Journal of Dairy Research (2002) **69** 243–254. © *Proprietors of Journal of Dairy Research 2002*
DOI: 10.1017/S0022029902005460 *Printed in the United Kingdom*

Manothermosonication of heat-resistant lipase and protease from *Pseudomonas fluorescens*: effect of pH and sonication parameters

By ANTONIO VERCET, JUSTINO BURGOS and PASCUAL LOPEZ-BUESA*
Food Technology Laboratory, Faculty of Veterinary Science, University of Zaragoza, Miguel Servet 177, 50013 Zaragoza, Spain

(*Received 18 March 2001 and accepted for publication 19 December 2001*)

Summary. The effect of different parameters (pH, ultrasonic amplitude and pressure) on the resistance to heat and manothermosonication (MTS) treatments of heat resistant lipase and protease produced by *Pseudomonas fluorescens* B52 and NCDO 2085, respectively, were studied. Lipase B52 thermoresistance decreases with an increase of pH. However, inactivation by MTS seems to be pH independent. There were only slight increases in the MTS efficiency when increasing pressure at UHT temperatures and the effect of amplitude was different depending on treatment temperature. Protease NCDO 2085, which was very resistant to MTS at 30 °C, was very sensitive to MTS at 76 °C. Increases in applied pressure had no effect on MTS efficiency at 140 °C and its inactivation by MTS was almost temperature independent between 76–109 °C. Data obtained are compared with previous published data and inactivation mechanisms are discussed.

Keywords: Manothermosonication, lipase, protease, *Pseudomonas fluorescens*.

Food preservation requires destruction or inhibition of microorganisms and enzymes, and generally, heat treatment is the method most widely used. But heating can also have a negative impact on nutritional value and organoleptic properties of foods. For this reason, there is an increased interest in new procedures able to inactivate enzymes and destroy microorganisms with little or no heat. Some of these methods combine heat with other physical or chemical agents in order to enhance the effects of heat, so that the intensity of heat treatment applied can be reduced. Manothermosonication (MTS), which consists of the simultaneous application of heat and ultrasound under moderate pressure (100–700 kPa), is one of these new technologies (Lopez *et al*. 1994; Burgos, 1999) that could be applied to liquid foods.

Introduction of refrigerated tanks for bulk storage of milk has reduced the problems linked with spoilage by mesophilic microorganisms but favours the growth of psychrotrophic bacteria. Thermoresistant enzymes produced by psychrotrophic microorganisms can reduce the quality and shelf-life of heat-treated milk and dairy products (Søraugh & Stepaniak, 1997). Development of a new technological procedure that would be as efficient as UHT in destruction of microbial spores and more efficient in psychrotrophic enzyme inactivation would be of great advantage for the milk industry. Some of these new technologies have failed to inactivate enzymes,

* For correspondence; e-mail: plopezbu@posta.unizar.es

and it is difficult find a new technology able to inactivate both microorganisms and enzymes while reducing the heating conditions. Nevertheless, MTS has proved to be an efficient tool to inactivate both microorganisms (Raso *et al.* 1998) and enzymes (Lopez *et al.* 1994, 1998; Vercet *et al.* 1999). Previous work with the inactivation of a lipase and a protease from *Pseudomonas fluorescens* by MTS has been reported (Vercet *et al.* 1997) but it is necessary to know how treatment parameters can affect MTS efficiency.

Effects of ultrasound on liquid media are complex. The main effects are due to cavitation, which consists of the formation, growth and, in some instances, implosion of bubbles (Suslick, 1990). Several mechanisms can act due to cavitation. When implosion occurs, extremely high temperatures and pressures (5000 °C and 500 MPa) are reached in a small volume of the liquid, called 'hot spot' due to these extreme conditions. Water sonolysis also occurs leading to formation of free radicals. Finally, mechanical damage can be expected, not only due to high pressures reached at implosion, but also to shear stresses and microstreaming. Most of these mechanisms are related to inertial cavitation. This kind of cavitation is a threshold phenomenon which depends critically on several parameters (Mason & Lorimer, 1988). The magnitude of effects resulting from inertial cavitation depends on the number of bubbles and on the intensity of their collapse. The number of imploding bubbles increases with ultrasonic amplitude, and the formation of bubbles is facilitated by an increase in temperature and frequency and a decrease in viscosity and static pressure applied. The intensity of implosion is also affected by several factors. In general, implosion intensity increases with an increase in viscosity, static pressure and diminishes with increases in water vapour pressure. As can be deduced, temperature exerts a complex and indirect effect: an increase in temperature will facilitate the formation of cavitating bubbles (due to diminished surface tension and viscosity) but will diminish the collapse intensity (due to increases water vapour pressure and the reduced surface tension and viscosity). Static pressure also has a complex effect. An increase in pressure will reduce the number of cavitating bubbles but will increase intensity of implosion. In this complex scenario, it is necessary to evaluate how different parameters affect the effectiveness of MTS in order to discuss the inactivation mechanisms and to optimize treatment conditions.

The aim of this work was to study how different parameters like static pressure, ultrasonic amplitude and pH affect psychrotrophic enzyme inactivation by MTS.

Materials and methods

Bacterial Strains, culture condition and enzyme preparation

Bacterial strains used were *Ps. fluorescens* B52 and *Ps. fluorescens* NCDO 2085 for lipase and protease production, respectively. Culture conditions and enzyme preparation conditions were as previously described (Vercet *et al.* 1997).

