0273-2289/95/5002—0161$06.60

Effect of Polyvinylalcohols on the Thermostability of Lipase from *Candida rugosa*

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Received October 13, 1993; Accepted May 2, 1994

ABSTRACT

Lipase from *Candida rugosa* was stabilized against thermal inactivation in the presence of polyvinylalcohols (PVA) of different molecular weights. The apparent rate constant of the lipase inactivation, k_d, at 49°C is 0.049/min and 0.022/min in the absence and in the presence of PVA (mol wt 22,000), respectively. The improvement of the lipase thermostability by adding PVA was confirmed by differential scanning calorimetry. The presence of PVA had also an effect on the hydrolytic activity of the enzyme. Furthermore, lipase was modified by covalent linkage to PVA by means of an original procedure. With respect to the native enzyme, the modified lipase has a slightly lower specific activity, but it is more stable against heat denaturation (k_d 0.032/min at 49°C).

Index Entries: Lipase; thermostability; *Candida rugosa*; polyvinylacohols.

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INTRODUCTION

Several lipases (EC 3.1.1.3) from microbial sources have been isolated in the recent years (*1,2*) and their potential applications in pharmaceutical, textile, chemical, and food industry have been proposed (*3*). The lipase from *Candida rugosa* (formerly known as *Candida cylindracea*) has been widely used in organic synthesis owing to its versatility. The effects on the enzyme activity induced by chemical modification of amino acid residues (*4*), covalent linkage to synthetic (*5,6*), and natural (*8–10*) polymers and immobilization on various supports (*7*) have been investigated.

The stabilization of enzymes against thermal inactivation is of great interest either from a theoretical point of view, i.e., a better understanding of the mechanisms of protein denaturation, and from a practical point of view when enzymes are to be employed in industrial processes. In fact, the large-scale use of a thermostable biocatalyst, rather than a thermosensitive one, has several advantages such as:

1. The possibility to operate at high temperature, which affords high reaction rates;
2. The increase of the storage and operational stabilities; and
3. The eventual increase of the resistance to protein denaturing agents.

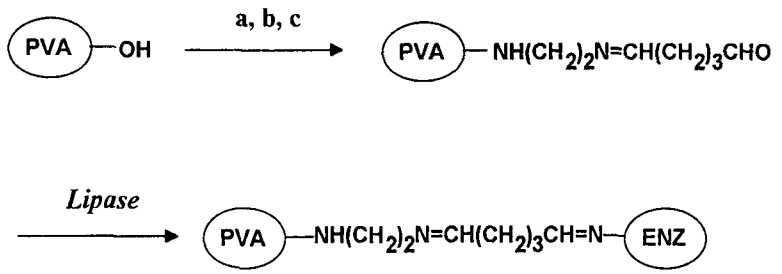
Polyalcohols have a protective effect against enzyme heat denaturation (*11–13*) through a mechanism of mutual interactions among solvent, additives, and enzyme molecule, in the native and denaturated form. Stabilization is not general but depends on the nature of the enzyme and on the extent of the interaction with the biocatalyst microenvironment.

In this work, the thermostability of *C. rugosa* lipase was studied by monitoring the residual activity at different temperatures in the presence of polyvinylalcohols (PVA) of different molecular weights (15,000, 22,000, and 70,000 Daltons). The inactivation is an apparent first-order kinetic process at all the temperatures studied. Differential scanning calorimetry experiments confirmed the stabilizing effect of PVA. PVA also affects the hydrolytic activity of the enzyme, as indicated by the dependence of the lipase activity on the polymer concentration. Furthermore, the lipase was chemically modified by covalently linking PVA to the enzyme molecule by means of an original procedure that allows an extensive modification of the lipase primary amino groups. The behavior of the modified enzyme was compared with that of the native enzyme.

MATERIALS AND METHODS

Chemicals

Lipase from *Candida rugosa* (Type VII), olive oil at low acidity, tributyrin, and arabic gum from *Acacia* tree were from Sigma Chem. Co.



Scheme 1. Covalent functionalization of lipase with PVA. (a) tosyl chloride, (b) 1,2-diaminoethane, (c) glutaraldehyde.

