Inactivation of Heat-Resistant Pectinmethylesterase from Orange by Manothermosonication

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Pectinmethylesterase of navel oranges shows two fractions greatly differing in thermostability. The most thermostable fraction accounts for ~10% of total activity. The thermal inactivation of this fraction follows first-order kinetics both in 5 mM, pH 3.5, citrate buffer and in orange juice at the same pH, showing a z value of 5.1 °C and an activation energy (E_a) of 435 kJ mol^{-1} K^{-1}. The heat resistance of the enzyme is ~25-fold higher in the juice than in citrate buffer. When ascorbic acid, sucrose, glucose, and fructose are added to the citrate buffer at the concentrations found in orange juice, the heat resistance of the enzyme increases 3-fold. The addition of pectin at 0.01% concentration multiplies it by a factor of 50. Manothermosonication (MTS), the simultaneous application of heat and ultrasound under moderate pressure (200 kPa), at 72 °C, increases the inactivation rate 25 times in buffer and >400 times in orange juice. MTS inactivation shows a higher z value (35.7 °C) and lower E_a (56.9 kJ mol^{-1} K^{-1}) than simple heating.

Keywords: *Orange pectinmethylesterase; manothermosonication; thermostable enzymes; orange juice*

INTRODUCTION

The worldwide orange crop amounts to >35 million tons each year (Nagy et al., 1993). More than half are processed to fruit juices and concentrates from which orange juice is later reconstituted (Chamarro et al., 1991). Usually, orange juices are heat treated to ensure both microbial and physicochemical stability, especially to maintain their cloudiness (Kimball, 1991). Orange juices are colloidal suspensions of cellular and polymer particles (Crandall et al., 1983). Colloidal stability is maintained by pectin molecules through a complex and not well understood mechanism. Cloud loss of citrus juices is one of the most intensively studied problems in food technology. It is due to the action of endogenous pectinmethylesterase (PME) on pectic substances. PME catalyzes the de-esterification of pectin molecules. De-esterified pectin molecules are able to interact through calcium bridges, leading to cloud loss and phase separation in single-strength orange juices and gelation in their concentrates (Pilnik and Voragen, 1991). Stabilization of the cloud in citrus juices requires the inactivation or inhibition of PME (Eagerman and Rouse, 1976; Kimball, 1991).

Several PME isoenzymes have been found in orange juice, but most of the problems this enzyme causes to the orange juice industry are due to (Versteeg et al., 1980) a thermoresistant isoenzyme of PME (TRS-PME), which constitutes a fraction of the total PME activity that varies with cultivar, degree of ripening, etc. (Snir et al., 1996a). Although the heat-sensitive PME isoenzymes are not entirely lacking clarifying capacity (Cameron et al., 1998), they show much lower activity in this respect, either because they are more sensitive to refrigeration temperatures or because they show a strong product inhibition (Versteeg et al., 1980). Temperatures of ~90 °C and holding times >1 min are needed to reduce sufficiently the activity of TRS-PME isoenzyme (Eagerman and Rouse, 1976; Versteeg, 1979). Heat treatment has a negative impact on orange juice quality because it causes losses in vitamins and volatile compounds and promotes browning reactions. The magnitude of these changes increases with the intensity (time and temperature) of the heat treatment.

Several strategies have been used to inhibit or inactivate PME avoiding the negative effects of intensive heat treatments. Inhibition of PME by polyphenols (Hall, 1966; Pilnik and Voragen, 1991), inhibition by specific proteic PME inhibitors (Castaldo et al., 1991), or inhibition by the oligogalacturonides produced by the action of added polygalacturonase or pectinlyase (Baker and Bruemmer, 1972; Krop and Pilnik, 1974; Termote et al., 1977) have been suggested as alternatives to the heat treatments. Other strategies rely on PME inactivation by nonthermal treatments, such as high pressure (Irwe and Olson, 1994; Donsi et al., 1996; Cano et al., 1997) low pH values (Owusu-yaw et al., 1988), or supercritical carbon dioxide (Arreola et al., 1991; Balaban et al., 1991; Ishikawa et al., 1996).

