Lipoxygenase Inactivation by Manothermosonication: Effects of Sonication Physical Parameters, pH, KCl, Sugars, Glycerol, and Enzyme Concentration

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Manothermosonication (MTS) efficiency in lipoxygenase (LOX) inactivation, in phosphate buffer, pH 6.5, increases with ultrasound amplitude in the range 0–104 μm at any temperature from 67.5 to 76.3 °C. Activation energy (E_a) is lower in MTS than in thermal enzyme inactivation and decreases with increasing ultrasound amplitude. Thermal and MTS resistance of the enzyme raises with decreasing pH in the range 5.2–8, but more so thermal than MTS resistance. Differences of E_a between thermal and MTS enzyme inactivation increase with decreasing pH. Glucose, sucrose, and glycerol increase both thermal and MTS LOX resistance. Neither of these enzyme stabilizers deviates the MTS inactivation course from first-order kinetics but all of them transform in biphasic the thermal inactivation course. LOX concentration does not affect thermal stability but increasing concentration of the enzyme increases MTS resistance. These results are discussed in terms of the possible inactivation mechanism added to heat by ultrasonic waves in MTS.

Keywords: *Manothermosonication; lipoxygenase; enzyme inactivation*

INTRODUCTION

Heat treatment is the most widely utilized method for stabilizing foods because of its capacity to destroy microorganisms and inactivate enzymes. However, since heat produces a negative impact in some organoleptic properties and accelerates the decay of some nutrients, a reduction in thermal inputs during preservation of food products is being sought. The increase in the demand for food with little heat-induced degradation of nutritional and organoleptic properties gave rise to the development of both nonthermal (Mertens and Knorr, 1992) or combined processes. In most combined processes other inactivating agents operate simultaneously or successively to heat, in such a way that their effects are either added or potentiated. One of the emergent combined methods is manothermosonication (MTS; Lopez et al., 1994; Sala et al., 1994), in which heat and ultrasound are simultaneously applied under pressure. This method increases synergistically the global bactericidal and enzyme inactivation efficiency.

Lipoxygenase (LOX; EC 1.13.11.12) is an iron-containing dioxygenase which catalyzes the oxidation of polyunsaturated fatty acids containing *cis,cis*-1,4-pentadiene units to the corresponding conjugated *cis,-trans* dienoic monohydroperoxides (Wong, 1989). This enzyme is usually involved in off-flavor development in many vegetable products (O'Connor and O'Brien, 1991); therefore, it must be destroyed by blanching before vegetable drying or freezing or by pasteurizing treatments in fruit juice concentration. LOX has been shown (Lopez et al., 1994) to be very sensitive to MTS inactivation in phosphate buffer at pH 6.5. The present work describes the effect of several MTS parameters, pH, enzyme concentration, neutral salts, sugars, and glycerol on LOX resistance to MTS inactivation.

EXPERIMENTAL PROCEDURES

Materials. LOX type 1B from soybean was a product of Sigma (St. Louis, MO). Other chemicals used were of reagent grade.

Enzyme Assay. LOX was assayed at 25 °C, in 50 mM borate buffer, pH 9, by a spectrophotometric method based on the methods of Ben Aziz et al. (1970) and Chen and Whitaker (1986), as described by Lopez et al. (1994).

Heat Treatments and Manothermosonication. Heat treatment were performed in a TR-SC thermoresistometer (Condon et al., 1989). Manothermosonication was carried out in a thermoultrasonic resistometer, built by a modification of the TR-SC instrument (Raso et al., 1993; Lopez et al., 1994). In all cases the treatment chamber (23 mL) was filled with the treatment medium and heated to the preset treatment temperature before 300 μL of the enzyme solution (usually 45 mg/mL in 20 mM borate buffer, pH 9.0) was injected. Dilution resulting from the entrance of treatment medium from the main vessel was taken into account when destroyed and residual activities were calculated.

Unless otherwise noted, the treatment medium consisted of 20 mM potassium phosphate buffer; pH was adjusted to 6.5 except for experiments on the effects of pH.

