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# Effect of temperature and/or pressure on lactoperoxidase activity in bovine milk and acid whey

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Summary. At atmospheric pressure, inactivation of lactoperoxidase (LPO) in milk and whey was studied in a temperature range of 69–73 °C and followed first order kinetics. Temperature dependence of the first order inactivation rate constants could be accurately described by the Arrhenius equation, with an activation energy of $635{\cdot}3 \pm 70{\cdot}7$ kJ/mol for raw bovine milk and $736{\cdot}9 \pm 40{\cdot}9$ kJ/mol for diluted whey, indicating a very high temperature sensitivity. On the other hand, LPO is very pressure resistant and not or only slightly affected by treatment at pressure up to 700 MPa combined with temperatures between 20 and 65 °C. Both for thermal and pressure treatment, stability of LPO was higher in milk than in diluted whey. Besides, a very pronounced antagonistic effect between high temperature and pressure was observed, i.e. at 73 °C, a temperature where thermal inactivation at atmospheric pressure occurs rapidly, application of pressure up to 700 MPa exerted a protective effect. At atmospheric pressure, LPO in diluted whey was optimally active at a temperature of about 50 °C. At all temperatures studied (20–60 °C), LPO remained active during pressure treatment up to 300 MPa, although the activity was significantly reduced at pressures higher than 100 MPa. The optimal temperature was found to shift to lower values (30–40 °C) with increasing pressure.

Keywords: Lactoperoxidase, bovine milk, diluted whey, pressure, temperature.

High hydrostatic pressure is clearly an emerging technology for nonthermal food preservation since it allows production of safe and high-quality food products (Knorr, 1993; Gould, 1995; Barbosa-Canovas *et al*. 1997). Hitherto, especially high pressure pasteurisation has received a great deal of interest since vegetative spoilage and pathogenic bacteria can be inactivated by relatively mild pressures (up to 600–700 MPa) at ambient temperature (Patterson *et al*. 1995; Smelt, 1998). As to sterilization, on the other hand, very high pressures are needed to inactivate bacterial spores at room temperature (Sale *et al*. 1970; Mills *et al*. 1998), making this technique, although technically feasible, not commercially profitable because of the very high cost. In this case, the use of a hurdle type approach by combining relatively mild

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pressure with mild temperature elevation or acidification would be worthwhile. Likewise the use of antimicrobial systems, whether naturally present or intentionally added to the food product, may offer some potentials. Indeed, pressure treatment of *Escherichia coli* MG1655 and pressure-resistant mutants in presence of antimicrobial peptides such as lysozyme and nisin, resulted in an increased lethality, the additional reduction being strongly dependent on the environment and the microbial strain (Hauben *et al.* 1996; Garcia-Graells *et al.* 1999).

Lactoperoxidase (LPO) has attracted special interest by many authors because of its antimicrobial activity in milk in presence of hydrogen peroxide ($H_2O_2$) and thiocyanate or halogens (Ravanis & Lewis, 1995). It catalyses the oxidation of thiocyanate ($SCN^-$), a compound widely distributed in animal tissues and secretions, by $H_2O_2$. The resulting hypothiocyanate ($OSCN^-$) or higher oxyacids are responsible for the antimicrobial activity, presumably because they react with protein sulphydryl groups, thereby causing inactivation of metabolic enzymes that depend on an active-site cysteine residue for their activity (Hernandez *et al.* 1990; Fuglsang *et al.* 1995). The natural occurrence of LPO in milk could likewise offer potential in pressure preservation (whether or not in combination with mild temperature elevation) of dairy products, provided the enzyme has a relative high pressure (-temperature) stability and maintains its activity under pressure. While some reports can be found in literature regarding thermal stability of LPO in milk, very little is known about its pressure stability. In this context, the effect of temperature and/or pressure on LPO in raw bovine milk and diluted whey has been focused on in this paper.

## Materials and methods

### *Milk source*

A single lot of fresh bovine milk was purchased from a local dairy farm (9/9/1999), characterized by a fat and protein content of, respectively, 14·2 g/kg and 31·0 g/kg. The milk was divided into small portions (30–50 ml) and stored under frozen conditions (−18 °C). For the preparation of acid whey, milk proteins were precipitated by adding successively 1·75 M-acetic acid and 1 M-sodium acetate, and removed by filtration (S589[3], Schluesser and Schnell GmbH, Germany). After filtration, the milk whey was diluted (1/5) in 0·1 M-phosphate buffer, pH 6·4, which corresponds to the optimal pH for LPO activity (Shahani *et al.* 1973; Hernandez *et al.* 1990). In this way, a stable solution was obtained which could be stored for several hours at room temperature without a change in LPO activity.

