Inactivation of Peroxidase, Lipoxygenase, and Polyphenol Oxidase by Manothermosonication

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The combined effects of heat and ultrasonic waves operating at absolute pressures between 1.5 and 7.2 kg/cm^2 on peroxidase, polyphenol oxidase, and lipoxygenase inactivation have been studied over a broad range of temperatures, static pressures, and ultrasound amplitudes. A synergistic effect which can substantially reduce enzyme resistance and the heat treatment required for inactivation was observed in all cases. The peroxidase z value, estimated at 26 °C, was not changed by the combined treatment, but that of lipoxygenase increased from 4.2 °C (heat treatment) to 6.8 °C (combined treatment). The enzyme destruction efficiency of the combined process greatly increases with ultrasonic wave amplitude; decimal reduction times at constant temperature decreased logarithmically with increasing amplitudes. Static pressure does not seem to affect much the destructive effect of the combined process. This combined treatment could help to solve, in milk, juices, and other drinks, problems caused by thermostable enzymes.

INTRODUCTION

Heat treatment is the most utilized method for stabilizing foods because of its capacity to destroy microorganisms and inactivate enzymes. However, since heat can alter as well many organoleptic properties of foods and diminish the contents or bioavailability of some nutrients, there is a growing interest in searching for methods able to reduce the intensity of the heat treatments needed for food preservation.

It has been proved (García et al., 1991) that the simultaneous use of heat and ultrasonic waves could reduce substantially, at atmospheric pressure and temperature in the range 70–90 °C, the heat resistance of two strains of *Bacillus subtilis*. Recent experiments performed in the authors' laboratory (Raso et al., 1993) show that this increase in the global bactericidal efficiency extends to temperatures up to 140 °C, if the combined treatments are applied under the appropriate pressure. For this procedure the term manothermosonication (MTS) has been coined.

The work here described aims to explore if MTS would result as well in a substantial reduction of the heat resistance of three enzymes wich cause deleterious effects on foods: peroxidase (EC 1.11.1.7), lipoxygenase (EC 1.13.11.12) and polyphenol oxidase (EC 1.14.18.1 and 1.10.3.1).

Peroxidase is a heme-containing enzyme which can catalyze a large number of reactions in which a peroxide is reduced while an electron donor is oxidized and is considered to have an empirical relationship to off-flavors and off-colors in raw and unblanched vegetables. Because of its relatively high thermal stability, peroxidase activity is frequently used to evaluate the efficiency of vegetable blanching (Richardson and Hyslop, 1985).

Lipoxygenase 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, 1987). This enzyme is involved in off-flavor development in many vegetable products (O'Connor and O'Brien, 1991).

Polyphenol oxidase is a copper-containing enzyme that functions as a monooxygenase in the *O*-hydroxylation of monophenols and as a two-electron oxidase in the oxidation of *o*-diphenols to *o*-quinones which undergo further modifications to give brown pigments (Mayer and Harel, 1991). It is one of the most damaging enzymes to quality preservation of fresh fruits and fruit juices.

EXPERIMENTAL PROCEDURES

Materials. Peroxidase type VI from horseradish and lipoxygenase type 1B from soybean were purchased from Sigma (St. Louis, MO). Polyphenol oxidase from mushrooms was a product from Serva (Heidelberg, Germany). Other chemicals used were of reagent grade.

Enzyme Assays. Peroxidase activity was determined by a method based in monitoring the decomposition of hydrogen peroxide by peroxidase with *o*-dianisidine as hydrogen donor by measuring the rate of color development at 460 nm. Spectrophotometric measurements were made in a Uvikon 810 from Kontron (Zurich) equipped with a recorder and a thermostated cell holder block.