Enzyme Inactivation Parameters. Since heat enzyme inactivation generally follows first-order kinetics, residual activity data are usually analyzed in accordance to the classical equations used in the study of first order chemical reaction, which allows the estimation of rate constants (k) and activation energy (E_a). Because of its advantage for heat treatments calculations, it is also common in working on thermostability of food enzymes to express enzyme inactivation in terms of D and z values (Versteeg et al., 1980; Christen and Marshall, 1985; Richardson and Hyslop, 1985; Diermayer et al., 1987). The D, or decimal reduction, value is defined as the time required to inactivate 90% of the original enzyme activity at a constant temperature, and it is obtained from plots of log (enzyme activity) vs time. The z value expresses the temperature dependence of thermal inactivation, and it is obtained from the plot of log D against temperature (thermal inactivation plots) as the number of degrees required for the thermal inactivation curve to traverse one log cycle. Both approaches have been followed in this work.

RESULTS

Effect of Pressure and Ultrasound Amplitude on LOX MTS Inactivation Efficiency. Semilog plots of residual activity versus time of MTS treatment at

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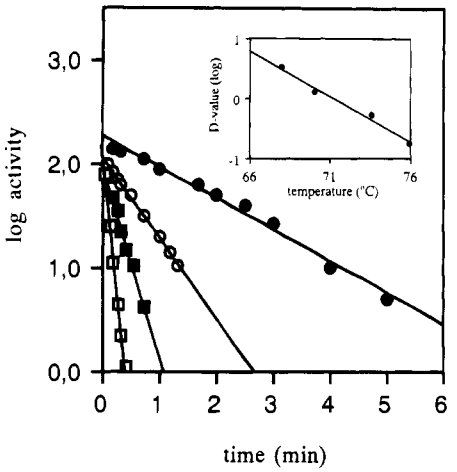

Figure 1. Lipoxygenase inactivation by MTS operating at 76 μM ultrasound amplitude and 4.5 kg/cm^2. Temperature: 68 °C (●), 70 °C (○), 73.6 °C (■), or 76 °C (□). Insert: temperature dependence of D value.

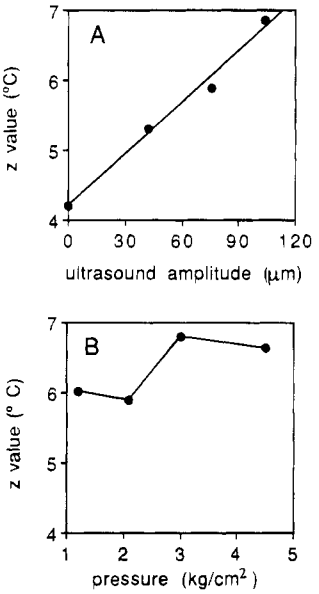

Figure 2. MTS inactivation of lipoxygenase: z value dependence on ultrasound amplitude (A) and pressure (B).

Table 1. Activation Energy (kJ mol^{-1} K^{-1}) of LOX MTS Inactivation at Different Pressures and Ultrasound Amplitudes

	amplitude (μm)			
pressure (kg/cm^2)	0	42	76	104
1.2			375 ± 45	
2.1	563 ± 30[a]	429 ± 36	383 ± 51	329 ± 48
3.0			320 ± 18[a]	
4.5			337 ± 31	

[a] Estimated from data previously reported (Lopez et al., 1994).

different temperatures (in the range 67.5–76.3 °C) ultrasound amplitude (0–104 μm) and pressures (1.2–4.5 kg/cm^2) were always linear and allowed to estimate D and z values and E_a. Figure 1 illustrates results obtained in one of these series of experiments. The dependence of z and E_a on pressure and ultrasound amplitude is shown in Figure 2 and Table 1, respectively.

As previously reported (Lopez et al., 1994), pressure changes in the range explored do not substantially affect either D, z, or E_a.

Table 2. D Values (Minutes) of LOX Inactivation under Heat and MTS Treatments at Different pHs and Temperatures

		pH			
temp (°C)	treatment	5.2	6.5	7.2	8
69	heat	111	51.8	11.6	1.64
72	heat	18.4	7.84	1.17	0.43
74	heat	6.58	2.89	0.50	0.20
67	MTS	5.07	4.21	1.60	1.03
70	MTS	1.83	1.64	0.51	0.26
72.5	MTS	0.71	0.45	0.13	0.07

Table 3. Effect of pH on the Activation Energy (kJ mol^{-1} °K^{-1}) of Thermal and MTS LOX Activity Destruction

	treatment	
pH	heat	MTS
5.2	546 ± 20	348 ± 13
6.5	563 ± 30	386 ± 23
7.2	627 ± 19	443 ± 18
8.0	680 ± 20	515 ± 13

On the contrary, the inactivation efficiency rises (D values diminish) as amplitude increases. Amplitude also strongly affects z and E_a. Increasing ultrasound amplitude brings about linear increases of z and linear decreases of E_a.