(St. Louis, MO). PVA 15,000 and PEG were from Fluka Chemie AG (Buchs, Switzerland); PVA 22,000 and 70,000 were from SERVA (Heidelberg, Germany). All other reagents were of analytical grade.

Purification of Lipase

Lipase was purified by ion-exchange chromatography on a DEAE-cellulose column, equilibrated with 20 m*M* bis/Tris buffer, 1 m*M* $CaCl_2$, pH 6.5. The protein was eluted with a linear gradient of NaCl from 0–300 m*M*. The active fractions were concentrated, dialyzed, and applied to a column of Sephadex G-100. The enzyme preparation contained 0.3 mg/mL of proteins. Proteins were assayed by the method of Bradford (*14*), by using bovine serum albumin as standard.

Modification of Lipase with Activated PVA

Five grams of PVA (15,000 Daltons) and 2 mL of pyridine were added to 30 mL of dichloromethane. Tosyl cloride (5 g) was added stepwise at 0°C and the mixture stirred for 24 h at 25°C. The modified PVA was recovered by filtration, washed with dichloromethane, and then added to 40 mL of dichlorometane containing 4 mL of 1,2-diaminoethane. The suspension was stirred for 48 h at room temperature. The aminoalkylated PVA was recovered by filtration, washed with ethanol, and dehydrated under vacuum. The amino groups present in the modified PVA were determined with the trinitrobenzenesulfonate (TNBS) method (*15*). It was found that 0.9 moles of $-NH_2$ per mole of PVA were present in the modified PVA.

Five hundred milligrams of the aminoalkylated PVA and 0.5 mL of glutaraldehyde (25% w/v) were added to 5 mL of dioxane. The suspension was stirred for 1 h at 0°C. Then, the activated PVA was filtered, washed with dioxane and added to a lipase solution at 4°C. This solution was previously prepared dissolving 1.5 g of *C. rugosa* lipase in 20 mL of Na/phosphate buffer 50 m*M*, pH 7.0, stirring for 30 min at room temperature and then removing the insoluble material by centrifugation (10,000*g*, 20 min). The reaction was carried out for 12 h at 4°C. The activation of PVA and the coupling reaction with the protein are represented in Scheme 1.

The modified lipase was purified by gel filtration chromatography on Sephadex G-100. The extent of modification was determined by titration of the free amino groups of the native and modified enzyme by using the TNBS method (*15*). Twenty residues out of 37 primary amino groups (*16*) were modified with this procedure. The lipase-modified preparation contained 0.8 mg/mL of proteins. It retained 81% of the hydrolytic activity of the native enzyme, assayed with olive oil as substrate.

Enzyme Assay

The assay of the lipase hydrolytic activity was derived from Henry et al. (*17*). An emulsion of olive oil (50 mL) in water (50 mL) containing 5% arabic gum and 0.2% sodium benzoate, was prepared by stirring the mixture in a Waring blendor for 15 min. The reaction mixture (3 mL H_2O, 2 mL of the substrate emulsion, 1.5 mL Na/phosphate buffer 100 m*M*, pH 7.0, and 0.02 mL of the enzyme) was incubated at 30°C for 30 min with shaking at 90 rpm. The reaction was stopped by adding 3 mL of ethanol 95%. The free fatty acids were measured by titration with standardized NaOH. The specific activity of lipase was expressed in units per mg of protein (U/mg), 1 U being the amount of enzyme liberating 1 μeq of fatty acid per minute under the described conditions.

Thermal Stability

The effect of the temperature on the stability of the native (with and without additives) and modified lipase was determined by as follows. Several samples (4.5 mL of phosphate buffer 50 m*M* pH 7.0) of the native (0.02 mL) and modified enzyme (0.01 mL) were incubated at a fixed temperature (from 40–53°C). At regular time-intervals a sample was withdrawn from the thermostat and rapidly cooled in an ice-bath to stop the inactivation reaction. Then, the residual activity was assayed, by adding 2 mL of substrate, as described above.

Calorimetry

Differential scanning calorimetry (DSC) experiments were performed with a Perkin Elmer DSC-7 instrument, equipped with high pressure stainless steel pans. The typical protein content was 2.5 mg in phosphate buffer 50 m*M* pH 7 in the sample pan. The reference pan was filled with buffer with or without PVA. The heat contribution from the reference was therefore automatically subtracted during acquisition. The scan rate was 2°C/min.