### *Lactoperoxidase activity assay*

LPO activity was measured spectrophotometrically at 412 nm (Biochrom 4060, u.v.-visible spectrophotometer, Pharmacia Biotech, UK) and 20 °C with 10 mM-ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) and 10 mM-$H_2O_2$ as substrate, according to an adjusted procedure of Hernandez *et al.* (1990). Instead of whole milk, acid whey was used for activity measurement, a procedure which is possible because LPO activity is mainly associated with the whey fraction (Shahani *et al.* 1973; Chavarri *et al.* 1998). Previous results showed that in this way, the accuracy/reproducibility of the assay method was improved. The activity was calculated from the slope of the linear part of the regression line when absorbance increase was plotted versus reaction time.

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*Lactoperoxidase inactivation study*

Isothermal inactivation experiments were performed in a thermostated water bath at temperatures between 69 and 73 °C. Milk or whey samples, filled in screw cap vials (4 ml, Vel Polylabo, Belgium) were treated at constant temperature for pre-set times. Isobaric-isothermal inactivation experiments were performed in a thermostated laboratory scale, multivessel high pressure equipment (HPIU-1000, Resato, The Netherlands), using an oil-glycol mixture (TR15, Greenpoint Oil, The Netherlands) as pressure transmitting fluid. Milk or whey was filled in microcentrifuge tubes (500 $\mu$l, Elkay, UK) and pressure treated for pre-set times. Pressure was built up slowly ($\pm$100 MPa/min) to minimize the temperature increase resulting from adiabatic heating (Kalichevsky *et al.* 1995). Besides, only isobaric-isothermal conditions were taken into account for data analysis by excluding the initial phase of variable pressure and temperature from the experiment. The initial activity ($A_0$, $t = 0$) was defined as the activity of the enzyme sample when entering the time domain where pressure and temperature remain constant in time (Weemaes *et al.* 1997). The use of this 'zero-point' approach is only acceptable for zero and first order reactions. A first set of experiments was performed at constant pressure (700 MPa) in combination with temperatures between 15 and 65 °C. Secondly, pressure inactivation (150–750 MPa) was studied at 73 °C, a temperature where inactivation at atmospheric pressure occurs.

After thermal and/or pressure treatment, the samples were transferred to ice water to stop inactivation and stored there until activity measurement. In a preliminary experiment it was verified that no reactivation of the enzyme took place during storage (2 h). Both thermal and/or pressure inactivation experiments were performed in duplicate.

*Lactoperoxidase activation study*

Since there is no method available to determine the progress of the LPO catalysed reaction *in situ*, a quench method was used to study the effect of temperature and/or pressure on the activity of LPO in acid whey. For thermal activation studies, the reaction was initiated at room temperature by mixing diluted whey solution with 0·1 M-phosphate buffer, pH 6·4, containing 10 mM-ABTS and 10 mM-$H_2O_2$. Subsequently, 1 ml aliquots were pipetted into individual tubes and after 3 min, incubated in a water bath at constant temperature between 20 and 80 °C for pre-set times. At the respective times, the reaction was stopped (quenched) using a heat shock (2 min at 90 °C). As to pressure-temperature activation, the reaction was likewise initiated at room temperature (0·1 MPa) and flexible microtubes were filled with the solution. After 4 min (time to prepare the samples and to enclose them in the pressure vessels), the samples were pressurized and treated for pre-set time periods. Pressures between 0·1 and 300 MPa in combination with temperatures between 20 and 60 °C were selected for this study. Analogous to the inactivation experiments, the initial phase of variable pressure and temperature was excluded from the experimental data set. Immediately following depressurisation of the individual vessels, the reaction was quenched using a heat shock (2 min at 90 °C). In both cases, the samples were transferred to ice water after quenching and stored there until absorbance measurement (412 nm). All experiments were carried out in duplicate.