Effect of the pH of the Treatment Medium on Heat and MTS Resistance of LOX. It is well known that the pH of the medium may cause alterations in protein conformation and in most cases affects the thermal inactivation rate constants of enzymes (Stauffer, 1989). The pHs for maximum stability of the enzymes are generally close to the so-called pH optimum (Webb, 1964).

Although it has been reported for LOX from peas (Farkas and Goldblith, 1962) and asparagus (Ganthavron et al., 1991) that pH in the range 4–7 does not affect heat resistance, our results (Tables 2 and 3) demonstrate that in the pH range explored (5.2–8) the resistance of soybean type 1B LOX against heat and MTS is strongly pH dependent. Under both type of treatments, D values increase with decreasing pH, but much more so in heat than in MTS, as shown in Figure 3A, where the ratio D value/D value at pH 8 is plotted against pH. This reveals that the synergistic effect of heat and ultrasonic waves (and the potential advantages of MTS versus heat treatment for LOX inactivation) is higher at the acidic pHs characteristic of most fruits and vegetables than at neutral or alkaline conditions.

The pH of the medium substantially affects as well the temperature dependence of the inactivation rate. The E_a of the MTS inactivation is at any pH lower than that corresponding to heat inactivation. On both processes E_a increases with increasing pH, but E_a is more pH dependent in MTS than in heat inactivation.

As shown in Figure 3B, the pH dependence (expresed as the ratio D value at pH 5.2/D value at pH 8) of the heat inactivation decreases and that of inactivation by MTS increases when temperature is raised.

Influence of KCl, Sugars, and Glycerol on Heat and MTS Resistance of LOX. Farkas and Goldblith (1962), Svennson and Eriksson (1972), and Alsoe and Adler-Nissen (1988) reported that pea and soybean solids had a protective effect on LOX against thermal inactivation. We have studied the effects of KCl, sugars, and glycerol on heat and MTS resistance, mainly for comparative purposes.

The effect of neutral salts on LOX resistance to heat and MTS inactivation was studied at 72.5 and 71.5 °C

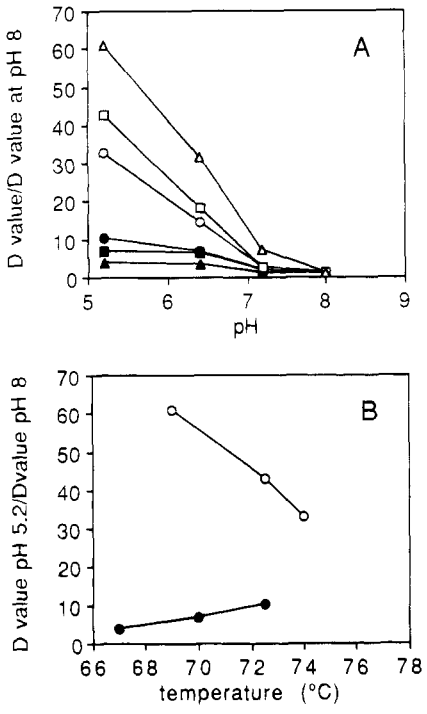

Figure 3. Influence of pH on lipoxygenase resistance to heat and MTS treatments. (A) Relative changes of lipoxygenase D values with pH in heat and MTS treatments; plot of the ratio D values at different pHs/D value at pH 8. Heat treatment at 69 °C (△), 72.5 °C (□), and 74 °C (○). MTS treatments at 90 μM ultrasound amplitude, 3 kg/cm^2, and 67 °C (△), 70 °C (■), and 72.5 °C (●). (B) Influence of temperature on the relative changes of D with pH in heat (○) and MTS (●) treatments.

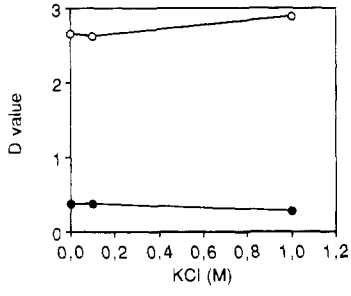

Figure 4. Influence of KCl concentration on lipoxygenase heat and MTS resistance. D value dependence on KCl concentration in MTS (●) and heat treatments (○). Heat treatments at 72.5 °C; MTS treatments at 71.5 °C, 3 kg/cm^2, and 90 μm ultrasound amplitude.

respectively, by measuring residual activity after treatments in the presence of different KCl concentrations. Semilog plots of activity versus time were always linear. As shown in Figure 4, changes in KCl concentration scarcely affect either heat or MTS resistance. Nevertheless, since the small effects produced by increasing KCl concentration are enzyme protective in the heat treatment and destructive in MTS, they result in a small enhancement of the synergistic effect.

