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Striking activation of oxidative enzymes suspended in nonaqueous media

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ABSTRACT **The catalytic activity of four lyophilized oxidative enzymes—horseradish peroxidase, soybean peroxidase, *Caldariomyces fumago* chloroperoxidase, and mushroom polyphenol oxidase—is much lower when directly suspended in organic solvents containing little water than when they are introduced into the same largely nonaqueous media by first dissolving them in water and then diluting with anhydrous solvents. The lower the water content of the medium, the greater this discrepancy becomes. The mechanism of this phenomenon was found to arise from reversible denaturation of the oxidases on lyophilization: because of its conformational rigidity, the denatured enzyme exhibits very limited activity when directly suspended in largely nonaqueous media but renatures and thus yields much higher activity if first redissolved in water. Two independent means were discovered for dramatically minimizing the lyophilization-induced inactivation, both involving the addition of certain types of excipients to the aqueous enzyme solution before lyophilization. The first group of excipients consists of phenolic and aniline substrates as well as other hydrophobic compounds; these presumably bind to the hydrophobic pocket of the enzyme active site, thereby preventing its collapse during dehydration. The second group consists of general lyoprotectants such as polyols and polyethylen glycol that apparently preserve the overall enzyme structure during dehydration. The activation effects of such excipients can reach into the tens and hundreds of fold. Moreover, the activations afforded by the two excipient groups are additive, resulting in up to a complete protection against lyophilization-induced inactivation when representatives of the two are present together.**

When placed in nonaqueous solvents instead of the natural, aqueous milieu, enzymes exhibit remarkable new properties, including the ability to catalyze reactions impossible in water, enhanced thermostability, molecular "memory," and radically altered selectivity (1). This holds a promise of substantially broadening the biotechnological utility of enzymes (1).

The main drawback of enzymes functioning in organic solvents is their drastically reduced catalytic activity compared with that in water (2). Ironically, one of the principal causes of this inactivation is not an adverse effect of the organic solvent itself but rather denaturation of the enzyme brought about by its prior dehydration (2), usually through lyophilization (3). Although a number of approaches have been proposed to minimize this denaturation, they have been tested only with relatively simple, hydrolytic enzymes (2). And yet, the greatest practical potential rests with more complex, oxidative enzymes, e.g., peroxidases (4, 5).

In the present work, we have expanded the scope of both the mechanistic investigation of the lyophilization-induced inactivation and the search for effective remedies to oxidative enzymes, including peroxidases. Their catalytic performance in organic solvents containing from <1% to a few percent of water has been greatly improved by simple and rational means as a result of this quest.

MATERIALS AND METHODS

Materials. All enzymes used herein were obtained from Sigma: horseradish peroxidase (HRP; type II), soybean peroxidase, chloroperoxidase (from *Caldariomyces fumago*), and mushroom polyphenol oxidase. Hydrogen peroxide (30% solution, analytical grade) was purchased from Mallinckrodt. Isopropyl alcohol (99.9+% pure) and *N*,*N*-dimethylformamide (99.8+% pure) were purchased from EM Science. Acetone (99.5+% pure), polyethylene glycol (PEG; average M_r of 10,000 Da), all substrates, and other chemicals were purchased from Aldrich and were of the highest purity available.

Enzyme Preparation. Enzymes were lyophilized by quickly freezing their aqueous solutions in liquid nitrogen and then drying under vacuum (10–20 μm Hg, −50°C, in a Labconco model 8 freeze drier) for 24 hours. When enzymes were lyophilized in the presence of excipients, the latter were added to an aqueous 10 mM phosphate buffer solution, and the pH was adjusted to the desired value before the addition of the enzyme (pH 7.0 for HRP and soybean peroxidase, pH 5.0 for chloroperoxidase, and pH 6.5 for polyphenol oxidase). Enzyme concentrations before lyophilization were 5 mg/ml except for 1 mg/ml for chloroperoxidase. The fraction of the enzyme in lyophilized powder was determined by dividing the weight of the enzyme added to the aqueous solution by the total weight of the powder obtained after lyophilization.

Enzyme suspensions in largely nonaqueous reaction media were prepared by using two distinct methods. In the first method, the enzyme was dissolved in an aqueous solution and then diluted with an anhydrous organic solvent. In the other method, the lyophilized enzyme powder was directly suspended in the nonaqueous reaction medium with a low water content, and then a 20-sec ultrasonication was applied to make the enzyme suspension homogenous. In both cases, the same final water content [always expressed as % (vol/vol)] and enzyme concentration were achieved.