The fitting and deconvolution of the experimental DSC curves were carried out according to the mathematical treatment of Freire and Biltonene (*18,19*). The calorimetric enthalpy change of unfolding, ΔH, was calculated by integration of the area underneath the curve, after baseline subtraction. The van't Hoff enthalpy change, ΔH_{vH}, was calculated through the van't Hoff relationship by considering the progression

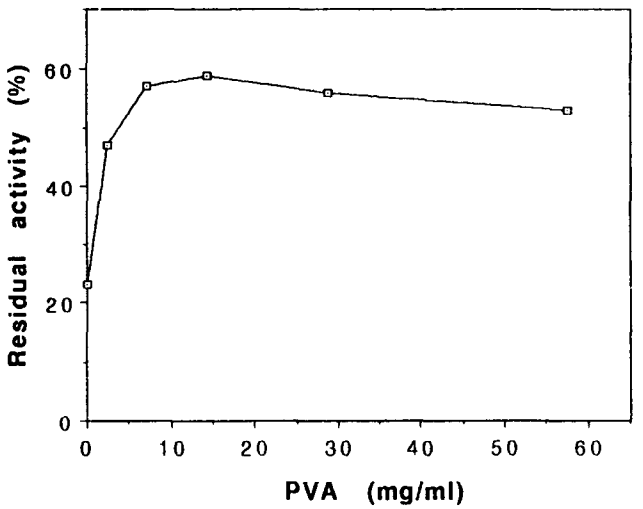


Fig. 1. Residual activity of lipase after 30 min incubation at 49°C in the presence of scalar concentration of PVA 22,000.

of the transition as a function of the temperature. The temperature of the transition middle point, T_m, was also evaluated from the fitting analysis.

RESULTS

The effect of PVA 22,000 on the lipase thermal inactivation was determined by incubating the enzyme at 49°C for 30 min in the presence of scalar concentrations of the polymer. As shown in Fig. 1, the residual activity of the enzyme in the absence of PVA was 24% of the initial one; whereas, in the presence of PVA, the residual activity increased as a function of PVA concentration, reaching a maximum at 15 mg/mL. Residual activity was referred to the activity of the native enzyme assayed at 30°C in the presence of the same concentration of PVA. In this way, it was possible to evidentiate only the effect of the polyol on the enzyme stability since the presence of PVA influenced the lipase hydrolytic activity. In fact, PVA enhances the enzyme activity towards the oily substrate at low concentrations (Fig. 2), probably by improving the quality of the substrate emulsion.

By considering the time course of the enzyme inactivation a first order monomolecular reaction, the apparent rate constant of the enzyme inactivation reaction (k_d) was calculated from the slope of the curve obtained by plotting ln E/E_o vs time (E and E_o are the activities of the partially inactivated and native enzyme, respectively). The lipase inactivation kinetics at 49°C in the absence and in the presence of PVA 22,000 (28 mg/mL) are shown in Fig. 3. At this temperature, PVA significantly stabilizes the protein. The k_d values calculated from the best linear fitting of the data are 0.049/min and 0.022/min, respectively. The inactivation kinetics were

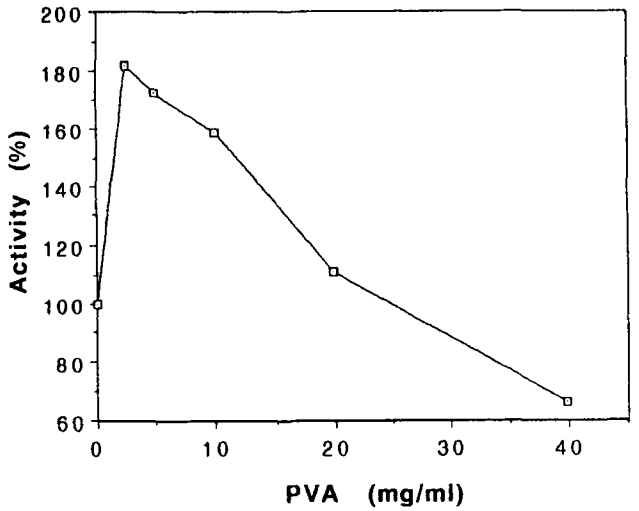


Fig. 2. Lipase activity at 30°C in the presence of scalar concentration of PVA 22,000.