Heat and MTS treatments

Heat and MTS treatments were performed in a batch thermoresistometer as previously described (Lopez *et al.* 1994; Raso *et al.* 1998). Protease treatments were applied in milk whey obtained from skimmed milk by acid precipitation and subsequently re-adjusted to selected pH values. Lipase was treated in 0·1 M-phosphate buffer at the selected pH. Enzyme solutions (30–40 mg lyophilized protease powder or 10–15 mg lipase powder dissolved in 200 μl treatment medium)

https://doi.org/10.1017/S0022029902005460 Published online by Cambridge University Press

were injected into the treatment chamber containing 23 ml of the treatment medium equilibrated at the set treatment conditions. Sonication was carried out with a Branson device operating at 20 kHz at different amplitudes and gauge pressures.

Protease assay

Protease activity was determined as described by Beynon & Jay (1978). Azocasein was supplied by the Sigma Chemical Co (St. Louis, MO). Absorbance measurements were performed on a Spectrophotometer Uvikon 820 (Kontron Analytical, Zurich, Switzerland). Incubations were performed at 35 °C.

Lipase assay

Lipase activity was determined by the fluorimetric method originally reported by Dooijewaard-Klosterziel & Wouters (1976) and used by Stead (1983) for *Ps. fluorescens* lipase. Methyl umbeliferyl oleate was supplied by Sigma and 2-methoxyethanol was supplied by Aldrich-Sigma (Stenheim, Germany). Substrate preparation was as follows: 10·9 mg methyl umbeliferyl oleate were dissolved in 1·5 ml 2-methoxyethanol. Of this solution, 0.3 ml was then added to 4·7 ml water to obtain an emulsion. Assay mix consisted of 3 ml assay buffer (0·1 M-Tris.HCl-5 mM-$CaCl_2$, pH 7·1) and 0·1 ml substrate emulsion. Reactions, which were performed at room temperature, were started by addition of 2–50 μl lipase-containing solution. Fluorescent intensity was measured periodically. Activity was based on initial rates that were obtained from plots of fluorescent intensity versus time. Corrections were made for non-enzymic hydrolysis by recording changes in fluorescence in the absence of lipase solution. Fluorescence measurements were made in a fluorescence spectrophotometer (F-1200 Hitachi, Tokyo, Japan) and excitation and emission wavelengths were set at 325 and 450 nm, respectively.

Enzyme inactivation parameters

Enzyme resistance at different temperatures was expressed both as D values (D_T) and as inactivation rate constants (k). Temperature dependence of inactivation reactions was expressed both as z values and activation energies (E_a). D value is the decimal reduction time for any fixed temperature. z Value is the increase in temperature needed to reduce to one tenth the D value or the increase in temperature required for the D value to drop 1 log cycle.

Statistical analysis

Statview program (Abacus concepts Inc., Berkeley, CA, USA) was used for linear regression analysis. Comparison of slopes was performed with Prism program (Graphpad Software).

RESULTS

Inactivation of Ps. fluorescens *B52 lipase*

Effect of pH. D values obtained from inactivation plots of lipase B52 by heat treatment, at different pH and temperature, are shown in Table 1. Thermoresistance of lipase B52 diminishes with increasing pH. The enzyme was more sensitive to thermal inactivation at pH 8, which is close to its optimal pH for activity. MTS (350 kPa and 117 μm) inactivation behaviour (Table 1) was different from thermal inactivation. Lipase B52 resistance to inactivation was similar at pH 5·8 and 8·0, which was lower than the resistance at pH 6·6. pH dependence of *Ps. fluorescens* B52 lipase inactivation can be expressed as the ratio of D value at pH studied/D value at

https://doi.org/10.1017/S0022029902005460 Published online by Cambridge University Press

Table 1. D *values for* Pseudomonas fluorescens *B52 lipase inactivation by heat and manothermosonication (MTS) treatments in phosphate buffer at different pH. MTS conditions: amplitude, 117* μm *and gauge pressure, 350 kPa*

(*D* values are means ± SEM, for $n = 3$†)

	Heat treatment		MTS treatment	
	Temperature, °C	*D*, min	Temperature, °C	*D*, min
pH 5·8	110	8·55 ± 0·14	110	1·16 ± 0·07*
	120	4·40 ± 0·17	121	0·89 ± 0·04*
	130	2·41 ± 0·07	131	0·58 ± 0·01*
	140	1·40 ± 0·04	140	0·46 ± 0·03*
pH 6·6†			30	7·52
			72	2·8
			100	1·67
	110	8·85	110	1·39
	120	3·7	120	1·5
	130	2·06	130	1·53
	140	1·27	140	0·76
pH 7·2	110	5·55 ± 0·06	110	1·42 ± 0·02*
	120	3·11 ± 0·17		
	130	1·52 ± 0·06		
	140	0·85 ± 0·02	140	0·52 ± 0·03*
pH 8·0	110	2·86 ± 0·05	110	1·29 ± 0·07*
	120	1·73 ± 0·05	117	0·84 ± 0·03*
	130	0·98 ± 0·17	131	0·49 ± 0·02*
	140	0·53 ± 0·02	139	0·40 ± 0·03*

† *D* values at pH 6·6 are reproduced from Vercet *et al.* 1997.
* Values significantly different from the equivalent heat treatment value, $P < 0·05$.