Kinetic Measurements. Enzymatic activities were assayed spectrophotometrically following the procedures described in the literature: HRP and soybean peroxidase in the guaiacol (*o*-methoxyphenol) oxidation with H_2O_2 (6); HRP in the guaiacol, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) oxidation with H_2O_2 (7); chloroperoxidase for *p*-cresol oxidation with H_2O_2 (8); and polyphenol oxidase in the *p*-methylcatechol oxidation with molecular oxygen (9). An enzyme concentration of 0.10 μg/ml was used for all the enzymatic activity measurements in aqueous solutions, whereas that of 10 or 100 μg/ml was used for nonaqueous

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Abbreviations: PEG, polyethylene glycol; HRP, horseradish peroxidase.

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media. In comparing the activities of a given enzyme in aqueous solution and in nonaqueous media, the reaction product whose absorbance change was followed to determine the initial rate was assumed to have the same extinction coefficient in the two media.

RESULTS AND DISCUSSION

Conceptually, there are two distinct ways of preparing an enzyme suspension in an organic solvent containing a small amount of water. In one, a concentrated aqueous enzyme solution is diluted with a much larger volume of a neat organic solvent. In another, a solid (e.g., lyophilized) enzyme is directly suspended in the corresponding predominantly nonaqueous medium. In a purely aqueous solution, the enzymatic activity is independent of the enzyme's history (which is why enzyme assays are inherently reproducible). This is not the case, however, in nonaqueous media where, because of the scantiness of the "molecular lubricant" water, which leads to conformational rigidity of enzyme molecules (10), the enzymatic activity in a given system may be profoundly affected by how the enzyme is introduced, i.e., by its history (11). For instance, the proteases α-chymotrypsin and subtilisin placed in 99% organic solvents by the first route (hereafter for brevity referred to as "added via water") were up to 10 times more active than those directly suspended in the same 99% solvents (12). In the present work, this phenomenon was explored with a more complex and biotechnologically important group of enzymes, namely oxidases.

HRP, a thoroughly researched hemoprotein enzyme that catalyzes the oxidation of numerous phenols and aromatic amines with hydrogen peroxide as well as many other reactions (13, 14), was selected for the initial study. This enzyme is known to possess a diminished but still significant catalytic activity even in quite concentrated organic solvents (15, 16). We dissolved 0.33 mg/ml HRP in a 10 mM aqueous acetate buffer (pH 5.0) and then diluted this solution 1:33 with neat acetone (which led to enzyme precipitation); after addition of the classical peroxidase substrate guaiacol and H_2O_2, the enzymatic reaction was monitored spectrophotometrically. The rate of the enzymatic oxidation in this 97% acetone medium was found to be $\approx$0.8% of that in the otherwise identical purely aqueous buffered solution (pH 5.0). This 130-fold drop in the peroxidase activity on transition from a purely aqueous solution to 97% acetone can be readily rationalized by such factors as unfavorable energetics of substrate desolvation in acetone because of the stabilization of the guaiacol ground state, acetone penetration into the HRP's active site, and acetone-induced conformational distortions in the enzyme (17).

When lyophilized HRP was directly suspended in the 97% acetone medium, its enzymatic activity was found to be some 140 times lower still than that of the enzyme added via water. In other words, the same two suspensions of HRP in 97% acetone displayed vastly different catalytic activities depending on how they had been prepared. Importantly, we demonstrated that this difference was not due to an irreversible inactivation of HRP on lyophilization and/or subsequent sonication of the enzyme suspension in 97% acetone (to make it homogeneous): when assayed in a purely aqueous solution, the lyophilized enzyme was only 12%, and after the subsequent sonication only 20%, less active than HRP that had been neither lyophilized nor sonicated.

The uncovered marked difference in the catalytic activity between the directly suspended HRP and that added via water was not limited to the acetone medium. The enzyme introduced by the former method was also 140 times less active in 94% dimethylformamide and 260 times less active in 99.4% isopropyl alcohol.

To explain these peculiar observations, we hypothesized that HRP, like many other proteins (3), undergoes a significant reversible denaturation on lyophilization, leading to a plunge in enzymatic activity. When subsequently redissolved in water, where protein molecules are highly flexible (10, 18), the denatured enzyme reverts to the thermodynamically favored, catalytically active conformation. However, this reactivation should be greatly retarded, or even impossible, in organic solvents containing only little water because of the conformational rigidity of enzymes in such media (11). This hypothesis predicts that the lower the water content in a given organic solvent, the greater should be the discrepancy between the rate of the peroxidatic reaction catalyzed by the enzyme added via water (v_{avw}) and that directly suspended (v_{ds}). We confirmed this prediction experimentally, thereby supporting the underlying hypothesis. Although the v_{avw}/v_{ds} value in 97% acetone is 140, it is only 7.4 in 95% acetone but 650 in 98% acetone. Likewise, when the water content in dimethylformamide is raised from 6% (see above) to 10%, the v_{avw}/v_{ds} ratio plummets from 140 to 9.6. Finally, in the case of isopropyl alcohol, the v_{avw}/v_{ds} ratio grows from 83 at 2% water to >670 at 0.2%. (Note that in a purely aqueous buffer, $v_{avw}/v_{ds} = 1$.)