The assay medium consisted of 0.1 M sodium phosphate buffer, pH 6.5, containing 0.01% *o*-dianisidine, 0.005% H_2O_2, and 2% methanol (*o*-dianisidine was incorporated to the assay medium dissolved in methanol). Enzyme activity was determined at 25 °C using 1 mL of the above medium and 5–20 μL of enzyme sample, consisting of 0.28–0.43 mg of the enzyme preparation/mL of 20 mM potassium phosphate buffer, pH 6.5.

Lipoxygenase was assayed by a spectrophotometric method based on a modification of the methods of Ben Aziz et al. (1970) and Chen and Whitaker (1986). Substrate stock solutions were prepared by mixing 10 μL of "pure" linoleic acid, 32 μL of Tween 20, and 125 μL of 1 N NaOH and diluting to 12.5 mL with water. The stock solution was diluted before assay 1:10 with 50 mM borate buffer, pH 9.0. The reaction was initiated by adding 2–20 μL of the enzyme sample (2–4 mg of the enzyme preparation/mL of 20 mM potassium phosphate buffer, pH 6.5) to 1 mL of the diluted substrate. Hydroperoxide formation was monitored by measuring the absorbance at 234 nm at 25 °C.

Polyphenol oxidase activity was spectrophotometrically determined by monitoring the rate of dopachrome formation from DL-Dopa at 475 nm (Savagaon and Sreenivasan, 1978). The assay mixture consisted of 10 mM DL-Dopa and 0.05 M potassium phosphate buffer, pH 7.0; the reaction was initiated by adding to 1 mL of the assay mixture 100 μL of the enzyme sample (1.3–

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1.7 mg/mL of potassium phosphate buffer, pH 6.5). The assay was performed at 40 °C.

Heat Treatment and Manothermosonication. Heat treatments were performed in a TR-SC thermoresistometer (Condon et al., 1989). Manothermosonication was performed in a thermoultrasonic resistometer, built by a modification of the TR-SC instrument (Raso et al., 1993). Inside the main vessel a 23-mL treatment chamber fitted with an ultrasonic horn, Model 450 from Branson (Danbury, CT), was located; this ultrasonic horn irradiates at a fixed frequency of 20 kHz. An injection port allowed the introduction of the enzyme solution in the treatment chamber containing the treatment medium (20 mM potassium phosphate buffer, pH 6.5) at a preset temperature.

A solenoid valve located in the treatment chamber allowed periodical removal of samples (100–200 μL) which were collected on small glass tubes introduced in ice. A thermocouple connected to a recorder allowed measurement of the actual temperature in the treatment chamber. The treatment chamber was connected to the main vessel, containing as well the treatment medium, by means of a pressure equilibrating valve which allowed the entrance of a volume of the treatment medium equal to the volume of sample removed. The resulting dilution factor was taken into account when destroyed and residual enzyme activities were calculated. A paddle stirrer inside the treatment chamber continuously mixed its contents. Pressure was applied to the main vessel from a nitrogen cylinder.

The enzyme solution volume injected into the treatment chamber was 300 μL, and the solutions injected were as follows: peroxidase, 6.6–10 mg/mL of water; lipoxygenase, 50–100 mg/mL of 20 mM, pH 9.0, sodium borate buffer; polyphenol oxidase 30–40 mg/mL of 10 mM, pH 6.5, sodium phosphate buffer.

Enzyme Inactivation Parameters. Because of its advantage for heat treatment calculations, it is common in working on thermostability of food enzymes to express enzyme inactivation in terms of the pertinent parameters (D and z values) of the inactivation plots (Versteeg et al., 1980; Christen and Marshall, 1985; Richardson and Hyslop, 1985; Diermayer et al., 1987). This was the approach followed in this study.

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.

RESULTS AND DISCUSSION

Peroxidase Inactivation. Thermal inactivation of the peroxidase preparation used, in the temperature range 110–142.6 °C, and the combined destructive effects of heat and ultrasonic waves are shown in Figure 1.