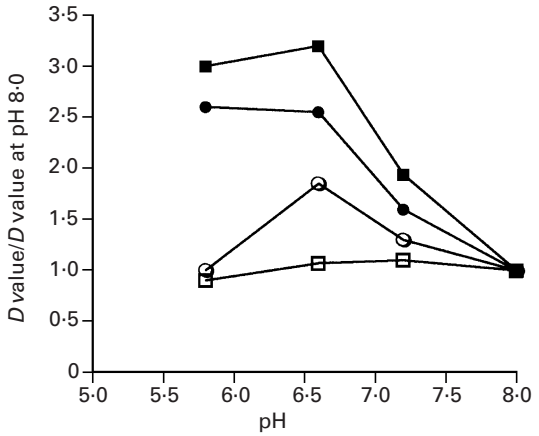

Fig. 1. Effect of pH on *Pseudomonas fluorescens* B52 lipase inactivation in phosphate buffer by heat treatment at 110 °C (■) and 140 °C (●) and by manothermosonication (MTS) at 110 °C (□) and 140 °C (○). MTS conditions: amplitude, 117 μm and gauge pressure, 350 kPa.

pH 8·0. Figure 1 shows the difference between MTS and thermal inactivation. This was especially clear at low pH and 110 °C, where the difference between the two treatments in the ratio $D_{pH5·8}/D_{pH8·0}$ was greatest. Table 2 shows z and E_a values for inactivation by heat and MTS.

Effect of Pressure. Effect of pressure was studied at 140 °C, pH 6·6, and amplitude 117 μm, at gauge pressures from 350 to 600 kPa. Under these conditions, increasing

https://doi.org/10.1017/S0022029902005460 Published online by Cambridge University Press

Table 2. E_a *and* z *values for the inactivation of* Pseudomonas fluorescens *B52 lipase and* Pseudomonas fluorescens *NCDO 2085 protease by heat and manothermosonication* (*MTS*) *at different pH. Lipase treatments were carried out in phosphate buffer and protease treatments in milk whey*

(Values are means ± SEM, for $n = 3$)

	Heating		MTS	
pH	z, deg C	E_a, kJ/mol	z, deg C	E_a, kJ/mol
Lipase, *Ps. fluorescens* B52				
5·8	38·5 ± 1·4	77·6 ± 1·2	76·9 ± 6·0*	39·6 ± 4·0*
7·2	35·7 ± 1·3	81·7 ± 3·0		
8·0	41·7 ± 1·7	72·3 ± 3·5	55·5 ± 6·3*	52·7 ± 4·5*
Protease, *Ps. fluorescens* NCDO 2085				
5·8	23·26 ± 0·5	126 ± 6·5	27·8 ± 4·5	106·3 ± 18·0
7·2	31·25 ± 5·0	93·5 ± 14·1		
8·0	32·26 ± 1·0	90·5 ± 1·4	45·4 ± 2·1*	65·0 ± 2·5*

* Values significantly different from the equivalent heat treatment value; $P < 0·05$.

Table 3. D *values for the inactivation of* Pseudomonas fluorescens *B52 lipase and* Pseudomonas fluorescens *NCDO 2085 protease by manothermosonication* (*MTS*) *at pH 6·6, 140 °C, amplitude 117* μm *and different pressures. Lipase treatments were carried out in phosphate buffer and protease treatments in milk whey*

(Values are means ± SEM, for $n = 3$)

Pressure, kPa	*Ps. fluorescens* B52 lipase D, min	*Ps. fluorescens* NCDO 2085 protease D, min
350	0·74 ± 0·03	0·90 ± 0·20
400	0·73 ± 0·02	1·13 ± 0·04
500	0·61 ± 0·02	0·85 ± 0·03
550	0·58 ± 0·02	—
600	0·57 ± 0·01	0·82 ± 0·02

pressure from 350 to 500 kPa resulted in < 20 % reduction in D (Table 3). Further pressure increase to 600 kPa had no additional effect on D values (Table 3).

Effect of amplitude. The effect of amplitude, ranging from 60 to 150 μm, was investigated at 76, 350 and 500 kPa (140 °C, pH 6·6). Figure 2 shows the ratio of MTS inactivation rate constants at different amplitudes. The behaviour at both temperatures is similar, and the relationship with amplitude was not linear. At both temperatures, inactivation seems to be more amplitude dependent at amplitudes above 120 μm.

Inactivation of Ps. fluorescens *NCDO 2085 protease*

Inactivation at low temperatures. In order to complete the NCDO 2085 protease inactivation carried out at pH 6·6 and already published (Vercet *et al*. 1997), thermal and MTS inactivation was performed at temperatures between 30 and 140 °C. It is necessary to indicate that MTS treatment at 300 kPa, 117 μm and 30 °C did not cause protease inactivation, but MTS treatments at higher temperatures (76 °C) produced a relatively high inactivation rate ($D_{76\,°C} = 3·27$ min). The thermal destruction curve for both treatments is shown in Fig. 3. Inactivation patterns appear different for both treatments: thermal inactivation follows Arrhenius law (z value 31 deg C, E_a 95 kJ/mol) but for MTS two different steps can be seen: from 76 to 110 °C in which

https://doi.org/10.1017/S0022029902005460 Published online by Cambridge University Press

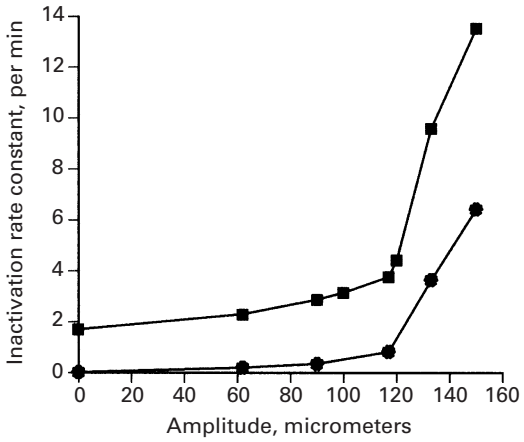

Fig. 2. Effect of ultrasonic amplitude on *Pseudomonas fluorescens* B52 lipase inactivation by manothermosonication (MTS) treatments in phosphate buffer, pH 6·6 and at different ultrasonic amplitudes. MTS conditions: 76 °C gauge pressure 350 kPa (●); 140 °C gauge pressure 500 kPa (■).