Addition of HRP (or other enzymes) via water has a major drawback of requiring a very high enzyme concentration in aqueous solution before dilution with a neat organic solvent to attain a sufficient concentration thereafter. The lower the desired water content in the eventual organic medium, the more severe this problem becomes. Directly suspending the enzyme in a nonaqueous medium, in addition to being more straightforward, does not suffer from this restriction. Thus this method would be clearly preferred if it resulted in a comparable enzymatic activity, i.e., if the v_{avw}/v_{ds} ratio could be pushed toward unity.

To this end, we explored lyophilizing HRP from aqueous solutions containing various phenolic and aromatic-amine substrates of this enzyme. The rationale behind this was that a substrate bound to the active site could protect the enzyme from the dehydration-induced inactivation during lyophilization. Indeed, as can be seen in Table 1 (the first 11 entries), all of the different substrates tested greatly activated HRP directly suspended in 97% acetone (while having no appreciable influence on the catalytic activity of HRP when measured in water): the activation effect varied from 4.0- to 62-fold depending on the substrate.

It is worth noting that some of the substrates listed in Table 1 are quite volatile and exerted their activating effect even though very little (for *p*-cresol and aniline) or virtually no (for guaiacol and *o*-cresol) substrate was left after the lyophilization, as determined by comparing the weight of the enzyme sample lyophilized in the absence and in the presence of the substrate. This confirms our rationale that the substrate plays its protective role during the lyophilization process by preventing HRP's denaturation.

Phenolic substrates are known to bind to a hydrophobic pocket in the active site of HRP (19–21). Therefore, we thought that perhaps even hydrophobic compounds that are not substrates of HRP could still bind to this pocket and protect the enzyme against denaturation during lyophilization, thereby increasing the enzymatic activity on direct suspension in nonaqueous solvents. The data presented in Table 1 (entries 12–19) confirm this hypothesis. One can see that a number of hydrophobic compounds, both aromatic and aliphatic, bearing various functional groups, activated the enzyme directly suspended (but not added via water) in 97% acetone when present in aqueous HRP solution before lyophilization. The magnitude of the activation effect observed was comparable to that attained in the case of substrates (Table 1). Once again, even a volatile compound, *trans*-1,2-cyclohexanediol, was found effective (27-fold activation) even though only 14% of its original amount remained in the solid HRP sample after

Table 1. Activation of horseradish peroxidase suspended in 97% acetone by various excipients colyophilized with the enzyme

Entry	Excipient*	Activation effect†
1	*o*-Methoxyphenol (guaiacol)	4.0
2	*o*-Methylphenol (*o*-cresol)	8.0
3	*p*-Methylphenol (*p*-cresol)	7.7
4	*o*-Aminophenol	15
5	*o*-Hydroxybenzoic acid	18
6	*o*-Hydroxybenzyl alcohol	62
7	*m*-Hydroxybenzyl alcohol	15
8	*p*-Hydroxyphenethyl alcohol	10
9	Aniline	5.8
10	*p*-Aminobenzoic acid	20
11	*p*-Methoxyaniline (*p*-anisidine)	6.6
12	Benzyl alcohol	4.0
13	Benzoic acid	16
14	*p*-Nitrophenol	15
15	Benzylamine	7.0
16	1-Phenyl-1,2-ethanediol	18
17	*trans*-1,2-Cyclohexanediol	17
18	*cis*-1,2-Cyclohexanedicarboxylic acid	4.7
19	Cyclohexylamine	4.0
20	PEG	6.5
21	Sucrose	4.0
22	Tris	27

The enzymatic activity in all instances was measured in the oxidation of 25 mM guaiacol with 0.25 mM hydrogen peroxide. The reaction medium, in which these substrates were dissolved, was formed by mixing an aqueous acetate buffer (10 mM, pH 5.0) with anhydrous acetone in a 3:97 (vol/vol) ratio. HRP was lyophilized from an aqueous buffered solution containing a given excipient as described in *Materials and Methods,* suspended in the acetone medium at 10 mg/ml, briefly sonicated, and stirred at 25°C. For other experimental conditions, see *Materials and Methods.*