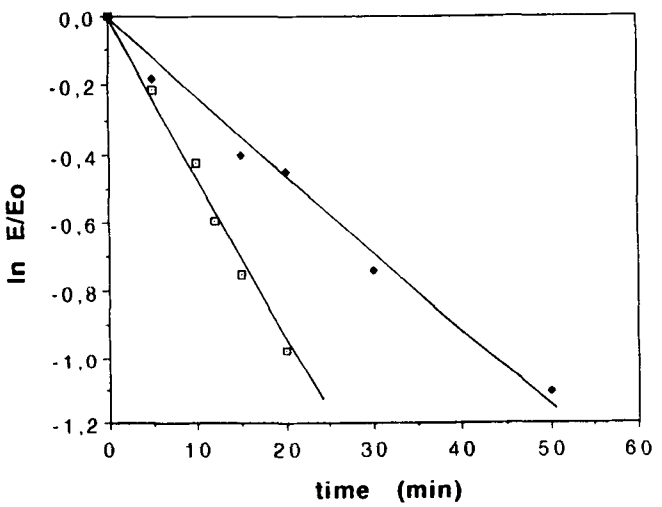


Fig. 3. Inactivation kinetics of native lipase (□) and of lipase in the presence of PVA 22,000 (◆) at 49°C. For all the experiments as a function of the temperature PVA concentration was 28.8 mg/mL in the incubation mixture, corresponding to a concentration of 20 mg/mL in the assay volume; at this PVA concentration the activity at 30°C was 100% of that without PVA, as shown in Fig. 2.

studied at different temperatures, from 40–53°C, with and without PVA. The results are listed in Table 1. The data were all fitted by first-order kinetics mechanism. The corresponding Arrhenius plot is shown in Fig. 4. As it can be seen, in the whole temperature range, the values of k_d obtained in the presence of PVA are lower than those in the absence of PVA. It is worth to note that, within the experimental uncertainty, the two sets of data cannot be fitted by a straight line, although linear behavior is often observed (*7,20*). The nonlinearity of the Arrhenius plot may be obtained

Table 1
pparent Rate Constants of Lipase
Inactivation as a Function of the Temperature[a]

Temperature, °C	k_d of Native lipase				k_d of Modified lipase[e]
	No additive	PVA 15,000[b]	PVA 22,000[c]	PVA 70,000[d]	
53	0.086 ± 0.010	0.049	0.081	0.067	0.065
49	0.049 ± 0.007	0.015	0.022	0.023	0.032
45	0.020 ± 0.002	0.007	0.007	0.011	0.012
42		0.0012	0.005	0.007	
40	0.017 ± 0.001				0.010

[a]The concentrations of PVA were selected in order to keep constant the hydroxyl groups concentration.
[b]41 mg/mL.
[c]28 mg/mL.
[d]9 mg/mL.
[e]Lipase chemically modified with PVA 15,000.

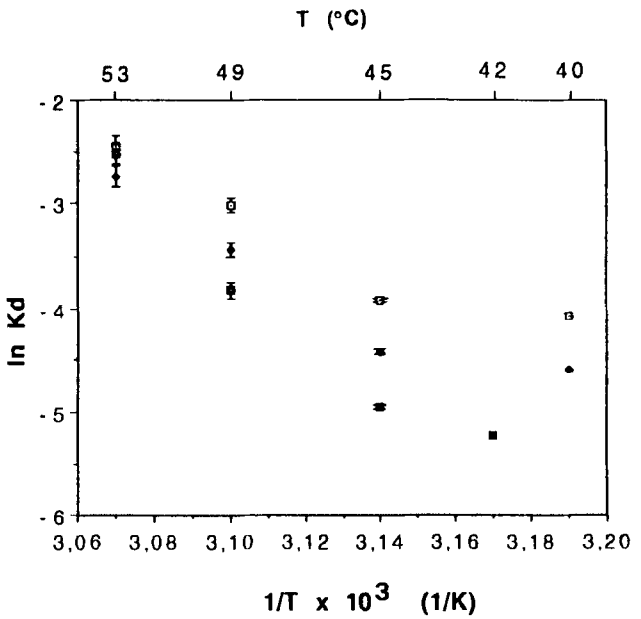


Fig. 4. Arrhenius plot for native lipase (□), for native lipase stabilized by PVA 22,000 in solution, 28 mg/mL, (■) and for lipase chemically modified with PVA 15,000 (◆).