To define an appropriate experimental set-up for the activation study, some preliminary experiments were carried out. At first it was verified that the measured

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absorbance remained constant during storage on ice for at least 2 h. Besides, it was checked that in absence of milk whey, no increase in absorbance was noted during thermal and/or pressure treatment, indicating the substrate to be stable under all pressure-temperature conditions studied and its conversion to be merely caused by the enzymatic reaction. Secondly, some preliminary experiments were performed with different enzyme and substrate concentrations to set forth the final modus operandi. Concentrations inducing a linear increase in absorbance with time during at least 15 min were chosen in order to estimate accurately the activity from the slope of the regression line. For all experiments discussed below, the following reaction mixtures were used: 50 $\mu$l ABTS (10 mM), 50 $\mu$l $H_2O_2$ (10 mM), 10 $\mu$l diluted whey and 750 $\mu$l phosphate buffer, pH 6·4. In order to account for the variability in absolute LPO activity between different whey samples (about 19 %), results were re-calculated relative to the activity of the same sample at 20 °C and atmospheric pressure.

*Data analysis*

Isothermal and isobaric-isothermal inactivation of LPO could be described by a first order model (1), allowing the inactivation rate constant ($k$) to be determined from a semilogarithmic plot (2) of the activity retention ($A/A_0$) as a function of treatment time ($t$)

$$\frac{dA}{dt} = -kA, \tag{1}$$

$$\ln\left(\frac{A}{A_0}\right) = -kt. \tag{2}$$

Temperature dependence of the inactivation rate constant, expressed as the activation energy ($E_a$), is given by the Arrhenius relationship (3), in which $R$ is the universal gas constant, $T$ the absolute temperature and $T_{ref}$ the absolute reference temperature (343 K). The $E_a$ value can hence be calculated from a semilogarithmic plot of $k$ versus reciprocal temperature.

$$\ln k = \ln k_{ref} - \left(\frac{E_a}{R}\left(\frac{1}{T} - \frac{1}{T_{ref}}\right)\right) \tag{3}$$

On the basis of the experimental data, kinetic parameter values ($k, E_a$) were calculated successively from equations (2) and (3) by an individual two-step linear regression approach (SAS, 1989) from the logarithmic data. This approach was preferred over a global one-step non-linear regression approach (Claeys *et al*. 2001), in which the kinetic parameters are estimated in a global fit of the entire data set using non-linear regression analysis of relative activity retentions according to equation (4). The latter approach does not allow calculation of individual $k$ values in areas where the Arrhenius equation is not applicable (Ludikhuyze, 1998).

$$\ln\left(\frac{A}{A_0}\right) = -k_{ref}\exp\left(\frac{-E_a}{R}\left(\frac{1}{T} - \frac{1}{T_{ref}}\right)\right)t \tag{4}$$

The reaction catalysed by LPO was followed by measuring the absorbance increase due to conversion of ABTS in the presence of $H_2O_2$. The activity of LPO ($\Delta$OD/min) was estimated from the initial linear part of the curves obtained when plotting the absorbance as a function of reaction time under conditions of constant temperature and/or pressure. Linear regressions were again performed using the SAS package (SAS, 1989).

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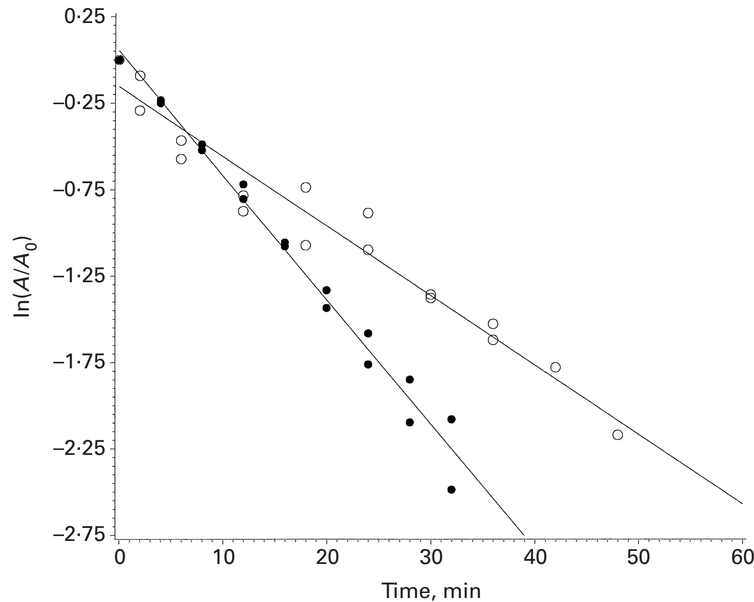


Fig. 1. Residual lactoperoxidase activity ($A/A_0$) as a function of treatment time in raw milk (○) and diluted whey (●) treated at 70 °C. $A$, enzyme activity at time $t$; $A_0$, initial enzyme activity. Duplicate measurements are represented.