Another possible alternative is manothermosonication (MTS). MTS is an emergent technology that efficiently combines the inactivating effect of heat and ultrasonic waves (Burgos, 1998). The enzyme's inactivation and microbial destruction effects of ultrasonic waves are thought to be related to the intensity of the implosion of "inertially cavitating" bubbles induced by ultrasound waves. This implosion generates microscopical hot spots (temperatures estimated at 5000 K) and local pressures of ~500 bar (Suslick, 1988). Under these conditions water is decomposed (sonolysis) and free radicals are produced. Bubble implosion also generates very high

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shear forces, which can even break covalent bonds and split polymeric molecules. Cavitation intensity decreases with increasing temperature and disappears when the boiling point is reached. In MTS, moderate pressures are applied to allow cavitation at temperatures close to or above the boiling point. This also increases the implosion intensity, which is related to the difference between the static pressure and the vapor pressure inside the bubble.

MTS has proved to be an efficient tool to inactivate some other enzymes, such as lipoxygenase, peroxidase, and proteases and lipases from psychrotrophic bacteria (Lopez et al., 1994; Sala et al., 1995; Vercet et al., 1997). The aim of the work described in this paper was to explore the ability of MTS to inactivate the heat-resistant fraction of PME both in model systems and in orange juice.

MATERIALS AND METHODS

Materials. Navel oranges and commercial orange juice were purchased in a local market.

PME Extraction. Navel oranges were cut in halves and pressed using a household orange squeezer. The juice was filtered through several layers of nylon gauze. PME was extracted from the retained pulp as described by MacDonell et al. (1945) with 2 volumes of 0.25 M NaCl in pH 8.2 borate−acetate buffer (0.45 M boric acid, 0.1 M sodium tetraborate, 0.3 M sodium acetate) by stirring at room temperature during 1 h. The extract was filtered through nylon gauze, and the pulp was re-extracted as before. The pooled extracts were submitted to ammonium sulfate selective precipitation, collecting the protein fraction precipitating in the 30−80% ammonium sulfate saturation range. The precipitate was redissolved in 10 mM maleate buffer, pH 7.1, and 0.1 M NaCl and dialyzed overnight against the same buffer solution. The dialyzed extract was heat treated in preheated glass tubes (o.d. 1.25 cm, i.d. 1.5 cm; 5 mL in each tube) at 70 °C for 5 min to inactivate heat labile PME isoenzymes. The solution was immediately frozen, lyophilized, and stored at −20 °C. Typical yield of the extraction procedure was ∼1 g of lyophilysate/100 g of orange pulp. The PME activity of the lyophilysate averaged 0.5 (± 0.02) unit/mg. A unit of activity is defined as the amount of PME that liberates 1 μmol of carboxyl groups/min.

PME Assay. PME was assayed, at room temperature, by acid−base titration using 100 mL of substrate [1% citrus pectin (60% of esterification degree, Sigma, St. Louis, MO) in 0.15 M NaCl, adjusted to pH 7.0 with concentrated NaOH]. Five milliliters of the enzyme solution was added to the substrate, and the solution was kept at pH 7 by dropwise addition of 10 mM NaOH. PME activity was expressed in units.

Heat Treatments. Heat treatments were performed in a water bath equilibrated at the desired temperature. Prewarmed thin-wall test tubes (1 cm i.d.) were filled with 5 mL samples. Three tubes each time were removed from the bath at different time intervals, immediately cooled by immersion in ice water, and assayed for PME activity. Heating times began to be computed when the sample reached the treatment temperature, measured by means of a thermocouple. Three tubes were removed at that moment to measure initial activity. Each heat treatment was performed in duplicate.