when the inactivation process is accompanied by the concurrent denaturation of the protein molecule because of the large and positive heat capacity change associated with the protein unfolding (*21,22*). In this situation, the rate of denaturation should overwhelm the rate of any other inactivation process. In order to verify whether the temperature interval of the kinetic experiments overlaps the thermal range of the lipase unfolding transition, the enzyme stability was studied by Differential Scanning

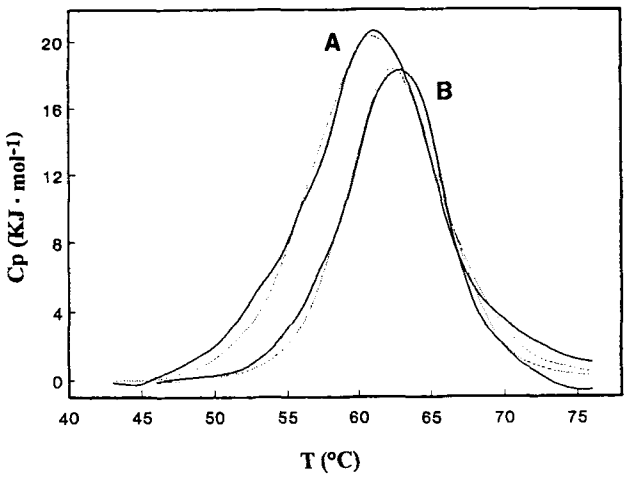


Fig. 5. Excess molar heat capacity of *C. rugosa* lipase as a function of the temperature. (A) phosphate buffer 50 m*M*, pH 7. (B) same buffer and PVA 22,000 20 mg/mL. Scan rate 2°C/min.

Table 2
Thermodinamic Parameters of Lipase Unfolding Transition

Additive	ΔH, KJ/mol	T_m, °C	Cu
None	501.6 ± 40	60.3 ± 0.3	1.09
PVA 22,000, 20 mg/mL	430.5 ± 40	62.0 ± 0.3	1.19

Calorimetry (DSC). Lipase, purified as described in the experimental section, undergoes a single cooperative unfolding transition, with and without PVA, as it is shown in Fig. 5. The thermodynamic parameters (the enthalpy change, ΔH, the temperature of the transition middle point, T_m, and the ratio of ΔH and ΔH_{vH}, Cu) associated with the process are listed in Table 2. It is clear from the curves shown in Fig. 5 that above 45°C the native lipase in the absence of PVA, and, to a lesser extent, in the presence of PVA 22,000 (20 mg/mL), already begins to unfold. In the presence of PVA, ΔH decreases, whereas T_m slightly increases, suggesting a small improvement of the thermal stability. It is interesting to note that the unfolding process can be reasonably well approximated by a two-state transition, as indicated by the values of Cu close to 1 (*23,24*).

In order to assess to what extent the stabilizing effect of PVA was caused by the presence of hydroxyl groups in the molecule or the length of the carbon chain, PVAs of 15,000 (41 mg/mL) and 70,000 (9 mg/mL) were tested at the same hydroxyl concentration as PVA 22,000. With both polymers, the values of k_d calculated in the same range of temperature (43–53°C), were similar to those of the enzyme incubated in the presence of PVA 22,000, except at 53°C (Table 1). These results indicate a small dependence on the length of the carbon chain, suggesting a more relevant contribution of the hydroxyl groups to the stabilization effect of PVA.