Table 1. *Kinetic parameters, reaction rate constant* ($k$) *and activation energy* ($E_a$) *for thermal inactivation of lactoperoxidase in raw bovine milk and acid whey* (*diluted 1/5 in 0·1* M*-phosphate buffer, pH 6·4*)

| | Raw bovine milk* | | Diluted whey | |
|---|---|---|---|---|
| Temperature (°C) | $k$-value, min$^{-1}$ | $r^2$ | $k$-value, min$^{-1}$ | $r^2$ |
| 69 | $(1·22 \pm 0·17) \times 10^{-2}$ | 0·778 | $(2·89 \pm 0·19) \times 10^{-2}$ | 0·934 |
| 70 | $(4·02 \pm 0·22) \times 10^{-2}$ | 0·953 | $(7·20 \pm 0·23) \times 10^{-2}$ | 0·984 |
| 71 | $(5·92 \pm 0·41) \times 10^{-2}$ | 0·921 | $(14·43 \pm 0·93) \times 10^{-2}$ | 0·942 |
| 72 | $(11·58 \pm 0·88) \times 10^{-2}$ | 0·911 | $(34·93 \pm 3·16) \times 10^{-2}$ | 0·938 |
| 73 | $(18·61 \pm 0·93) \times 10^{-2}$ | 0·961 | $(55·49 \pm 4·00) \times 10^{-2}$ | 0·946 |
| | $E_a$: 640·6 ± 72·5 kJ/mol<br>$r^2 = 0·963$ | | $E_a$: 736·9 ± 40·9 kJ/mol<br>$r^2 = 0·991$ | |

* Results on bovine milk are taken from Claeys *et al.* 2001.

## RESULTS

### *Inactivation of lactoperoxidase in bovine milk and diluted whey*

Thermal inactivation (69–73 °C) of LPO in milk (Claeys *et al.* 2001) and diluted acid whey could be accurately described by a first order kinetic model (Fig. 1), as regression coefficients were situated between 0·911 and 0·984 (except for bovine milk at 69 °C) and no systematic deviations from the semi-logarithmic linear behaviour were observed. Our results agreed with several literature reports on inactivation of LPO in milk and/or whey (Hernandez *et al.* 1990; López-Fandiño *et al.* 1996; Barrett *et al.* 1999). Olszewski & Reuter (1992), on the other hand, noted the order of the thermal inactivation reaction of LPO in raw milk to be 1·5.

The kinetic parameters corresponding to the first order model are presented in Table 1. From this table it becomes clear that the thermostability of LPO is higher in raw bovine milk than in diluted whey, the rate constants being about half in the