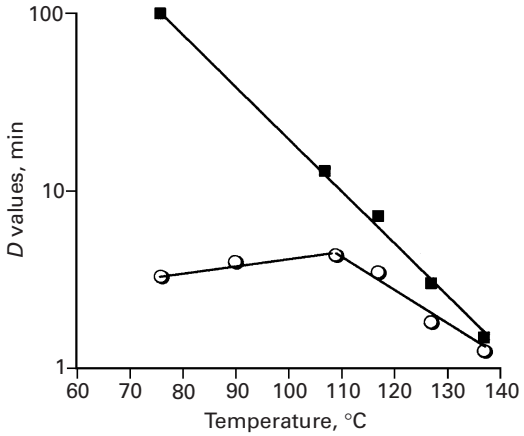

Fig. 3. Thermal destruction curve for the inactivation of *Pseudomonas fluorescens* NCDO 2085 protease by heat (■) and manothermosonication (MTS; ○) treatments in milk whey pH 6·6. MTS conditions: amplitude, 145 μm and gauge pressure, 350 kPa.

inactivation rate was almost independent of temperature, and at higher temperatures in which D values for MTS follow a similar trend to thermal inactivation as temperature increases, leading to higher z values and lower E_a (47 °C; 63 kJ/mol).

Effect of pH. The effect of pH on protease NCDO 2085 inactivation rate was different for heat and MTS for the treatment conditions applied (temperatures between 110–140 °C; amplitude, 117 μm; gauge pressure, 350 kPa). D values obtained from inactivation plots are shown in Table 4. Protease NCDO 2085 reached maximum resistance to thermal inactivation at pH $\approx$ 6·6. E_a and z values are shown in Table 2: these values are similar for heat and MTS inactivation at pH 5·8 but slightly different for pH 6·6 and 8·0.

Effect of pressure. The effect of static pressure on MTS inactivation was studied with pressures ranging from 300–600 kPa (amplitude, 117 μm at 140 °C, pH 6·6). Pressure had almost no effect on protease inactivation by MTS; an increase of 200 kPa only changed D from 1·13 to 0·82 min (Table 3).

Effect of amplitude. The effect of amplitude ranging from 62–150 μm, was studied

https://doi.org/10.1017/S0022029902005460 Published online by Cambridge University Press

Table 4. D *values for protease* (Pseudomonas fluorescens *NCDO 2085*) *inactivation by heat and manothermosonication* (*MTS*) *treatments in milk whey at different pH. MTS conditions amplitude, 117* μm *and gauge pressure 350 kPa*

(*D* values† are means ± SEM, for $n = 3$)

	Heat treatment		MTS treatment	
	Temperature °C	*D*, min	Temperature, °C	*D*, min
	110	6·06 ± 0·21	110	3·30 ± 0·11*
pH 5·8	120	2·55 ± 0·12	120	1·46 ± 0·03*
	130	0·94 ± 0·02	130	0·80 ± 0·04
	140	0·33 ± 0·02	140	0·24 ± 0·01
pH 6·6†	107	13	109	4·3
	117	7·2	117	3·45
	127	3·02	127	1·80
	137	1·49	137	1·24
pH 7·2	110	7·75 ± 0·25	110	3·00 ± 0·09*
	120	5·59 ± 0·12		
	130	1·79 ± 0·07		
	140	1·01 ± 0·06	140	0·6 ± 0·03*
pH 8·0	110	6·54 ± 0·12	110	2·59 ± 0·08*
	120	3·29 ± 0·08	120	1·41 ± 0·06*
	130	1·57 ± 0·05	130	0·90 ± 0·06*
	140	0·82 ± 0·05	140	0·56 ± 0·03*

† *D* values at pH 6·6 are reproduced from Vercet *et al.* 1997.
* Values significantly different from the equivalent heat treatment value; $P < 0·05$.

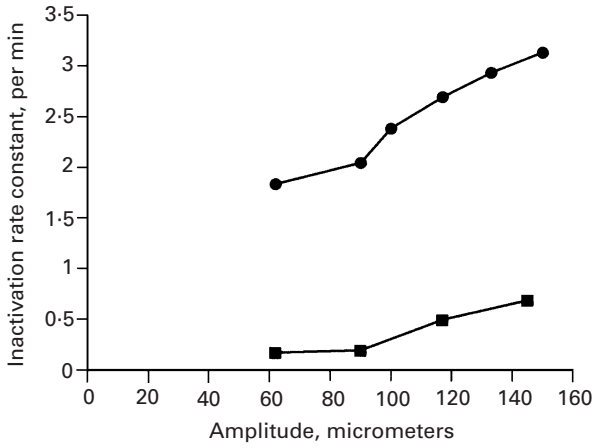

Fig. 4. Effect of ultrasonic amplitude on *Pseudomonas fluorescens* NCDO 2085 protease inactivation by manothermosonication (MTS) in milk whey, pH 6·6, at different MTS conditions: 76 °C and gauge pressure, 350 kPa (■); 140 °C and gauge pressure, 500 kPa (●).