MTS Treatment. MTS treatments were performed in a continuous MTS apparatus built in our own laboratory. It consisted of a stainless steel cylindrical treatment chamber (i.d., 2 cm; length, 2 cm) to which a sonication horn (model 450; from Branson, Danbury, CT) was attached. The treatment chamber was surrounded by an external one used as a refrigeration unit. The temperature of the treatment chamber contents during the experiments was adjusted at the desired value by pumping ice-cold water through the external chamber at the appropriate flow rate by means of a peristaltic pump (M. Winter, München, Germany). Solutions to be MTS treated were forced through the treatment chamber by a Nemo screw pump (Netzsch Mohnopumpen GmbH, Waldkraiburg, Germany). Pressure was maintained at the fixed value (200 kPa, gauge pressure) by a valve situated at the end of the treatment circuit. Temperature was monitored at the outlet of the treatment chamber by means of a thermocouple. Treatment time was regulated by adjusting the flow rate. Treatment times were calculated by dividing the chamber volume (6.28 mL) by the flow rate.

Ultrasonic irradiation was performed at a frequency of 20 kHz and an ultrasound amplitude of 117 μm. Once treatment time and temperature had been adjusted and after twice the void volume (50 mL) of enzyme solution was passed through the treatment circuit, three different samples were recovered at the exit from the treatment chamber, cooled on ice-cold water, and immediately assayed for enzyme activity. Each MTS treatment was performed in duplicate.

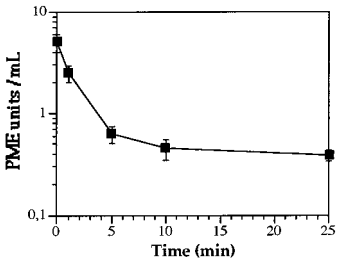

Figure 1. Heat inactivation of PME in whole orange juice at 65 °C.

RESULTS

Heat Resistance of Orange PME Extracts. Heat treatments of whole orange juice at 65 °C resulted, as expected, in biphasic PME inactivation curves (Figure 1), confirming the presence in the samples of PME isoenzymes with different heat resistances. The heat-stable fraction accounted for ∼10% of the total PME. Through similar experiments at different temperatures (data not shown), it was proved that the procedure used by Seymour et al. (1991) to selectively inactivate the heat-sensitive isoenzymes of the PME from grapefruit (70 °C for 5 min) was equally valid for the PME from navel orange extracts.

Heat Inactivation of Thermoresistant Fraction of Orange PME. The freeze-dried extracts containing only TRS-PME activity were redissolved in 5 mM, pH 3.5, citrate buffer at a concentration of 10 mg mL^{-1}, and the heat resistance of the enzyme was measured at different temperatures. The results of these experiments are plotted in Figure 2. From these plots, a z value of 5.1 °C (see inset) for the heat inactivation reaction was calculated, and from the Arrhenius plot of the inactivation rate constant (Figure 6) was calculated an E_a of 435 (± 5) kJ mol^{-1} K^{-1}. The D values obtained were much lower than reported by Versteeg et al. (1980) for the TRS-PME from the same orange cultivar. The reason for this discrepancy could be that Versteeg et al. heated their enzyme preparation in orange juice. To settle this question, we also heat treated our TRS-PME fraction in orange juice at different temperatures. The results of these experiments are shown in Figure 3. A dramatic increase in the D values (25-fold at 72 °C) but no significant difference in z value (or E_a) could be observed. The D and z values obtained in orange juice were similar to those reported by Veersteg et al. (1980).

To investigate which of the orange juice components was responsible for the PME protection against heat inactivation, we treated 5 mL of PME samples (5 PME

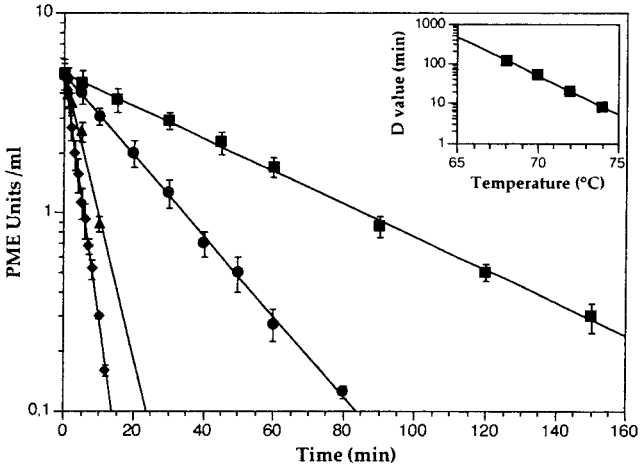

Figure 2. Inactivation of heat-resistant PME by heat treatment in 5 mM, pH 3.5, sodium citrate buffer, at 68 (■), 70 (●), 72 (▲), and 74 °C (◆).