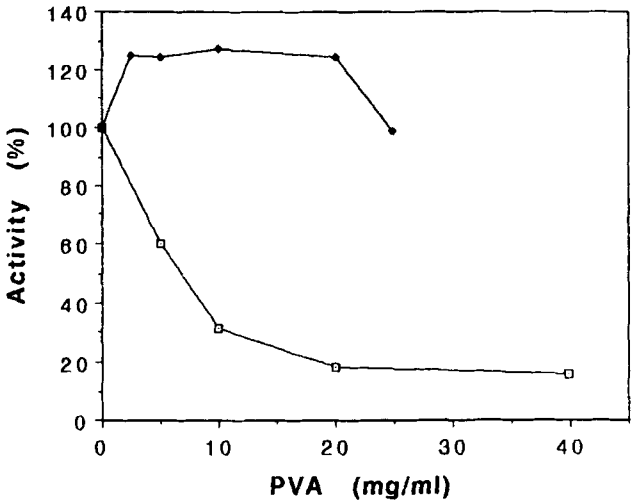


Fig. 6. Lipase activity measured at 30°C in the presence of scalar concentration of PVA 15,000 (□) and 70,000 (◆).

As far as the hydrolytic activity of the enzyme is concerned, it was observed that PVA 70,000 enhanced the enzyme activity even at much lower concentration than PVA 22,000 (less than 20 mg/mL, Fig. 6) Conversely, lipase activity decreased in the presence of PVA 15,000 at any concentration of the polymer. The commercial origin of the polymers (PVA 15,000 was from a different source with respect to PVA 22,000 and 70,000) might account for the observed effects, probably owing to the different influence on the formation of the substrate emulsion.

The apparent inactivation rate constant of the lipase covalently modified with PVA is 0.032/min at 49°C (Table 1). The presence of the free polymer in solution was more effective (k_d 0.022/min) against enzyme unfolding than the covalent modification of the enzyme. However, k_d of the modified enzyme is still lower than that of the native lipase (k_d 0.049/min), suggesting that the chemical modification, which did not affect significantly the enzyme activity (the modified lipase retained 81% of the activity of the native form) did have a slight beneficial effect on the lipase thermal stability. As shown in Fig. 4, the modified lipase is more stable than the native enzyme in the whole temperature interval studied. The values of k_d at different temperatures are reported in Table 1. Considering that the extent of lipase modification was 54%, i.e., 17 of the free amino groups of each molecule were linked to PVA molecules, and that in each experiment 8 mg of the modified enzyme were used, it can be calculated that only 1.32×10^{-3} millimoles of PVA were present (the molecular weight of the lipase native form is 120,000 Daltons [25]). That is, the concentration of PVA in the sample was about 6500-fold lower than that used in the experiments where PVA was just used as additive to the lipase solution.

In order to verify whether other polymers had a similar effect on the lipase stability, polyethyleneglycol (PEG) of different molecular weight

(4000, 20,000, and 40,000) was tested at the same ponderal concentration of PVA at 49°C. From the data reported in the literature, PEG has a controversial action against thermal denaturation that depends on the protein (*26–28*). The apparent inactivation rate constants calculated in the presence of PEG were very similar (k_d = 0.02 ± 0.001/min), regardless of the PEG molecular weight. In any case, they were significantly lower than that of the native enzyme in the absence of PEG. Therefore, this polymer has also an apparent stabilization effect against the thermal inactivation of lipase.

DISCUSSION

The lipase from *C. rugosa* undergoes a time-dependent thermal inactivation process which can be described by a first-order kinetics. On the basis of the results obtained in this study, polymeric additives such as PVA do not change the apparent first-order kinetics of lipase inactivation, suggesting that in the presence of the polymer the mechanism of inactivation is not modified. This is not a general situation because in the case of other enzymes, including other lipases, several mechanisms may be involved in the inactivation process, such as irreversible structural changes, temperature induced conformational transitions, or chemical changes, with the consequence that simple first-order kinetic models are not enough to describe the observed behavior (*9*). In our case, in spite of the apparent first-order process at each temperature, thermodynamic observations suggest that in the higher temperature range of the kinetic study, inactivation is accompanied (or caused) by unfolding of the protein molecule. This conclusion is based on the inspection of the DSC curves, i.e., the temperature interval where unfolding occurs, as well as on the behavior (the change in slope) of the ln k_d vs 1/T plot, whose nonlinearity cannot be accounted for by the experimental uncertainty. In the presence of PVA, the unfolding process slightly shifts to higher temperatures, but not enough to be excluded from the temperature interval of the kinetic experiments.