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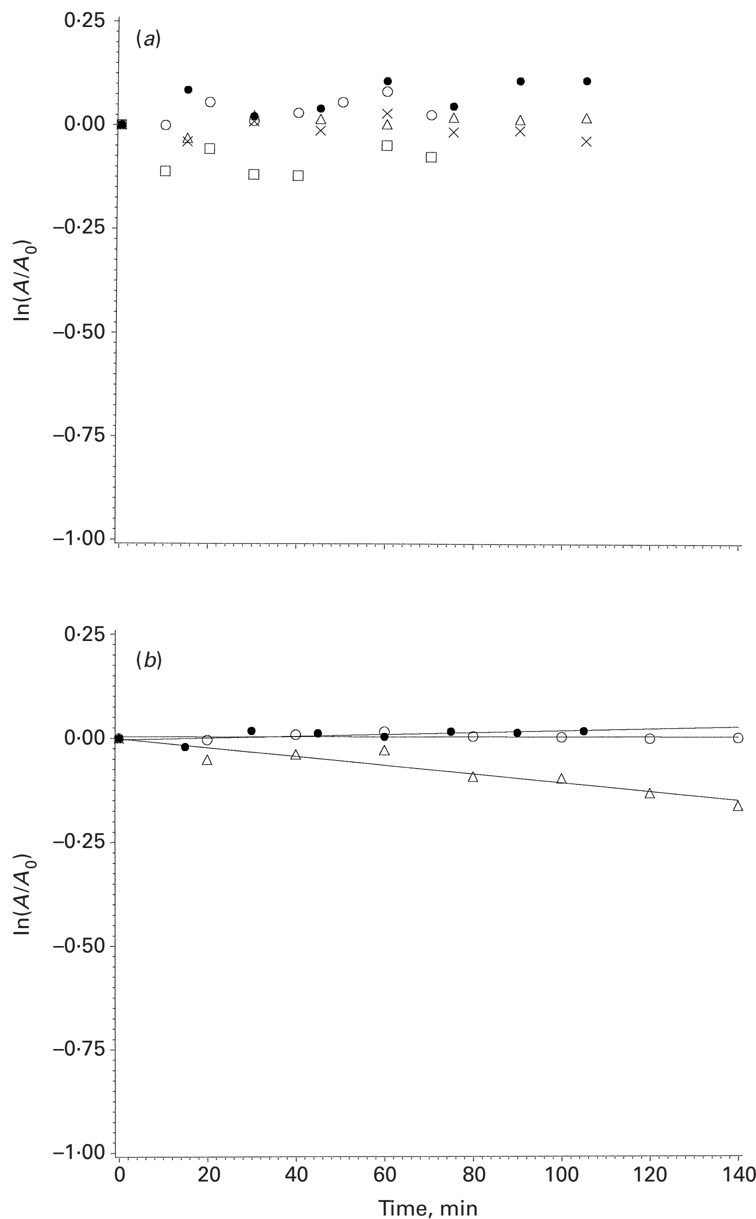


Fig. 2. Residual lactoperoxidase activity ($A/A_0$) as a function of treatment time at 700 MPa in (*a*) raw milk treated at 15 (○), 25 (●), 35 (△), 45 (□), and 65 (×) °C and (*b*) acid whey (1/5 diluted in 0·1 M-phosphate buffer, pH 6·4) treated at 10 (○), 40 (●) and 65 (△) °C. $A$, enzyme activity at time $t$; $A_0$, initial enzyme activity. Values are means of duplicate determinations.

former case. The activation energy for thermal inactivation in diluted whey seemed somewhat higher when compared to milk, but no significant differences were noted on a 95% confidence level.

As to pressure treatment at 700 MPa, no substantial inactivation of LPO in milk was observed, even after treatment times of 140 min at temperatures as high as 65 °C (Fig. 2*a*). In whey, minor inactivation of LPO was induced by a treatment at 65 °C

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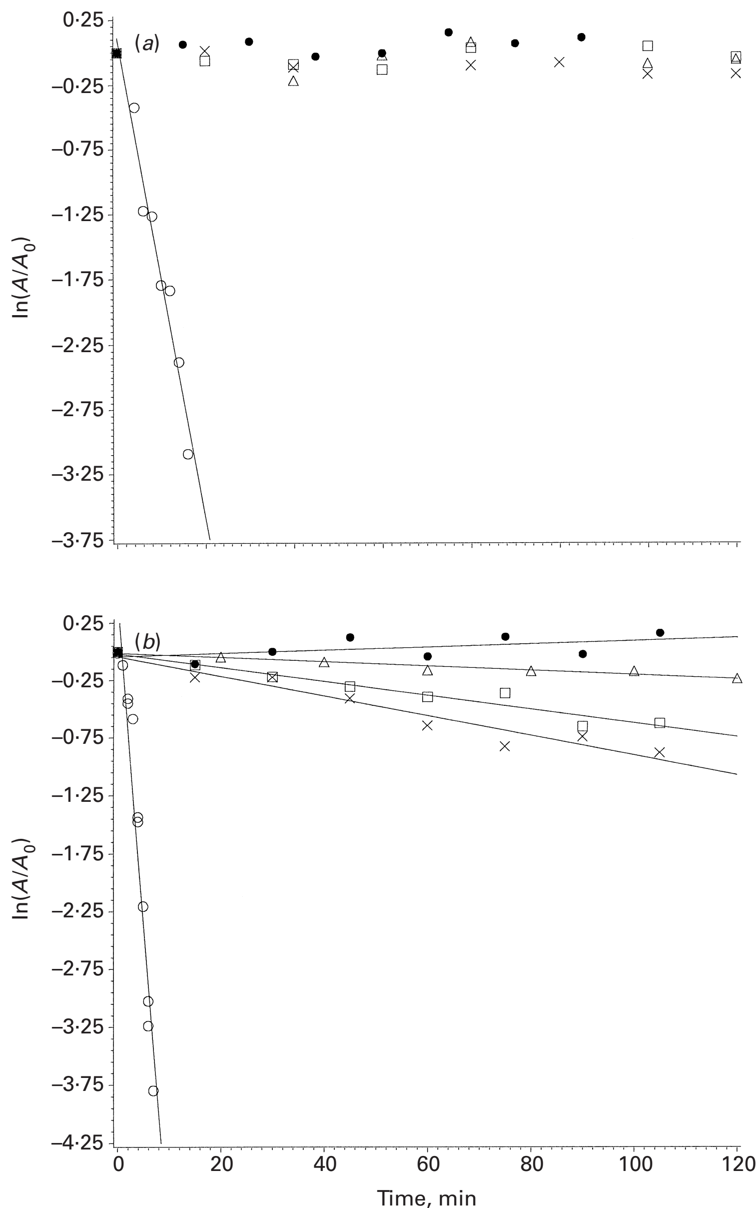