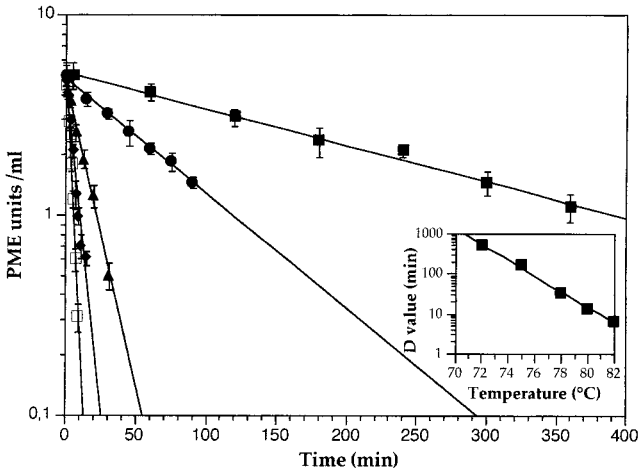

Figure 3. Inactivation of heat-resistant PME by heat treatment in orange juice, pH 3.5, at 72 (■), 75 (●), 78 (▲), 80 (◆), and 82 °C (□).

units mL^{-1}) in 5 mM citrate buffer, pH 3.5 (the same as the orange juice), to which sugars, ascorbic acid, and pectin were added. Addition of sugars and ascorbic acid at their normal concentrations in orange juice (3% glucose, 3% fructose, 6% sucrose, 0.5 mg mL^{-1} ascorbic acid; Kimball, 1991) resulted in a 3-fold increase of $D_{72°C}$. Addition of pectin (0.1 g L^{-1}), either in the presence or in the absence of sugars and ascorbic acid, resulted in a dramatic increase of the $D_{72°C}$ values. The D values for the heat treatments at different temperatures and in different heating medium compositions are shown in Table 1. From these data the E_a of heat inactivation was estimated as 451 (± 6.7) kJ mol^{-1} K^{-1}, a value not significantly different from that estimated for the inactivation in simple citrate buffer.

MTS of the Thermoresistant PME Fraction. TRS-PME was treated by MTS at 33 and 72 °C in citrate buffer and at 38 and 72 °C in orange juice. The results of these experiments are shown in Figures 4 and 5, where the heat resistance of the enzyme at 72 °C is also plotted for comparative purposes. D values for the inactivation are presented in Table 2. The rate of TRS-PME inactivation by MTS is at 72 °C higher than that of heat inactivation in both media, but especially in orange juice, and higher also than the theoretical inactivation rate obtained by adding the rate of MTS inactivation at 33 °C (treatment in citrate buffer) or 38 °C (treatment in orange juice) and that of simple heating at 72 °C (dashed lines of Figures 4 and 5).

The efficiency of MTS, expressed as the ratio $D_{72°C}$ of

Table 1. *D* Values for the Heat Inactivation of the Thermostable Fraction of PME from Orange at Different Temperatures in 5 mM, pH 3.5, Citrate Buffer; in 5 mM, pH 3.5, Citrate Buffer with Sugars,[a] Ascorbic Acid,[b] and Pectin[c] Added; and in Orange Juice

heating medium	temp (°C)	*D* value (min) ± SE
citrate buffer	68	125 ± 2.0
	70	50 ± 0.8
	72	20 ± 1.3
	74	8 ± 0.2
citrate buffer plus sugars and ascorbic acid	72	58.82 ± 4.5
citrate buffer plus sugars, ascrobic acid, and pectin	72	1000 ± 57
citrate buffer plus pectin	72	1000 ± 57
orange juice (pH 3.5)	72	500 ± 23
	75	200 ± 6
	78	33.3 ± 1.2
	80	15.4 ± 1.2
	82	7.2 ± 0.5

[a] Sucrose 6%, glucose 3%, fructose 3%. [b] Ascorbic acid 0.5 mg mL^{-1}. [c] Pectin 0.01%.