at pH 6·6 at 76 °C, 350 kPa (gauge pressure) and at 140 °C, 500 kPa (gauge pressure). Figure 4 shows ratio MTS inactivation rate at different amplitudes/inactivation rate by heat treatment for 76 °C and 140 °C amplitude. Inactivation appeared to be amplitude dependent above 90 μm. The same behaviour was observed at both temperatures studied, although protease MTS inactivation dependence on amplitude was higher at 76 °C.

https://doi.org/10.1017/S0022029902005460 Published online by Cambridge University Press

discussion

Inactivation of Ps. fluorescens B52 *lipase*. The significant change in the MTS inactivation temperature dependence at temperatures near 110 °C (Vercet *et al*. 1997) suggests that different inactivation mechanisms are operating at the two temperature ranges (above and below 100 °C). Our suggestion is that these two mechanisms are the separate effects of ultrasound and thermal inactivation. Total inactivation results from the additive effect of both mechanisms. The presence of two different mechanisms during MTS inactivation is corroborated by the results obtained at different pH values. Lipase thermo-inactivation has a high dependence on pH, but MTS inactivation is almost pH independent. This fact is clearly shown in Fig. 1 at 110 °C in which the relative contribution of heat to total inactivation is minimal. However, this is unclear for MTS treatments at 140 °C, a temperature at which both heat and MTS contributions to total inactivation will be near to 50% (because inactivation rate constant by simple heating at 140 °C was approximately half the MTS inactivation rate constant at the same temperature). This difference in the contribution of both mechanisms to the total effect, depending on pH, was the reason for the lowest z values for MTS at pH 8·0, in which the contribution of the inactivating effect of heat was higher than at pH 5·8. At low temperature and pH, ultrasound effects predominated in the inactivation, but as temperature increased and pH effect on thermal inactivation became more relevant, the contribution of heat to the overall inactivating effect of MTS was more important.

In the inactivation of lipoxygenase by MTS, a mechanisms based on an 'encaged' Fenton reaction, has been proposed (Lopez & Burgos, 1995). These mechanisms can be discounted in the inactivation of lipase B52 by MTS because its active site has a catalytic triad formed by serine, histidine and aspartic acid, without an Fe atom. Added to this, its incubation with H_2O_2 did not cause any activity loss (Vercet *et al*. 1997). However, this is not a reason to discard free radical production as a mechanism for lipase B52 inactivation by MTS. Sonolysis can lead to formation of hydroxyl radicals which can react with one of the amino acids participating in the active site; histidine is the most free radical-sensitive of the catalytic triad (Davies *et al*. 1987; Neuzil *et al*. 1993). However, the comparison of inactivation data obtained in this work and free radical production obtained in similar treatment conditions (Vercet *et al*. 1998) gives a different picture. Amplitude exerts different influence on both parameters: for lipase inactivation an increase in amplitudes above 120 μm produced a large increase in the inactivation rate, while these amplitudes had almost no effect on free radical production. Whatever the mechanism operated by ultrasound, it is highly dependent on amplitude. Of all the mechanisms proposed for inactivation of enzymes by ultrasound the most likely is mechanical damage. Several authors cited by Price (1990) show that mechanical efforts generated in non-inertial cavitation are able to break DNA and cellular membranes; Kashkooli *et al*. (1980) demonstrated the inactivation of malate dehydrogenase by this mechanism. In addition, bubble collapse in inertial cavitation produce shear stresses which are able to break polymers like polystyrene, dextran, proteins or DNA (El Pinner, 1964; Basedow & Ebert, 1977). Probably, for the inactivation of an enzyme, it is not necessary that the intensity of mechanical damage should be high enough to break covalent bonds; it would be sufficient to break the structures stabilizing the native conformation of the enzyme. The sensitivity to mechanical damage is related to the molecule conformation; a higher flexibility would result in a higher ability to cushion mechanical damage (Barteri *et al*. 1996). Extracellular hydrolytic enzymes produced

by psychrotrophs have 'flexible structures' (Mitchell *et al.* 1986; Kroll, 1989) that would not make them especially sensitive to mechanical damage.

However, it is well known that denaturation and inactivation of proteins and enzymes by unfolding occurs in interfaces, liquid-gas or liquid-liquid (Volkin & Klibanov, 1989; Makhzoum *et al.* 1996). A liquid under the effect of ultrasonic irradiation has a large number of bubbles that cavitate and therefore has a high air/water interfacial area, in which hydrophilic protein groups are exposed to water and the hydrophobic groups to air. Due to its relatively high molecular mass (50000 Da; Tan & Miller, 1992), and the flexibility of the structure of lipases (Kroll, 1989; Mitchell *et al.* 1986; Owusu *et al.* 1991, 1992; Makzhoum *et al.* 1993), an inactivating mechanism operating by unfolding at interfaces could also be expected. Interface unfolding and shear stresses can exert a combined denaturating effect (Charm & Wong, 1981), as reported for human growth factor (Maa & Hsu, 1997).