A comparison of the results depicted in this figure reveals that the combined treatments have no additive but synergistic effects. This synergy does not alter the z value (Figure 2), which was estimated to be 26 °C. The fact that the z value of peroxidase inactivation remains unchanged when ultrasonic waves are simultaneously applied must mean that, in the temperature range explored, the temperature dependence of the reaction(s) responsible for the ultrasonic waves' destructive effects on peroxidase is equal to the temperature dependence of the reaction responsible for its thermal inactivation.

Thermal inactivation of peroxidase has been studied for many years with enzyme preparations from different sources and various degrees of purification. Generally, a deviation of first-order kinetics is observed (Winter, 1971; Ling and Lund, 1978; Delincee et al., 1979; Henley and Sadana, 1985; Chang et al., 1988; Ganthavorn, 1991). In the experiments here reported, in which we used the same horseradish preparation used by Chang et al. (1988), this biphasic course of inactivation was also observed when no

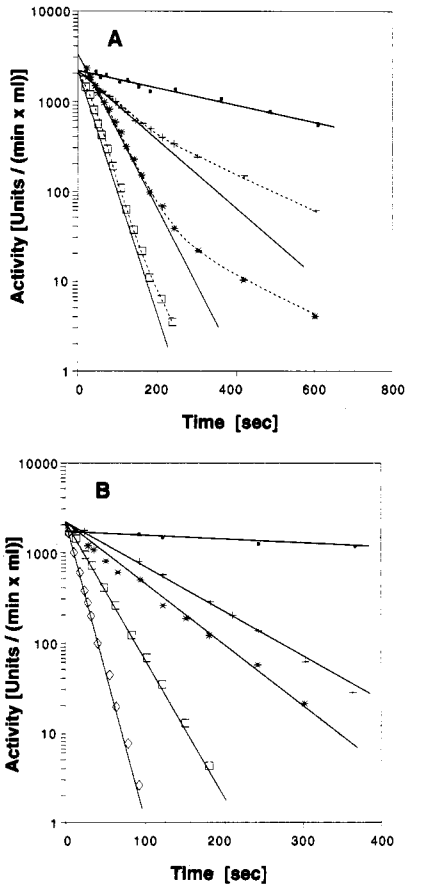

Figure 1. Heat and MTS inactivation of horseradish peroxidase in 20 mM potassium phosphate buffer, pH 6.5. (A) Heat resistance at 110.4 (⊡), 124.5 (+), 134.8 (*), and 140.8 °C (□). (B) Inactivation by MTS under the following conditions: absolute pressure, 4.8 kg/cm²; ultrasound amplitude, 145 μm; temperature 37 (⊡), 121.5 (+), 126.5 (*), 136.6 (□), and 142.6 °C (◇).

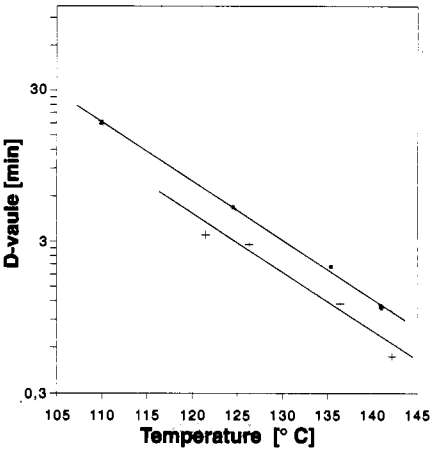

Figure 2. Peroxidase z values for heat (⊡) and MTS treatments (+). MTS operating conditions were as in Figure 1.

ultrasonic waves were applied but failed to appear when the preparation was manothermosonicated, as shown in Figure 1.