The effect of sugars and polyhydric alcohols, usually considered protein structure stabilizers (Timasheff and Arakawa, 1989), on enzyme inactivation by heat and MTS was studied with sucrose, glucose, and glycerol at 10% concentration (2.9, 5.6, and 10.9 M, respectively).

As shown in Figure 4, all of them protect LOX against both MTS and heat treatments. Plots of log of residual activity versus time in the presence of these solutes are linear on MTS, as corresponds to a first order kinetics. In heat treatment the presence of either sucrose,

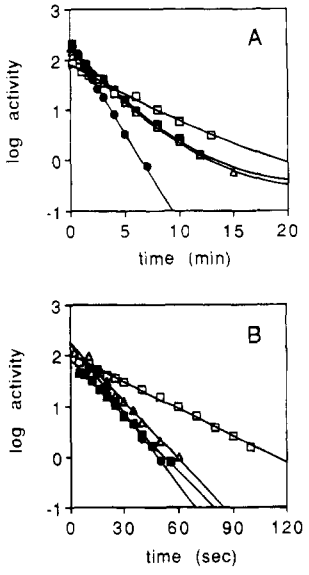

Figure 5. Effect of sugars and polyols on the heat and MTS resistance of LOX. (A) Heat treatments at 72.5 °C in the absence (●) or presence of 10% sucrose (■), 10% glucose (△), or 10% glycerol (□). (B) MTS treatments at 90 μM ultrasound amplitude, 3 kg/cm^2 pressure, and 71 °C in the absence (●) or presence of 10% sucrose (■), 10% glucose (△), or 10% glycerol (□).

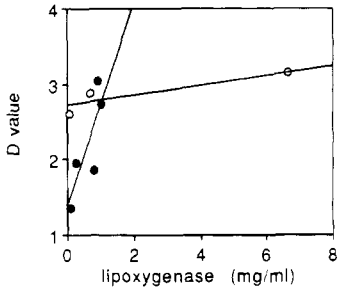

Figure 6. Effect of enzyme concentration on lipoxygenase heat and MTS resistance. Plots of D values in MTS (●) and heat treatments (○) versus concentration of the enzyme preparation in the treatment medium.

glucose, or glycerol produces strong deviations from first-order kinetics.

Influence of Protein Concentration on Heat and MTS Resistance of LOX. Proteins are known to be much more stable against heat and other physical denaturating agents in concentrate than in dilute solution. In general, the protective effect is nonspecific and has been ascribed to various causes (Putman, 1953), although in some cases more specific mechanisms can be in operation. Coakley et al. (1972) observed that the heat inactivation rate of alcohol dehydrogenase and lysozyme decreases with increasing enzyme concentration.

To study the effect of protein concentration on thermal and MTS inactivation of LOX, different quantities of the enzyme preparation were submitted to both treatments (between 0.11 and 1.03 mg/mL for MTS and between 80 μg/mL and 6.6 mg/mL for heat treatment). As shown in Figure 6, heat resistance was nearly independent of LOX concentration whereas MTS resistance increased linearly with increasing LOX concentration.

DISCUSSION

Evidence for Two Different Mechanisms Operating along MTS Inactivation of LOX. In peroxidase

and other enzymes a single mechanism seems responsible for heat and MTS inactivation (Lopez et al., 1994; Sala et al., 1994). In LOX inactivation by MTS two different mechanisms seem to be in operation. One of them due to the direct effect of heat and the other added when ultrasonic waves are applied. The following facts argue in favor of this statement: (i) the different temperature dependence of heat and MTS inactivation; (ii) the changes in the E_a of MTS inactivation when ultrasonic amplitude is varied; (iii) the differences observed on the influence of pH, on both heat resistance and E_a of the inactivation reaction(s); and (iv) the different effects of cosolutes and enzyme concentration upon both processes.