For this enzyme, the effect of pH and treatments at UHT temperatures, point to an additive effect, but inactivation rate by MTS from 30 °C (Vercet *et al.* 1997) to 110 °C increased five fold, which would mean that the inactivating effect of ultrasound would also be temperature dependent. One of the reasons used, and mentioned previously, to sustain this assumed effect is that effects of ultrasound diminish with temperature. The reason for this decrease in some of the effects is an increase in water vapour pressure, and therefore higher vapour content inside the bubbles. This vapour would act like a cushion when implosion occurs, producing a less intense collapse together with a decrease of viscosity and surface tension that would also diminish implosion intensity. But, on the other hand, increasing temperature would facilitate the formation of more cavitating bubbles. This is the same phenomenon occurring with some chemical effects that increase with ultrasonic amplitude [like sonolysis (Mason *et al.* 1994), iodine oxidation (Petrier *et al.* 1992; Entrezari & Kruus, 1994) and carbon tetrachloride degradation (Francony & Petrier, 1996)] and that was explained with more numerous collapses but with less intensity (Crum, 1995). This could be the reason why lipase B52 inactivation by MTS would also be temperature dependent, and this will discard a free radical-mediated inactivation, because for free radical production by ultrasound high intensity collapses are necessary (Riesz & Kondo, 1992).

Inactivation of Ps. fluorescens *NCDO 2085 protease*. MTS was more efficient than heat in the inactivation of protease NCDO 2085. However, the behaviour of this enzyme in MTS treatments was different, in some aspects, from lipase B52 behaviour. There are two surprising effects:

(1) Protease NCDO 2085 was resistant to an MTS treatment at 30 °C, 350 kPa, and 117 μm (Vercet *et al.* 1997), nevertheless it was MTS sensitive in the same conditions but at a higher temperature (76 °C) in which its inactivation by heat is still slow ($D_{75\,^{\circ}C}$ for thermal inactivation of 100 min).

(2) D value for MTS increased slowly with an increase in treatment temperatures in the range of 76–109 °C (Fig. 3). This indicates that efficiency of ultrasonic waves decreased progressively, especially since the contribution of heat to global inactivation by MTS increased in this temperature range.

To explain the substantial change in sensitivity to MTS between 30 and 76 °C, the peculiar behaviour of extracellular lipases and proteases from psychrotrophic microorganisms during thermal inactivation should be considered. As mentioned before, most hydrolytic enzymes produced by psychrotrophs have flexible structures, a low denaturation temperature and their themoresistance is due to a very efficient refolding (Mitchel *et al.* 1986; Owusu *et al.* 1991). The change in sensitivity

to MTS may be due to unfolding of the enzyme at 76 °C, which may facilitate the ultrasound inactivating mechanisms. It is difficult to explain why inactivation rate by MTS diminished when temperature increased in the range 76–110 °C. In a previous work (Vercet *et al.* 1997) it has been shown that MTS of NCDO 2085 protease at 30 °C had no inactivating effect. So, if the MTS inactivation rate at 30 °C is considered, we have to conclude that the combination of effects is synergistic at all temperatures studied. However, a detailed analysis of results shows that the effect of ultrasound and heating are not synergistic and the contribution of ultrasound to global MTS inactivation decreases with temperature, as shown at the temperature range 76–110 °C, in which contribution of heating to overall inactivating effect of MTS is almost negligible. This loss in the contribution of ultrasonic waves could be explained by the decrease of effectiveness of cavitation with growing temperatures (Mason *et al.* 1992, 1994; Didenko *et al.* 1994) due to the effect of water vapour pressure on viscosity and surface tension. This would mean that the inactivating effect added by ultrasound will have a higher dependence on the intensity of implosion rather than on the number of bubbles undergoing cavitation, as shown for free radical production by ultrasound (Riesz & Kondo, 1992). The fact that D values for MTS decrease with temperature at temperatures higher than 110 °C can be explained because heating is becoming the predominant inactivating mechanism as temperature increases, with a higher contribution to the overall MTS inactivation (40 % at 109 °C, 48 % at 117 °C, 60 % at 127 °C and 83 % at 137 °C). This fact also explains why the MTS inactivation rate constant of protease NCDO 2085 was not affected, at least at high temperatures (140 °C), by changing treatment-pH (in contrast to lipase B52), because thermal inactivation effects predominate.

Unlike lipase B52, the inactivation mechanism of protease by ultrasound seems to be related to free radical production. Comparison of the effects of pressure and amplitude on free radical production rate by MTS (Vercet *et al.* 1998) under similar conditions to those used in this work, showed that the evolution of free radical production rate and protease NCDO 2085 inactivation rate by ultrasound show the same dependence on both static pressure and ultrasonic amplitude. Added to this the increase in D values for MTS inactivation observed in the temperature range 76–109 °C could be related to a loss in free radical production rate with increasing temperature (Vercet *et al.* 1998).

This enzyme is a metalloprotease with Zn in its active site (Fairbairn & Law, 1986) so a mechanism like the one proposed for lipoxygenase could be again discounted. Free radical interaction with proteins can produce the oxidative modifications of the amino acids participating in the active site and other changes (i.e. denaturation, covalent aggregation, fragmentation) that hydroxyl radicals induce on proteins (Davies, 1987; Davies & Delsignore, 1987; Davies *et al.* 1987; Hunt *et al.* 1988). Effects of free radicals on proteins vary widely depending on the protein, not only on its primary structure but also secondary and tertiary structures (Davies, 1987; Le Marie *et al.* 1990). It is possible that at temperatures near 30 °C in which the enzyme maintains its native structure, protease NCDO 2085 could resist exposure to free radicals (that have a short lifetime) for several minutes. Following unfolding at higher temperatures (70 °C) target points for free radicals would be more accessible.