Although other mechanisms have been proposed to explain the biphasic course of peroxidase thermal inactivation (Winter, 1971; Henley and Sadana, 1985), this phenomenon is generally accepted to be due to the presence of isozymes of different heat stability. Chang et al. (1988) demonstrated the presence in horseradish peroxidase of no less than seven isoforms of isoelectric points in the pH range 4.0–9.0 and suggested that the more alkaline the isoelectric point, the higher the heat resistance. Were this so, we should conclude that the synergism of heat and

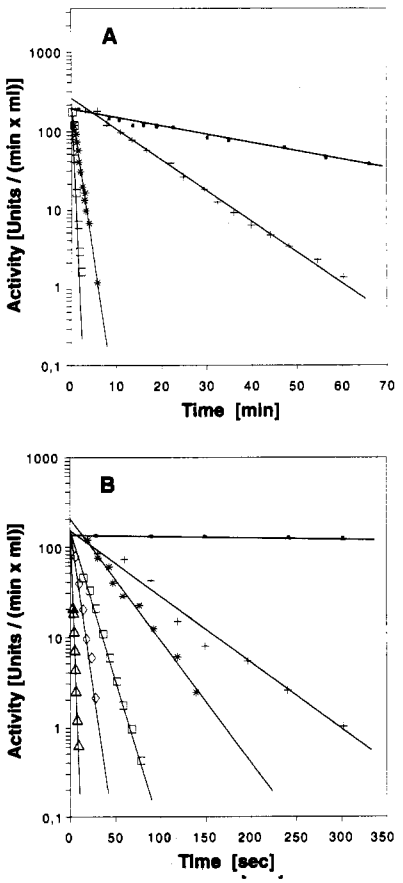

Figure 3. Heat and MTS inactivation of lipoxygenase (soybean, type 1B) in 20 mM potassium phosphate buffer, pH 6.5. (A) Thermal inactivation at 67 (⊡), 70 (+), 74 (*), and 76 °C (□). (B) Loss of activity under MTS treatment at an absolute pressure of 4 kg/cm² and ultrasound amplitude of 76 μm at 37 (⊡), 67 (+), 69 (*), 71.3 (□), 74.2 (◇) and 77 °C (△).

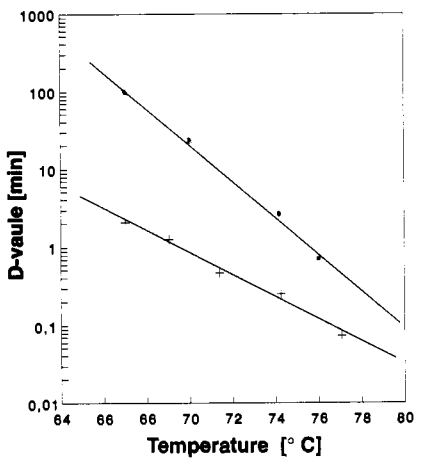

Figure 4. Change induced by MTS in lipoxygenase z value: heat treatment (⊡); MTS (+) under the operation conditions stated in Figure 3.

ultrasonic waves is greater when acting on the isozymes of more alkaline isoelectric point. On the other hand, if the deviation of peroxidase thermal inactivation from first-order kinetic were due, as suggested by Winter (1971), to the formation of enzyme aggregates with different heat stabilities, the monophasic inactivation of peroxidase under MTS could be attributed to the well-known dissociation effect of ultrasonic waves on aggregates.

Inactivation of Lipoxygenase and Polyphenol Oxidase. Lipoxygenase heat and MTS resistances are compared in Figures 3 and 4. The same is shown in Figure 5 for polyphenol oxidase. On both enzymes, a synergistic

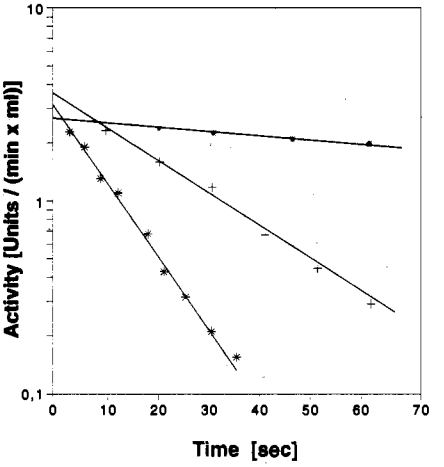

Figure 5. Polyphenol oxidase heat and MTS inactivation in 20 mM potassium phosphate buffer, pH 6.5: heat resistance at 70.7 °C (+); MTS inactivation at an absolute pressure of 4 kg/cm² and ultrasound amplitude of 35 μm at 37 (⊡) and 70.7 °C (*).

effect of heat and ultrasonic waves, as previously demonstrated for peroxidase, was observed.