Effects of Ultrasound Amplitude. Although Kashkooli et al. (1980) have proved that enzyme inactivation by ultrasounds at room temperature can be in some instances related to acoustic microstreaming, it is generally accepted that ultrasounds effects are mainly sonochemically initiated and therefore related to cavitational collapse. The intensity of these phenomena is strongly dependent on ultrasound amplitude, since the ultrasonic irradiation intensity is proportional to the square of the amplitude, and the higher the ultrasonic irradiation intensity the higher the temperature during collapse and consequently the sonochemical reaction rate (Mason and Lorimer, 1988). Therefore the relative contribution of the mechanism added by the ultrasonic waves to heat inactivation must increase with amplitude. Tables 1 and 3 strongly suggest that the inactivation mechanism added by ultrasonic waves to thermal denaturation has an substantially lower E_a.

pH Effects. The fact that thermal stability keeps growing as the pH of the medium moves away from the optimum toward more acidic values, at least until pH 5.2, although the optimum pH of soybean lipoxygenase 1 is about 9.5, seems a rather unusual phenomenon. It suggests that electrostatic interactions between amino acid residues located in parts of the molecule away from the active site play important roles in protecting the native structure against heat, but not against the inactivation mechanism added by the ultrasonic waves, since overall MTS inactivation is scarcely pH dependent. The different pH dependence of each of these two inactivation mechanisms brings about a progressive decrease in the contribution of ultrasonic waves to the overall inactivation as pH increases, as revealed by the progressive increase of the E_a of the MTS inactivation.

Nature of the Inactivation Mechanism Added by the Ultrasonic Waves in MTS. The differences observed in the influence of pH on thermal and MTS inactivation may be related to the specific mechanisms involved in the inactivation and allow some insight in their nature.

It is well known that sonication gives rise to H· and OH· free radicals by decomposition of water inside the oscillating bubbles (El'pinner, 1965). Hydroxyl radicals are very reactive and can induce the initial formation of peroxy radicals on amino acid residues, producing great losses of tryptophan, tyrosine, and other amino acids (Davies et al., 1987). So it is initiated a chain of reactions which in the presence of oxygen usually ends in protein fragmentation and under anaerobic conditions in the formation of higher molecular weight aggregates by cross-linking (Stadtman, 1993). Manothermosonication of LOX in the presence of a variety of OH· scavengers does not result in increase of LOX resistance (Lopez and Burgos, unpublished data). Nevertheless this does not discard the OH· free radical intervention in lipoxygenase inactivation. Hydroxyl radicals have very short half-lifes and their range of action is very short, but can undergo radical–radical combination reactions to give hydrogen peroxide. Hydrogen peroxide is known to be an efficient LOX inactivating factor even at very low concentration and room temperature (Mitsuda et al., 1967). Furthermore, hydrogen peroxide could be decomposed by means of the Fenton reaction with the Fe^{2+} of the LOX active site generating new hydroxyl radicals as it has been proved by several metalloenzymes (Stadtman, 1993). Free radicals produced through metal-catalyzed reactions of this type cannot be easily scavenged (Samuni et al., 1983; Stadtman, 1993) because they react with the amino acid residues at metal-binding sites, which are specific targets. These so called "caged" metal-catalyzed oxidation processes (Stadtman, 1993) have been shown to be responsible for enzyme inactivation of other metalloenzymes (Hodgson and Fridovich, 1975; Levine, 1983; Szweda and Stadtman, 1991).

For the Fenton reaction to happen iron must be in its reduced form, while catalytically active lipoxygenase must have its iron atom in the ferric state (Schilstra et al., 1994). The native fraction of lipoxygenase remained along heat and MTS treatments in our experiments in the ferrous form since a lag period to reach maximum activity was always observed in its assay.

If we assume that lipoxygenase MTS inactivation takes place through such a mechanism it was expected to decay in its efficiency with increasing pH for several reasons: (1) As it is well known, H^+ is a reactant in the Fenton reaction. (2) In the presence of oxygen by combination of H· with O_2, hydroperoxyl radicals, HO_2·, are also produced (Davidson, 1990). These are in equilibrium with superoxide anion radicals O_2^-. The pK_a of HO_2· is about 4.7 (Bielski and Allen, 1977) and therefore the HO_2· concentration shall be higher at low pH values. There are at least three H^+-dependent reactions involving these free radicals and leading to H_2O_2 so favoring increased H_2O_2 concentrations at acidic pHs:

$$HO_2\cdot + H^+ \rightarrow H_2O_2$$

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

$$HO_2\cdot + HO_2\cdot \rightarrow H_2O_2 + O_2$$

(3) Although O_2^- and its protonated form in equilibrium HO_2· are only moderately reactive in aqueous solution it has been reported that they are able to inactivate some enzymes (Kuo et al., 1987). An inactivation mechanism in which superoxide radicals were implied should be more efficient at low pH since the protonated form is substantially more reactive than the unprotonated one and as it has already been mentioned the lower the pH the higher the HO_2· concentration.