Additive or synergistic combination? Until now it has been suggested that the effects of ultrasound and heat in microbial destruction and enzyme inactivation combine synergistically (Lopez *et al.* 1994; Sala *et al.* 1995; Vercet *et al.* 1997; Lopez *et al.* 1998). This was based on the fact that the inactivation rate of the combined

method, at temperatures at which the effects of heating by itself are measurable, is greater than the addition of the rate of ultrasonic inactivation at room temperature plus the inactivation rate by simple heating at the same temperature. But no temperature dependence of the inactivating effect added by ultrasound has been considered. On these bases, both lipase and protease inactivation by MTS would be a synergistic combination of effects. But, as happens for thermoresistant pectinesterase from orange (Vercet *et al*. 1999), there are some reasons to question this simplistic approach. Heat inactivation of these enzymes at temperatures below 110 °C is negligible or very slow so its contribution to the global inactivating effect of MTS will be negligible. Our suggestion for the MTS inactivation of both enzymes is that there is no real interaction between the inactivating effects of ultrasound and thermal inactivation. Variations measured in the temperature range at which no effect of heat by itself can be observed, are due to the temperature dependence of the effect added by ultrasound. This variation will be different depending on the predominant inactivating mechanism operated by ultrasound. If it is a free radical-mediated reaction, then a higher intensity of collapse will be important, rather than a larger number of cavitating bubbles. Conversly, for inactivation mechanisms like interface unfolding, it will be more important to have a high number of nuceli undergoing cavitation.

Thanks are give to Instituto Danone for a fellowship granted to Antonio Vercet and to Spanish Comision Interministerial de Ciencia y Tecnología (CICYT) for financing this work as a part of the project ALI 98-0589 CP.

REFERENCES

Barteri, M., Fioroni, M. & Gaudiano, M. C. 1996 Oxidation of Fe (II) horse heart cytochrome c by ultrasound waves. *Biochimia et Biophysica Acta* **1296** 35–40

Basedow, A. M. & Ebert, K. H. 1977 Ultrasonic degradation of polymers in solution. *Advances in Polymer Science* **22** 83–148

Beynon, R. J. & Jay, J. 1978 The inactivation of native enzymes by a neutral proteinase from rat intestinal muscle. *Biochemical Journal* **173** 291–298

Burgos, J. 1999 Manothermosonication. In *Encyclopedia of Food Microbiology*, pp. 1462–1469 (Eds R. K. Robinson, C. A. Batt & P. D. Patel). New York: Academic Press

Charm S. E. & Wong, B. L. 1981 Shear effects on enzymes. *Enzyme and Microbial Technology* **3** 111–118

Crum, L. A. 1995 Comments on the envolving field of sonochemistry by a cavitation physicist. *Ultrasonics Sonochemistry* **2** 147–152

Davies, K. J. A. 1987 Protein damage and degradation by oxygen radicals. I. General aspects. *Journal of Biological Chemistry* **262** 9895–9901

Davies, K. J. A. & Delsignore, M. E. 1987 Protein damage and degradation by oxygen radicals. III. Modification of secondary and tertiary structure. *Journal of Biological Chemistry* **262** 9908–9913

Davies, K. J. A., Delsignore, M. E. & Lin, S. W. 1987 Protein damage and degradation by oxygen radicals. II. Modification of aminoacids. *Journal of Biological Chemistry* **262** 9902–9907

Didenko, Y. T., Nastich, D. N., Pugach, S. P., Polovinka, Y. A. & Kvochka, V. I. 1994 The effect of bulk temperature on the intensity and spectra of water sonoluminiscence. *Ultrasonics* **32** 71–76

Dooijewaard-Klosterziel, A. M. P. & Wouters, J. T. M. 1976 Some properties of the lipase of *Geotrichum candidum* evaluated by a fluorimetric assay technique. *Journal of Applied Bacteriology* **40** 293–299

El'Piner, I. 1964 Action of ultrasonic waves on biomacromolecules. In *Ultrasound, physical, chemical and biological effects*, pp. 149–230 (Ed. I. El'Piner). New York: Consultants Bureau

Entrezari, M. H. & Kruus, P. 1994 Effect of frequency on sonochemical reactions I: oxidation of iodine. *Ultrasonics Sonochemistry* **21** 75–80

Fairbairn, D. J. & Law, B. A. 1986 Purification and characterization of the extracellular proteinase of *Pseudomonas fluorescens* NCDO 2085. *Journal of Dairy Research* **53** 457–466

Francony, A. & Petrier, C. 1996 Sonochemical degradation of carbon tetrachloride in aqueous solutions at two frequencies: 20 kHz and 500 kHz. *Ultrasonics Sonochemistry* **3** 77–82

Hunt, J. V., Simpson, J. A. & Dean, R. T. 1988 Hydroperoxide-mediated fragmentation of proteins. *Biochemical Journal* **250** 87–93

Kashkooli, H. A., Rooney, J. A. & Roxby, R. 1980 Effects of ultrasound on catalase and malate dehydrogenase. *Journal of Acoustic Society of America* **67** 1798–1801

Kroll, S. 1989 Thermal stability. In *Enzymes of psychrotroph in raw food*, pp. 121–152 (Ed. R. C. McKellar). Boca Raton: CRC Press

Le Marie, M., Thauvette, L., Foresta, B., Viel, A., Beauregard, G. & Potier, N. 1990 Effects of ionizing radiations on proteins: evidence of non-random fragmentations and a caution in the use of the method for determination of molecular mass. *Biochemical Journal* **267** 431–439