There are two main differences in the loss of peroxidase and lipoxygenase activities under the inactivation treatments considered: (a) Lipoxygenase inactivation follows under both treatments a first-order kinetics. It has been proved (Park et al., 1988) that lipoxygenase thermal inactivation follows first-order kinetics if the preparation is not composed of several isozymes with different thermostabilities; therefore, either the enzyme activity of the preparation used here is due almost exclusively to a single isozyme or all of the lipoxygenase isozymes present in the preparation have very similar D and z values. (b) The z value for MTS under the experimental conditions above stated (6.8 °C) is greater than the corresponding value (4.2 °C) for heat inactivation. This means that the synergistic effect decreases when the temperature increases and would disappear at about 85 °C.

The increase of the z value of lipoxygenase when the ultrasonic waves are applied simultaneously to the heat treatment agrees with the fact that, in sonochemical reactions, rates are slowed as temperature is increased (Suslick, 1988) because the greater the solvent (water) pressure found within the cavitation bubbles generated by ultrasounds, previous to collapse, the less effective the collapse. It is more difficult to explain why peroxidase inactivation does not follow the same rule; most likely this reveals that a different inactivation mechanism operates on each of the two enzymes.

For peroxidase heat inactivation it has been suggested (Lu and Whitaker, 1974) that the mechanism involves the release of the heme moiety previous to the denaturation of the liberated apoprotein.

Effect of Ultrasound Amplitude and Hydrostatic Pressure in the Enzyme Inactivation Efficiency of MTS Treatments. It is well established that increased intensity of ultrasound iradiation increases the sonochemical reaction rates (Suslick, 1988). Figure 6 shows the changes in MTS enzyme destruction efficiency with ultrasound amplitude. The greater the amplitude, the higher the efficiency. This rule is obeyed by the three enzymes studied, and a plot of log (D values) vs irradiation amplitude demonstrates (Figure 6D) that log (D value) decreases linearly with increasing amplitude in all cases.

Changes in hydrostatic pressure have been shown to alter the rates of some sonochemical reactions by an order of magnitude (Verral and Shegal, 1988). In general, the reaction rate increases with increasing pressure up to a maximum, after which it begins to decrease. The pressure