Since free radical mechanisms are usually slowed down by increasing protein concentration, the increase in the decimal reduction time with increasing protein concentration is one more argument in favor of LOX inactivation by MTS occurring through free radicals amino acid oxidation. It should be noted that the protective effect of protein in ultrasound inactivation of some enzymes has been previously observed (Coakley et al., 1973).

Effect of Cosolutes. Several studies have shown that stability of proteins, in aqueous solution, may be increased by sugars and polyhydric alcohols (Back et al., 1979; Lee and Timasheff, 1981; Kella and Poola, 1985; Graber and Combes, 1989). This seems to be a general effect although it may be worth noting that Chang et al. (1988) have described a destabilizing effect of sugars on horseradish peroxidase submitted to thermal treatment and that the intensity of this effect increases with the increase of the reducing power of the sugars. They suggested as possible a mechanism for this inactivating effect the interaction of the reducing sugars with amino acids residues, which could affect the conformation of the active site. Back et al. (1979) attributed the sugars' stabilization of protein structure to an increase of hydrophobic interactions as a consequence of increasing the structure of water.

The protection of the native structure of proteins by cosolutes is now generally attributed to their preferential exclusion from the protein surface. The preferential exclusion is due in the case of sugars and KCl to the increase of the surface free energy of water, i.e. the surface tension, which deplete them from the surface layer. The predominant cause of exclusion of glycerol is a solvophobic effect; this is due to its ability to fit well in the water lattice and to form hydrogen bonds that reinforce water interactions, making contacts between the nonpolar residues of proteins and the glycerol solution even more unfavorable than contacts with water.

According to Timasheff and Arakawa (1989) and Timasheff (1993), preferential exclusion of compounds which are essentially inert toward the protein, like sugars and KCl, protects the native structure because the binding of the stabilizer would increase the chemical potential of the protein and therefore the free energy of the system. Since this unfavorable situation would be more so in the denaturated state, where the protein–solvent surface contact is increased, the equilibrium between the native and denaturated states would be pushed by these cosolutes toward the native conformation. The same would occur in the case of glycerol since the number of unfavorable interaction sites on the protein surface would increase upon unfolding.

It seems reasonable to argue for the plausibility of the lower protection efficiency of these neutral stabilizers cosolutes against MTS being due to distortion of the native enzyme molecule to the limit of its flexibility by the ultrasonic waves. This would increase the water–protein interface in the native state and so diminish the changes of the protein surface produced by the denaturation process.

On the other hand, it could be also argued that the microstreaming produced by the ultrasonic waves could make difficult the preferential exclusion of the cosolutes from the enzyme surface impairing the preferential exclusion phenomenon.

Mathematical analysis of the enzyme thermodestruction curve in the presence of sugars and glycerol (Figure 5) revealed that the experimental data are compatible with a two fraction first-order model of inactivation. Biphasic behavior of the heat inactivation curve has already been described for LOX from different sources (Borhan and Snyder, 1979; Christopher et al., 1970; Kim et al., 1987; Bhirud and Sosulski, 1993) but it has been always linked to the presence of different isozymes in the preparation. In the work reported here, the biphasic behavior has not been observed in the absence of sugar or glycerol, which argues against the presence in the enzyme preparation of several isozymes of different heat resistance in the experimental buffer.

It is well known nevertheless that the stabilizing effect of the same additive can be even contrary for different isozymes (Yoovidhya et al., 1986). It is therefore possible that our preparation had more than one isozyme equally stable in the experimental buffer but differently affected by sugars and glycerol. Otherwise this phenomenon could only be explained by the formation in the presence of these protectors of an intermediate partially denaturated protein with higher thermal stability. In fact, partial stabilization of proteins at temperatures close to that of maximum endothermic heat flow has been observed in calorimetric studies (Ma and Harwalkar, 1991). It seems worth noting that there is not any relationship between the reducing power of the sugars and the degree of deviation from first-order kinetics. This discards the involvement of the reaction between reducing groups and dibasic amino acids, to which Chang et al. (1988) attributed the destabilizing effect of sugars on horseradish peroxidase.

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