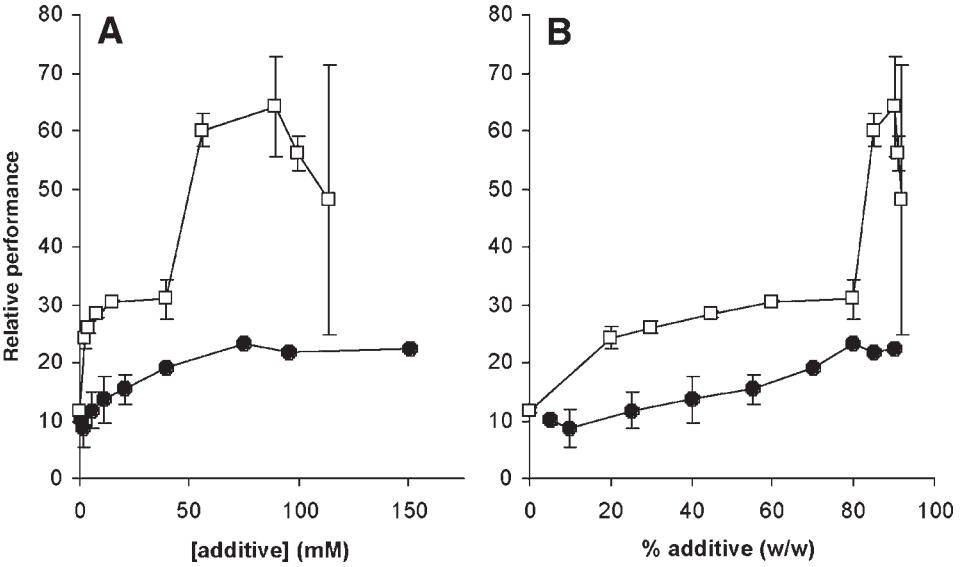

Fig. 4. RP of lipase treated with (●) KCl, pH 7.5, 17 h of lyophilization (n = 2); and (□) NaPhos, pH 8.5, 30 h of lyophilization (n = 2–4). The concentration of salt additive is plotted **(A)** in terms of concentration and **(B)** in terms of % (w/w). Lipase in KCl and NaPhos experiments came from different manufacturer's lots. Error bars are present for all points and represent ±1 SD.

tory pH-lowering effect described above. Conceptually, NaPhos can buffer the pH of the microaqueous layer surrounding the lipase, keeping it close to whatever pH it had before being exposed to the acetic acid. The reaction system of Triantafyllou et al. *(17)* did not have a short-chain organic acid or any other material that would significantly alter the pH of the microaqueous layer. Therefore, the benefit to be gained by buffering this layer is smaller compared with our reaction system. Triantafyllou et al. *(17)* did not observe a plateauing of the KCl enhancement effect, up to 90% KCl (w/w), the highest concentration that they tested. They observed that different salts, including both buffering and nonbuffering species, produce similar increases in performance when compared on a percent-by-weight scale. Figure 4B shows our data replotted on this scale; our results do not agree with the earlier observation. In the case of the earlier research, because buffering was unnecessary, KCl and NaPhos both may have been acting by the same mechanisms, leading to the same magnitude rate enhancements.

An interesting difference between the two types of salts is their RP vs concentration profiles; RP increases and plateaus with increasing [KCl], whereas increasing [NaPhos] leads to rapidly rising RP up to a peak beyond which RP drops off rapidly. We speculate that the drop-off of RP at high [NaPhos] is directly related to the buffering capacity of the NaPhos. At least three factors determine the pH of the microaqueous layer: the pH of the pretreatment solution, the concentration of buffering salts, and the concentration of acetic acid. There is some optimum pH for this layer. High pre-

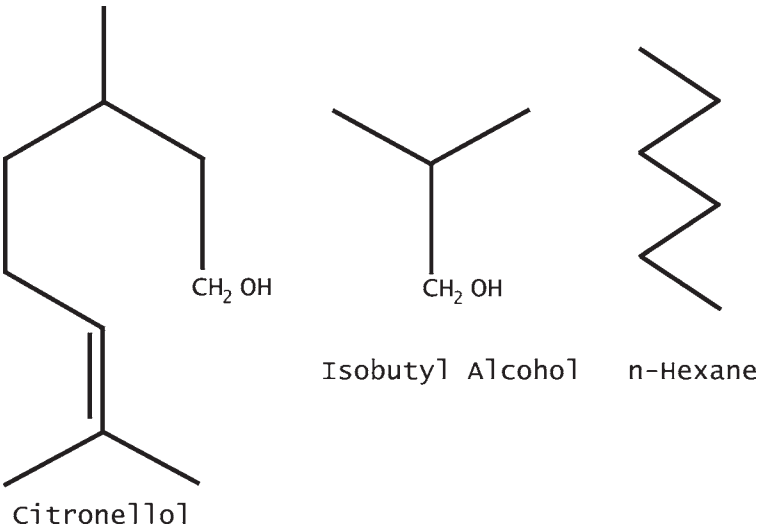

Fig. 5. Structures of organic additives used.

treatment pH and high NaPhos concentration will tend to increase performance by counteracting the effect of acetic acid pushing the pH below optimal. However, with very high NaPhos or very high pH, the pH of the microaqueous layer is actually above optimal, even after the addition of acetic acid, inhibiting performance.