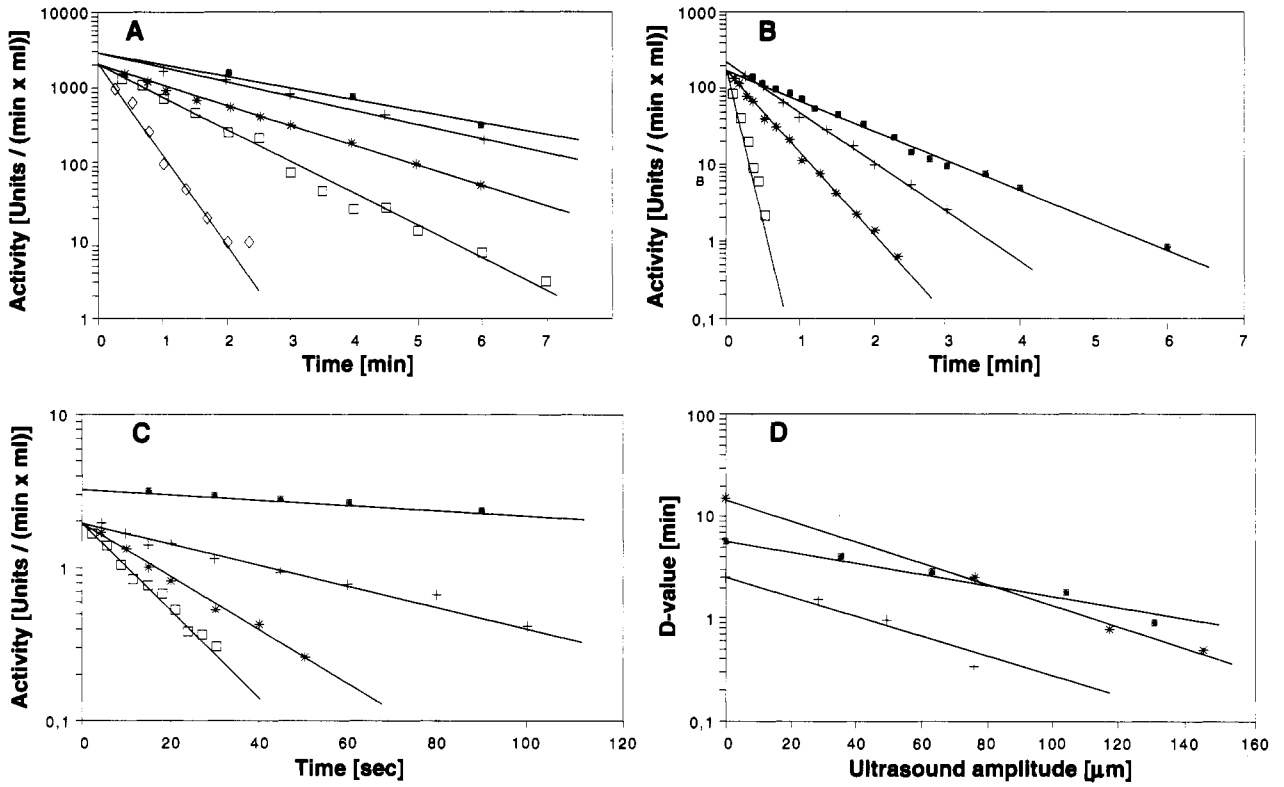

Figure 6. Effect of ultrasound amplitude in the enzyme inactivation efficiency of MTS in 20 mM potassium phosphate buffer, pH 6.5, at 4 kg/cm^2 of absolute pressure. (A) Peroxidase: temperature, 123 °C; amplitudes, 0 (⊡), 63 (+), 104 (*), and 134 μm (□). (B) Lipoxygenase: temperature, 74 °C; amplitudes, 0 (⊡), 28 (+), 49 (*), and 76 μm (□). (C) Polyphenol oxidase: temperature, 60 °C; amplitudes, 0 (⊡), 76 (+), 117 (*), and 145 μm (□). (D) Plots of D_{123} for peroxidase (⊡), D_{74} for lipoxygenase (+) and D_{60} for polyphenol oxidase (*), vs ultrasound amplitude.

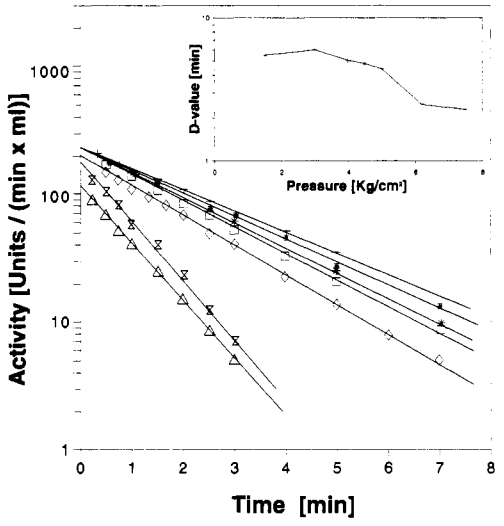

Figure 7. Dependence of MTS lipoxygenase inactivation efficiency on static pressure. Operation conditions: 20 mM potassium phosphate buffer, pH 6.5; temperature, 70 °C; ultrasonic wave amplitude, 63 μm; pressure, 1.5 (⊡), 3 (+), 4 (*), 4.5 (□), 5 (◊), 6.2 (△), and 7.2 kg/cm^2 (⧗). In the insert log of D is plotted vs hydrostatic pressure.