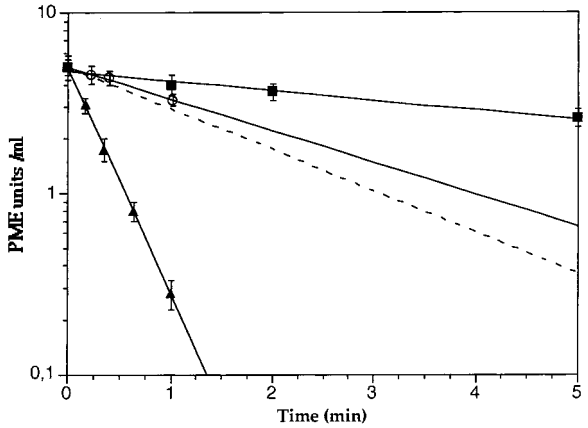

Figure 4. Inactivation of heat-resistant PMR in 5 mM, pH 3.5, sodium citrate buffer by heat treatment at 72 °C (■) and by MTS at 200 kPa, 117 μm of amplitude, and 33 (○) and 72 °C (▲). The dashed line represents the expected effect of the theoretical addition of enzyme inactivation by MTS at 33 °C and by heat at 72 °C.

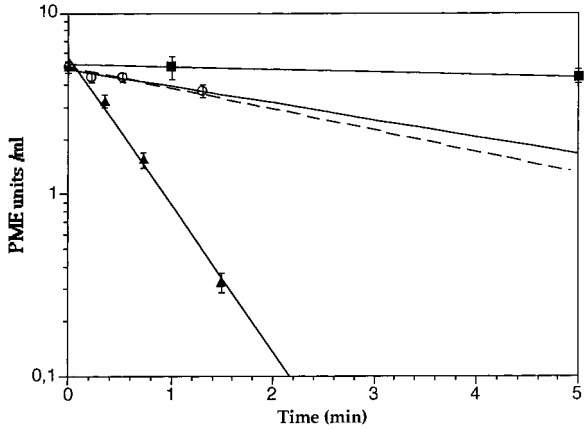

Figure 5. Inactivation of heat-resistant PME in orange juice, pH 3.5, by heat treatment at 72 °C (■) and by MTS at 200 kPa, 117 μm, and 38 (○) and 72 °C (▲). The dashed line represents the expected effect of the theoretical addition of enzyme inactivation by MTS at 38 °C and by heat at 72 °C.

Table 2. *D* Values for the MTS[a] Inactivation of the Thermostable Fraction of PME from Orange at Different Temperatures in 5 mM, pH 3.5, Citrate Buffer and Orange Juice (pH 3.5)

treatment medium	temp (°C)	*D* value (min) ± SE
citrate buffer	33	5.65 ± 0.42
	72	0.8 ± 0.02
orange juice	38	10.87 ± 1.94
	60	3.01 ± 0.1
	72	1.24 ± 0.08
	79	0.8 ± 0.02

[a] Gauge pressure, 200 kPa; amplitude, 117 μM.

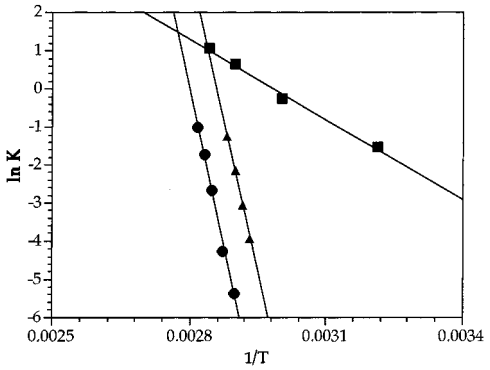

Figure 6. Arrhenius plot of the rate constant of the inactivation of thermoresistant PME by heating in 5 mM, pH 3.5, citrate buffer (▲) and orange juice (●) and by MTS in orange juice at 200 kPa and 117 μm (■).

simple heat treatment/$D_{72°C}$ of MTS, is ∼25 in citrate buffer and ∼403 in orange juice.