*The concentration of all excipients in the aqueous solution of HRP before lyophilization was 100 mM (on the monomer basis in the case of PEG) except for *o*-aminophenol which, being insoluble at this concentration, was 40 mM). For two of the excipients listed, *o*-hydroxybenzyl alcohol and Tris, we examined the dependence of the activation effect on the excipient concentration in the range from 20 to 400 mM. It was found that the maximal effect was attained at a 100 mM excipient concentration in the aqueous HRP solution before lyophilization, presumably because of a physical blockage of the active sites in the enzyme suspension by the excipient molecules at higher concentrations (2).

†Defined as the initial rate of the enzymatic peroxidation catalyzed by HRP lyophilized in the presence of an excipient divided by that catalyzed by HRP lyophilized in the absence of excipients. Several measurements conducted in duplicate revealed that the experimental error was typically in the 5–15% range and never exceeded 25%.

lyophilization. Likewise, benzyl alcohol afforded a 4-fold activation despite the fact that it completely disappeared as a result of lyophilization.

To our surprise, even hydrophilic nonsubstrate compounds, such as Tris, PEG, and sucrose afforded a 4- to 27-fold activation of HRP suspended in 97% acetone (the last three entries in Table 1). The mechanism of this activation is discussed below.

We found that the activation phenomenona described above were not limited to the acetone medium. Representative compounds from Table 1, when present in aqueous solutions of HRP before lyophilization, also markedly enhanced the subsequent catalytic activity of the lyophilized enzyme suspended in 99.4% isopropyl alcohol: the substrate *o*-hydroxybenzyl alcohol (65-fold), the nonsubstrate hydrophobic compound *trans*-1,2-cyclohexanediol (29-fold), and the hydrophilic nonsubstrates PEG and Tris (8- and 25-fold, respectively).

We next examined the generality of the observed activation with respect to the substrate. A common substrate of HRP structurally unrelated to guaiacol, ABTS (7), even at a 5 mM concentration in aqueous solution before lyophilization, led to a 5.8-fold activation of HRP suspended in 97% acetone and an 18-fold activation in 99.4% isopropyl alcohol when assayed against guaiacol (as all of the other activators in Table 1 were). Interestingly, the reverse was not the case—guaiacol did not significantly activate the enzyme suspended in 99.4% isopropyl alcohol against ABTS, and neither did the hydrophobic nonsubstrates from Table 1 *trans*-1,2-cyclohexanediol and 1-phenyl-1,2-ethanediol. On the other hand, ABTS activated HRP suspended in the isopropyl alcohol medium when it was used as its own substrate (rather than guaiacol). Likewise, all three hydrophilic nonsubstrate activators, entries 20–22 in Table 1, also activated the enzyme against ABTS (6.3-, 30-, and 67-fold, respectively).

To rationalize these seemingly puzzling findings, we hypothesized that there are two independent mechanisms of activation. One involves hydrophobic substrates and nonsubstrates that bind to the active site of HRP and thus prevent its collapse during lyophilization. The other involves general hydrophilic lyoprotectants (22), such as the polyols Tris or sucrose, which do not bind to the active site but instead replace the water solvent molecules departing during lyophilization, thereby protecting the overall native enzyme structure. This hypothesis readily explains how the hydrophobic compounds listed in Table 1, as well as ABTS, activate HRP toward guaiacol: because all of them are of the size comparable to, or greater than, that of guaiacol, the cavity that they preserve in the active site of the enzyme during lyophilization is sufficient to subsequently accommodate guaiacol. In contrast, the cavity in the enzyme active site preserved by guaiacol, *trans*-1,2-cyclohexanediol, and 1-phenyl-1,2-ethanediol, all single-ring compounds, is apparently too small to subsequently accommodate ABTS, which is a four-ring compound. On the other hand, hydrophilic lyoprotectants, including Tris, PEG, and sucrose, which protect the overall enzyme structure, have an activating effect toward both the small and large substrates.