Phase Interfaces and Active-Site Protectants

Organic substances added to a lipase pretreatment mixture can potentially increase the performance of the lipase, by two distinct mechanisms. The lid covering lipase's active site is closed in aqueous solution but opens when it encounters a phase interface (PI) such as the surface of a fat globule *(10–12)*. If lipase is lyophilized from an aqueous pretreatment solution without a PI, the lid is locked in the closed position, restricting the movement of substrates into and out of the active site. If lipase is placed in an emulsion (high PI area), the lipase molecules will tend to position themselves on the PI with their lids open. Following flash-freezing and lyophilization, the pretreated lipase has its lid locked in the open position, improving access to the active site.

The second mechanism by which organic substances can increase RP involves substrate or substrate-like molecules (active-site protectants [ASPs]) *(22)*. These substances are believed to sit in the active site of the enzyme and prevent it from being distorted during lyophilization.

To explore these effects, three organic substances were used in the pretreatment of lipase. Hexane has low water solubility and will form PI but it does not have any important structural resemblance to the substrate citronellol (*see* Fig. 5), so it would not be expected to act as an ASP. Isobutyl alcohol has structural similarities to citronellol; it has a single hydroxyl

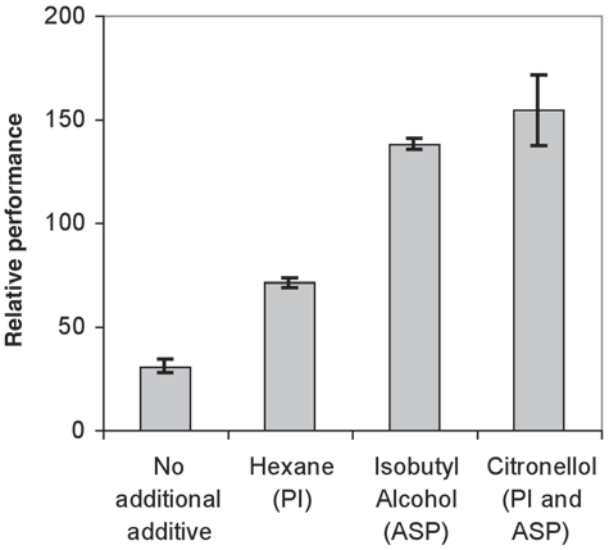

Fig. 6. Effect of adding 50 µL of different types of organic substances on RP. Base conditions were 80% NaPhos, pH 8.5, and 30 h of lyophilization ($n = 2$). Error bars represent ±1 SD.

group and a branched chain, so it might reasonably be expected to act as an ASP. Isobutyl alcohol is miscible with water, so it will not create a PI. Citronellol, the substrate, was also chosen to add to the pretreatment. It has ideal properties for an ASP and is insoluble in water, so it will create a PI. We believe that the use of substances with both PI and ASP properties is unprecedented in the literature.

A simple experiment was conducted to measure the increases in performance resulting from the different additives. Before dissolving the lipase, 50 µL of an organic substance was added to each pretreatment and emulsified, if necessary. The base conditions for each batch were 80% NaPhos, pH 8.5, and 30 h of lyophilization. Figure 6 shows the results of this experiment. Each of the additives had a significant performance-enhancing effect, relative to the pretreated lipase without any organic additive. The hexane treatment gave an RP of 70.9, which is comparable to the 90-fold increase in performance obtained by Gonzalez-Navarro and Braco *(12)* when pretreating lipase with PI. The fact that the RP of the citronellol treatment is higher than that of either the isobutyl or hexane treatments suggests that the two types of activating materials, PI and ASP, may be complementary. If this is the case, it may be possible to use a mixture of two materials, one that is solely an ASP and one that solely forms PI, to achieve a greater enhancement in performance. This could be important, e.g., when the substrate is water miscible or is too expensive to use in the pretreatment.