at which maximum rate occurs depends on the ultrasonic power used in the experiment. Figure 7 shows the static pressure dependence of the lipoxygenase MTS inactivation rate. It reveals that, under the experimental conditions used, the inactivation rate increases with increasing pressure. D values (Figure 7, insert) remained rather unchanged in the range of static pressures 1.5–5 kg/cm^2 but dropped to about one-third at 6–7 kg/cm^2.

Inactivation Mechanism. There are several possible ways through which ultrasonic waves could contribute to enzyme inactivation.

When applied to a liquid, ultrasonic waves promote acoustic cavitation. The bubble liquid interface continuously changes shape and size; acoustic streams occur in the liquid in the vicinity of the bubble, often resulting in severe shear stresses which can promote enzyme denaturation.

Cavitional collapse of the bubble in transient cavitation generates extremely high local pressures and temperatures. Maximum temperatures of 15 000 K and pressures of 1000 atm with residence times of less than 10 ns have been predicted, and liquid-phase effective temperatures of 1900 K in a boundary about 200 nm thick, with lifetimes of about 2 μs, have been calculated from data on sonochemical reactions experiments (Suslick, 1988). Inactivation of the enzymes in the hot spots so generated must be expected. Sonication promotes chemical reactions involving H· and OH· free radicals formed by the decomposition of water inside the oscillating bubbles (El'Piner et al., 1965). Free radicals so produced could be scavenged by some amino acid residues of the enzymes participating in structure stability, substrate binding, or catalytic functions. Proline, leucine, isoleucine, lysine, and glutamic acid have been proved to form easily peroxides by reacting with OH· radicals (Gebicki and Gebicki, 1993), and cysteine is quickly oxidated.

In some instances, when the enzyme is a hemoprotein, other mechanisms can contribute to inactivation since, on hemoglobin, splitting of heme from globin has been proven to occur in an ultrasonic field (Weissler, 1960).

Which of these mechanisms are responsible for the synergistic effect observed in MTS enzyme inactivation cannot be deduced from our experimental results, as the dependence of all sonochemical reaction rates on variables such as liquid temperature, ambient pressure, and ultrasonic intensity must be similar, because it reflects the effect of these parameters on bubble dynamics, number of

bubbles, and pressure and temperature inside the collapsing cavitation bubbles. Most likely, as already suggested, more than one mechanism is operative. In fact, the inactivation of some enzymes at low temperatures by long time exposures to an ultrasonic field has been known for more than 30 years (El'Piner, 1964). This low temperature inactivation has been generally attributed either to the splitting of low molecular weight polypeptides or individual amino acids or, more frequently, to oxidative mechanisms (Santamaria et al., 1953; Coakley et al., 1973).

Work in progress in the authors' laboratory aims to clarify which is the main mechanism responsible for the observed enzyme inactivation under MTS and to explore the possible contribution of this treatment to solve the problems caused in food technology by thermostable enzymes with high z values which can become the shelf-life-limiting factor for some foods microbiologically stablized by heat treatment. This is the case of the extracellular proteases and lipases from *Pseudomonas*, in UHT milk and several milk products (Cogan, 1977; Law, 1979; Burton, 1988), and of a small fraction of pectinesterases in concentrated citric juices, where the formation of gel occurs if this form of the enzyme has not been efficiently inactivated; from this gel no juice can be reconstituted (Versteg et al., 1978; Pilnik and Voragen, 1991).

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