MTS inactivation of TRS-PME in orange juice was also studied at 60 and 79 °C. The results of these experiments are presented in Table 2. The *z* value for MTS inactivation of TRS-PME in orange juice was estimated in 35.7 °C, ∼7 times higher than the value obtained for its heat inactivation. The Arrhenius plot of the inactivation rate constant at different temperatures (Figure 6) allowed us to calculate E_a as 56.9 (± 3.3) kJ mol^{-1} K^{-1} for MTS inactivation in orange juice.

DISCUSSION

Heat Resistance of Thermostable PME. Previous studies on the influence of soluble solids (SS) on the thermal stability of PME in orange juice are rather contradictory. The works of Atkins et al. (1956), Bissett et al. (1957), Carroll et al. (1957), and Tajchakavit and Ramaswamy (1997) show a decrease in thermal resistance with increasing SS, whereas that of Guyer et al. (1956) reveals an increase in thermostability with increasing juice concentration. More recently, Marshall et al. (1985), working with the TRS-PME fraction, have shown a steady increase of $D_{90°C}$ with the SS concentration of the juice in the range 10−35 °Brix.

Our experiments on the TRS-PME heat inactivation in citrate buffer with and without sucrose, glucose, fructose, and ascorbic acid at the concentrations ordinarily found in orange juice and traces of pectin clearly reveal that the thermostability of the TRS-PME in single-strength orange juice is due not only to the structure of the enzyme but also to the presence of these molecules in the orange juice. This is in accordance with the results of Marshall et al. (1985), although in their work an increase in SS from 10 to 30% did not significantly change the heat stability of the TRS-PME, whereas our work reveals a 3-fold elevation of $D_{72°C}$ due to the presence of sugars and ascorbic acid at a concentration equivalent to 11 °Brix. This elevation is most likely a nonspecific cosolute effect. Cosolute protection of enzymes or other proteins against heat denaturation has been frequently described, and it is probably due to their preferential exclusion from the protein surface to avoid the increase of the free energy of the system (Timasheff, 1993).

However, the most important factor in determining the TRS-PME heat resistance in citrus juice seems to be pectin. The presence of pectin, even at such a low concentration as 0.01%, in the citrate buffer containing the main components of the SS of orange juice produces a 17-fold additional increase in the thermal resistance of the TRS-PME fraction. This pectin concentration is only $\sim^1/_{10}$ of the usual pectin contents of single-strength orange juices (Hernandez et al., 1992). Pectin protection of TRS-PME against heat inactivation in the citrate buffer seems to be a substrate-mediated effect. Substrate-mediated protection of enzymes against heat and other inactivating agents has been described for several other enzymes, and it is due to impairment of the unfolding of the polypeptidic chain occasioned by the binding of the substrate (Klibanov, 1983; Kristjansson and Kinsella, 1991).

TRS-PME is more thermolabile in orange juice than in the citrate buffer containing the same sugar concentration and pectin, despite the orange juice containing more pectin. This is likely to be due to the different physicochemical states of pectin in both systems. In our model system, pectin is dissolved, whereas in orange juice pectin is present as suspended solids, contributing both to body and to cloud. In fact, PME is in orange juice bound to pectin. This binding to suspended pectin is responsible for the extreme difficulties found in the attempts to obtain pectin-free PME (Macdonald et al., 1993, 1994) and for the lack of enzyme permeability through membranes of a cutoff higher than the molecular weight of PME, unless the pH is increased and/or cations are added to promote the enzyme dissociation from pectin-containing particles (Snir et al., 1995, 1996b; Chen et al., 1998). Protection of the PME by pectin is obviously one of the reasons for the need of using high pasteurization temperatures for orange juice stabilization.