Lopez, P., Sala, F. J., de la Fuente, J. L., Condon, S., Raso, J. & Burgos, J. 1994 Inactivation of peroxidase, lipoxygenase and polyphenol oxidase by manothermosonication. *Journal of Agriculture and Food Chemistry* **42** 252–256

Lopez, P. & Burgos, J. 1995 Lipoxygenase inactivation by manothermosonication: effects of sonication on physical parameters. pH, KCl, sugars, glycerol and enzyme concentration. *Journal of Agriculture and Food Chemistry* **43** 620–625

Lopez, P., Vercet, A., Sanchez, A. C. & Burgos, J. 1998 Inactivation of tomato pectic enzymes by manothermosonication. *Zeitschrift für Lebensmittel-Untersuchung und-Forschung* **207** 249–252

Maa, Y. H. & Hsu, C. C. 1997 Protein denaturation by combined effect of shear and air-liquid interface. *Biotechnology and Bioengeneering* **54** 503–512

Makhzoum, A., Owusu, R. K. & Knapp, J. S. 1993 The conformational stability of a lipase from a psychrotrophic *Pseudomonas fluorescens*. *Food Chemistry* **46** 355–359

Makzhoum, A., Owusu-Apenten, R. K. & Knapp, J. S. 1996 Purification and properties of lipase from *Pseudomonas fluorescens* strain 2D. *International Dairy Journal* **6** 459–472

Mason, T. J. & Lorimer, J. P. 1988 General principles. In *Sonochemistry: theory, applications and uses of ultrasound in chemistry*, pp. 17–63 (Eds. T. J. Mason & J. P. Lorimer). Ellis Horwood: Chichester

Mason, T. J., Lorimer, J. P. & Baters, D. M. 1992 Quantifying sonochemistry: casting some light on a 'black art'. *Ultrasonics* **30** 40–42

Mason, T. J., Lorimer, J. P., Baters, D. M. & Zhao, Y. 1994 Dosimetry in sonochemistry: the use of aqueous terephthalate ion as a fluorescence monitor. *Ultrasonics Sonochemistry* **1** 91–95

Mitchell, G. E., Ewings, K. N. & Bartley, J. P. 1986 Physicochemical properties of proteinases from selected psychrotrophic bacteria. *Journal of Dairy Research* **53** 97–115

Neuzil, J., Gebicki, J. M. & Stocker, R. 1993 Radical-induced chain oxidation of proteins and its inhibition by chain-breaking antioxidants. *Biochemical Journal* **293** 601–606

Owusu, R. K., Makhzoum, A. & Knapp, J. S. 1991 The thermodynamic stability of lipases and proteases from psychrotrophic bacteria. *Food Chemistry* **39** 187–195

Owusu, R. K., Makhzoum, A. & Knapp, J. S. 1992 Heat inactivation of lipase from psychrotrophic *Pseudomonas fluorescens* P38: activation parameters and enzyme stability at low or ultra-high temperatures. *Food Chemistry* **44** 261–268

Petrier, C., Jeunet, A., Luche, J. L. & Reverdy, G. 1992 Unexpected frequency effects on the rate of oxidative processes induced by ultrasound. *Journal of American Chemical Society* **114** 3148–3150

Price, G. J. 1990 The use of ultrasound for the controlled degradation of the polymer solutions. In *Advances in Sonochemistry*, vol. 1, pp. 231–287 (Ed. T. J. Mason). London: Jai Press Ltd

Raso, J., Pagan, R., Condon, S. & Sala, F. J. 1998 Influence of temperature and pressure on the lethality of ultrasound. *Applied and Environmental Microbiology* **64** 465–471

Riesz, P. & Kondo, T. 1992 Free radical formation induced by ultrasound and its biological implications. *Free Radical Biology & Medicine* **13** 247–270

Sala, F. J., Burgos, J., Condon, S., Lopez, P. & Raso, J. 1995 Effect of heat and ultrasound on microorganisms and enzymes. In *New methods of food preservation*, pp. 176–204 (Ed. G. W. Gould). Glasgow: Blackie

Sørhaug, T. & Stepaniak, L. 1997 Psychrotrophs and their enzymes in milk and dairy products: quality aspects. *Trends in Food Science and Technology* **8** 35–41

Stead, D. 1983 A fluorometric method for the determination of *Pseudomonas fluorescens* AR11 lipase in milk. *Journal of Dairy Research* **50** 491–502

Suslick, K. S. 1990 Sonochemistry. *Science* **247** 1439–1445

Tan, Y. & Miller, K. J. 1992 Cloning, expression, and nucleotide sequence of a lipase gene from *Pseudomonas fluorescens* B52. *Applied and Environmental Microbiology* **58** 1402–1407

Vercet, A., López, P. & Burgos, J. 1997 Inactivation of heat-resistant lipase and protease from *Pseudomonas fluorescens* by manothermosonication. *Journal of Dairy Science* **80** 29–36

Vercet, A., López, P. & Burgos, J. 1998 Free radical production by manothermosonication. *Ultrasonics* **36** 615–618

Vercet, A., López, P. & Burgos, J. 1999 Inactivation of heat-resistant pectin methylesterase from orange by manothermosonication. *Journal of Agriculture and Food Chemistry* **47** 432–437

Volkin, D. B. & Klibanov, A. M. 1989 Minimizing protein inactivation. In *Protein structure: a practical approach*, pp. 1–24 (Ed. T. E. Creighton). Oxford: IRL Press