We next investigated the effect that the concentration of an organic additive has on RP (*see* Fig. 7) using the same base conditions as the previ-

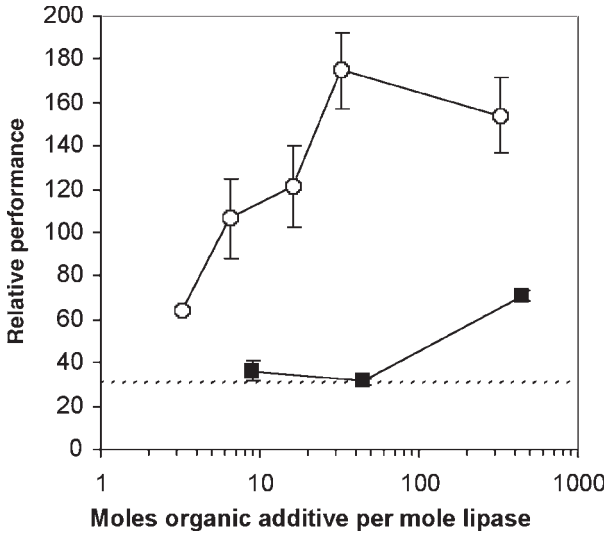

Fig. 7. Effect on RP of pretreatments with (○) citronellol or (■) hexane at a range of concentrations. Base conditions were 80% NaPhos, pH 8.5, and 30 h of lyophilization (n = 2). Error bars represent ±1 SD. The dashed line represents the RP of lipase pretreated with the base conditions but with no organic additive.

ous experiment and either citronellol or hexane. The upper limit for both additives is about 50 µL of additive/18 mL of pretreatment solution; higher concentrations will not form stable emulsions. The performance-enhancing effect of citronellol is highly concentration dependent, up to about 50 mol/mol of lipase, after which the effect plateaus. It is not surprising that this activating effect reaches a plateau at some level; once each active site is occupied by a molecule of citronellol and each molecule is sitting on an interface, higher concentrations should have no further effect. The actual concentration required to have every active site occupied likely depends on how the ASP partitions among the active site, the enzyme surface, and the surrounding water. The concentration required to have each lipase molecule sitting on an interface is likely mostly dependent on the size of the micelle.

The performance-enhancing effect of hexane was not apparent below 450 mol/mol of lipase (50 µL in 18 mL of water). A limitation in the experimental method may lead to an underestimate of hexane's effect. Hexane evaporates fairly rapidly at room temperature (boiling point = 68.7°C at 1 atm), and this evaporation may be accelerated in the ultrasonic bath. It is likely that some portion of the hexane is lost before the emulsion forms.

Finally, we examined the interaction and complementarity of an organic additive, citronellol, with a buffer salt pretreatment, NaPhos. We compared lipase treated with a range of NaPhos concentrations to a similar series that also had 5 µL of citronellol (32.4 mol of citronellol/mol of lipase) added to the pretreatment (*see* Fig. 8). Although the data are inadequate to demonstrate definitively a positive interaction (the effects of

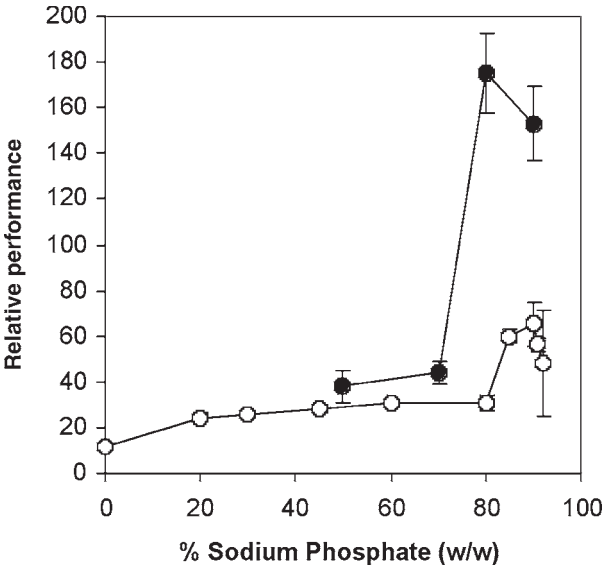

Fig. 8. Comparison of RP with 5 µL of citronellol added to pretreatment (●) (n = 2), and without any citronellol added (○) (n = 2–5) over a range of NaPhos concentrations. Base conditions were pH 8.5 and 30 h of lyophilization. Error bars represent ±1 SD.

the two treatments together is greater than the sum of each effect individually), the treatments are certainly complementary. The RP achieved with 80% (w/w) NaPhos and 5 µL of citronellol is greater than the RP achieved with any NaPhos concentration without citronellol. This suggests that the performance-enhancing mechanism(s) of citronellol is distinct from that of NaPhos.

Conclusion

The performance of ENPEs can be substantially improved by pretreatment of the enzyme. Appropriate pretreatment can increase lipase performance by at least 175-fold, compared with untreated lipase.

Pretreatment acts by alleviating one or more performance-inhibiting conditions. No single pretreatment additive or variable was able to alleviate all the inhibitory conditions. We have demonstrated the relative effects of several different pretreatment variables and shown that there is often interaction among pretreatment variables. The existence of multiple interactions complicates the task of developing an optimum pretreatment. Further work in our laboratory has demonstrated the use of response surface methodology to optimize pretreatments rapidly for a particular reaction system.

Acknowledgments

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