PME Inactivation by MTS. If the inactivation rate of PME by MTS at 33 °C in citrate buffer is compared with the data collected by El`Pinner (1964) on the effects of ultrasound waves on a number of enzymes, and with other more recently published data (Dubbs, 1966; Coakley et al., 1973; Kashkooli et al., 1980; Barteri et al., 1996), the TRS-PME fraction is found to be one of the enzymes most sensitive to the ultrasonic waves.

Different MTS enzyme inactivation mechanisms have been already suggested. In some cases a common inactivation mechanism for the MTS and the simple heat treatment have been proposed (Lopez and Burgos, 1995b). In other cases, in view of the differences in E_a and the pH dependence of the inactivation reaction(s), it was suggested that a new mechanism was added, in MTS, to heat denaturation (Lopez and Burgos, 1995a). The lower E_a of the PME inactivation by MTS and the substantial loss of the protection afforded by pectin point also to a different mechanism being added to heat denaturation in the MTS inactivation of PME.

Any of the known effects of the cavitation induced by the ultrasonic waves could theoretically be responsible for the added inactivation mechanism. However, it does not seem likely that free radicals generated by sonolysis play a main role, because in the temperature range 38−70 °C at which the simple heating contribution to the overall inactivation rate is negligible (as can be seen in Figure 6), the MTS inactivation rate grows with temperature while free radical production (Vercet et al., 1998) decreases. It is more reasonable to suggest that the mechanism added by MTS is linked to the shear stresses produced during the bubble implosion, because the loss of activity during ultrafiltration at low pH and high NaCl observed by Snir et al. (1995) suggests that PME is quite sensitive to shear.

The loss on MTS of the protection afforded by pectin against heat is in agreement with previous work in which it has been observed that MTS overcomes the effects that cosolutes (substrates or other molecules that physically interact with enzymes) have on the thermal stability of the enzymes (Lopez and Burgos, 1995a,b). "Loss of the protective effect of pectin to which PME is bound" is also related to inactivation during orange juice ultrafiltration by Snir et al. (1995).

The decrease in MTS enzyme inactivation efficiency as temperature increases seems to be a common phenomenon (Lopez and Burgos, 1995b; Vercet et al., 1997), and it appears to be mainly due to the decrease of the bubble collapse intensity, because of the elevation of the water vapor pressure inside the bubble, as temperature increases.

It has been suggested that the effects of heat and ultrasonic waves in microbial destruction (Ordóñez et al., 1987; Raso et al., 1994; Sala et al., 1995) and enzyme inactivation (Lopez et al., 1994, 1998; Vercet et al., 1997) combine synergistically. This suggestion is based on the fact that the destruction (inactivation) rate of the combined method at temperatures at which the effects of heating by itself are measurable is greater than the sum of the rate of ultrasonic inactivation at room temperature plus the rate of inactivation by simple heating. On these bases, orange PME inactivation by MTS would be also a synergistic combination of effects. There are, nevertheless, some reasons to question this rather simplistic approach, because the heat inactivation rate of TRS-PME at temperatures <70 °C is negligible, but the MTS inactivation rate of this PME fraction grows >3-fold between 38 and 60 °C, which reveals that the inactivation mechanism introduced by the ultrasonic waves is also temperature dependent.

The great increase in the rate of inactivation of the thermostable PME fraction from orange reached by ultrasonic irradiation while heating reveals that MTS could reduce dramatically the intensity (time and/or temperature) of the heat treatments used for orange juice stabilization. Temperatures of ∼65−70 °C seem most appropriate for this purpose, because the citric juice microflora is not particularly thermoresistant and a few seconds at these temperatures would be enough for its destruction (Kimball, 1991). Moreover, the ultrasonic waves enhance microbial heat destruction (Ordóñez et al., 1987). However, the introduction of MTS into the orange juice industry will require first the elucidation of its effects on nutrients and on the sensorial and rheological properties of the juice as well as the development of suitable industrial equipment.

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