The mechanism of the enzyme protection against inactivation or unfolding in aqueous solution containing polyols has been well studied in the case of low molecular weight compounds such as glycerol or sucrose (*29–31*). The positive effect on protein stability stems from the preferential hydration of the protein molecule in the aqueous medium containing these additives, i.e., polyols are preferentially excluded from the protein domain because of the inability of these compounds to interact with the protein surface. Thermodynamically, this means that the free energy of the solvent–solute mixture is raised: The protein has an unfavorable effect on the system. Exclusion effects are proportional to the superficial area of the protein (*29*). Therefore, the protein folded state is stabilized with respect to the unfolded state because, after unfolding, the

surface of contact between protein and solvent increases, leading to an even more unfavourable situation. The basis of such negative interactions are related with the exposure of nonpolar groups upon unfolding, which are repelled quite effectively by water-polyol mixture (*31*). However, other factors give contribution to the preferential hydration and to the enhancement of protein stability, such as excluded volume effects of the polyol molecule and the nature of the protein surface. In fact, the more hydrophobic and/or less charged the protein surface, the stronger the preferential hydration.

It is possible that PVA stabilizes the lipase native state through the same type of mechanism postulated for other polyols, such as sucrose and glycerol. This mechanism involves interactions between the hydroxyl groups of the additive and water molecules which are stronger than those between protein and additive, favoring preferential hydration. This can be also supported by our observation that another polyol, i.e. sorbitol, used at the same hydroxyl concentration employed in the case of PVAs, showed to be a stabilizing agent of the *C. rugosa* lipase (k_d = 0.030 ± 0.001/min at 49°C).

The proposed mechanism of interaction of PVA with the biocatalyst microenvironment may also explain why the effect is greater when the polyol is used as an additive in solution instead of being covalently linked to the protein molecule. In the latter case, even if the covalently bound PVA linkage can determine a certain degree of organization of solvent molecules (*32*), it cannot produce the degree of preferential hydration owing to solvent exclusion from protein domain to the same extent as PVA in solution. The chemical modification of the lipase amino acid side chains may play a stabilizing role by itself, similar, for example, to that observed in the case of the yeast invertase, a naturally glycosylated enzyme. In fact, invertase thermostability increases by increasing degree of glycosylation (*12*).

The observed modifications of hydrolytic activity in the presence of PVA can be associated with the formation of microemulsions that substantially differ from those formed by the oily substrate in water. In other words, PVA acts as an emulsifier, although the suspension tends to coalesce and gradually converts into water/oil type as the pH is lowered (*16*). Some authors have reported that the concurrent presence of proteins and PVA tends to stabilize the microemulsions by competitive adsorption of the macromolecules at interface (*33*).

There are controversial reports on the effect of PEG on protein inactivation rates. Protease from *Cucurbita ficifolia* is strongly stabilized by PEG at 80°C (*26*), as well as glucose-oxidase (*27*), whereas the addition of PEG, even at low molecular weights, is detrimental to the stability of α-amylase from *Bacillus stearothermophilus* at 90°C (*28*). Equilibrium thermodynamics experiments seem to confirm that PEG decreases protein stability, as judged by the decrease of the temperature of unfolding, as

studied by DSC. The magnitude of the destabilization depends on the hydrophobicity (or, conversely, on the charge density) of the protein studied (*34*). Although PEG does not seem to alter significantly the protein native state, which actually repels PEG molecules, it has been shown that it preferentially interacts with nonpolar protein groups in the denatured state, owing to its hydrophobic nature (*35*). The prevalence of the stabilization of the native state (through preferential hydration) or of the unfolded state (through specific hydrofobic interaction) is a delicate balance that depends on the specific protein studied.

In summary, the use of polyalcohols such as PVAs and sorbitol as well as polymers like PEG as stabilizing agent against thermal inactivation in solution, can be extended to lipase from *Candida rugosa*. In particular, it has been observed that PVA is more effective in solution rather than covalently linked to the protein molecule. Therefore, the use of the proper additives represents a simple strategy to obtain an enzymatic preparation with enhanced thermostability.

ACKNOWLEDGMENTS

This paper is dedicated to the memory of Vincenzo Scardi, who began the scientific collaboration of this group and the development of this research.

This work was financed by Ministero Ricerca Scientifica e Tecnologica, P.N.R. per le Biotecnologie (Rome, Italy).

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