This dual-mechanism hypothesis also predicts that the activating effects exerted by compounds acting via distinct mechanisms should be additive, whereas for those acting via the same mechanism they should not be. We verified these predictions experimentally. The following observations were made when using 97% acetone. A combination of *o*-aminophenol and Tris (concentrations and other conditions henceforth were the same as in Table 1) activated HRP 62-fold, whereas the individual compounds led to a 15- and 27-fold activation, respectively. Likewise, a mixture of guaiacol and PEG afforded a 14-fold activation, whereas the individual components activated 4.0 and 6.5 times, respectively. In contrast, a combination of Tris and PEG (the same mechanism) afforded only a 12-fold activation, whereas when used individually, the activation effects of these compounds were 27- and 6.5-fold, respectively. Even more dramatic additive effects were observed with 99.4% isopropyl alcohol. A combination of *o*-hydroxybenzyl alcohol and PEG activated HRP 118-fold, whereas separately, these compounds led to a 65- and 8-fold activation, respectively. Finally, whereas a mixture of *trans*-1,2-cyclohexanediol and PEG afforded a 129-fold activating effect, the individual compounds gave only 29- and 8-fold activations, respectively.

All of the results reported above were obtained with HRP. To test the generality of these findings, we expanded them to include two other peroxidases (14), one from soybean and the other from *C. fumago.* With respect to the former enzyme, we found that it too, like HRP, was far more catalytically efficient when placed in a nonaqueous medium via water than by directly suspending its lyophilized powder. This effect, although significant ($v_{avw}/v_{ds} = 24$) even in 96% acetone, rose to 100 in 98% acetone, and became quite striking ($v_{avw}/v_{ds} = 780$) in 99.5% acetone. The fact that the v_{avw}/v_{ds} ratio greatly

Table 2. Activation of soybean peroxidase suspended in 99.5% acetone by various excipients colyophilized with the enzyme

Excipient*	Activation effect†
o-Hydroxybenzyl alcohol	400
m-Hydroxybenzyl alcohol	100
o-Methoxyphenol (guaiacol)	33
Trans-1,2-cyclohexanediol	110
PEG	35
guaiacol + PEG	450
Trans-1,2-cyclohexanediol+PEG	800

The experimental conditions were the same as those outlined in Table 1 except that the reaction medium consisted of the aqueous acetate buffer and acetone in a 0.5:99.5 (vol/vol) ratio.

*The concentration of all excipients in the aqueous solution of HRP before lyophilization was 100 mM. When combinations of excipients were used (the last two entries), each was present at a 100 mM concentration.

†See footnote † in Table 1.

increases as the water content of the medium drops suggests the same underlying mechanism as in the case of HRP, i.e., the lyophilization-induced, reversible denaturation of soybean peroxidase.

Table 2 shows that the catalytic activity of soybean peroxidase directly suspended in 99.5% acetone could be dramatically enhanced by certain excipients present in aqueous solution of the enzyme before lyophilization. As with HRP (Table 1), *o*-hydroxybenzyl alcohol was the most potent single activator, yielding a 400-fold activation, and the hydrophobic nonsubstrate *trans*-1,2-cyclohexanediol was more effective than the assay substrate guaiacol. Moreover, the combinations of the latter two compounds with the hydrophilic lyoprotectant PEG afforded remarkable additive effects, with the mixture of *trans*-1,2-cylohexanediol and PEG giving the same catalytic activity of the directly suspended enzyme as that of the added-via-water soybean peroxidase (the last entry in Table 2).

C. fumago chloroperoxidase (23, 24) was also found to be much more active in 99.4% isopropyl alcohol when added via water than when directly suspended in that medium (v_{avw}/v_{ds} = 190). As in the case of the two plant peroxidases, this ratio could be dropped 27-fold to 7.0 if the enzyme was lyophilized in the presence of the substrate *o*-hydroxybenzyl alcohol.

Finally, it was found that mushroom polyphenol oxidase, which is not a peroxidase but instead oxidizes phenolic substrates with molecular oxygen, follows the same pattern of nonaqueous behavior as the three peroxidases. This enzyme added to 97% acetone via water was 180 times more reactive than when the lyophilized oxidase was directly suspended in this medium. However, this difference shrank to a mere 2-fold when polyphenol oxidase was lyophilized in the presence of 0.1 M *o*-hydroxybenzyl alcohol.

In closing, the present study demonstrates that the phenomenon of molecular memory (11) extends to oxidative enzymes. The catalytic activity of all four oxidases tested suspended in nonaqueous media (but not when dissolved in water) markedly depends on how the enzyme is introduced into the medium—whether via water or by direct suspension. The mechanism of this behavior was elucidated and led to an effective activation strategy. Specifically, the oxidases lyophilized in the presence of substrates and other hydrophobic compounds, as well as in the presence of hydrophilic lyoprotectants, exhibited a much higher activity when directly suspended in various nonaqueous media than those lyophilized without excipients. The activation effects exerted by two unrelated groups of excipients are mechanistically distinct and additive, thus resulting in up to a complete elimination of lyophilization-induced activity losses.

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