Fig. 3. Residual lactoperoxidase activity ($A/A_0$) as a function of treatment time at 73 °C in (*a*) raw milk treated at 0·1 (○), 150 (●), 400 (△), 700 (□), and 750 (×) MPa and (*b*) acid whey (1/5 diluted in 0·1 M-phosphate buffer, pH 6·4) treated at 0·1 (○), 150 (●), 400 (△), 700 (□), and 750 (×) MPa. $A$, enzyme activity at time $t$; $A_0$, initial enzyme activity. Values are means of duplicate determinations.

and 700 MPa for 140 min (Fig. 2*b*). In this case a first order rate constant of $(1{\cdot}07 \pm 0{\cdot}15) \times 10^{-3}$ min$^{-1}$ was calculated. Similar to thermal stability, pressure stability of LPO was higher in milk than whey.

At 73 °C, a temperature where inactivation at atmospheric pressure proceeds very rapidly, treatment at pressures between 150 and 700 MPa completely inhibited inactivation of LPO in milk, i.e. a very pronounced antagonistic effect of pressure and high temperature was observed (Fig. 3*a*). A similar antagonistic effect was noted

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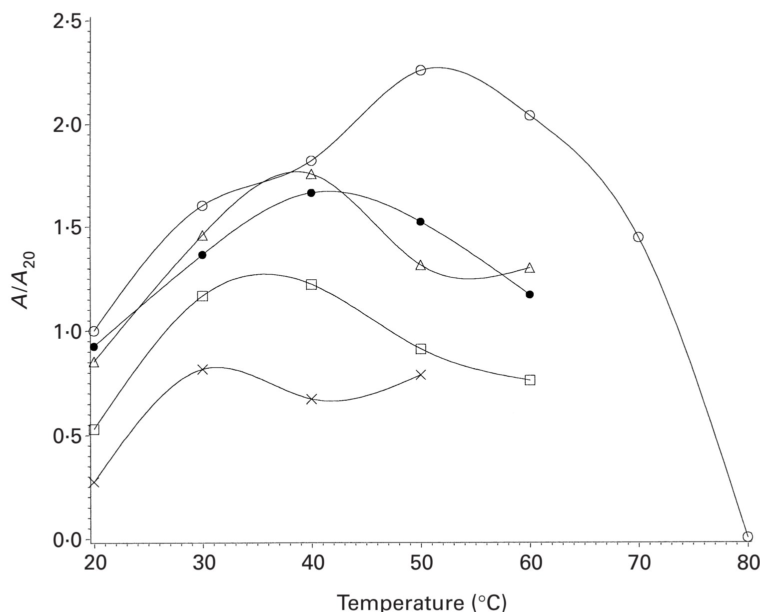


Fig. 4. Relative lactoperoxidase activity ($A/A_{20}$) in acid whey (diluted 1/5 in 0·1 M-phosphate buffer, pH 6·4) as a function of temperature at atmospheric and elevated pressure: 0·1 (○), 50 (●), 100 (△), 200 (□), and 300 (×) MPa. $A_{20}$, enzyme activity at 20 °C and atmospheric pressure; $A$, enzyme activity at the preset pressure-temperature condition. Values are means of duplicate determinations.

Table 2. *First order rate constants for (in)activation of lactoperoxidase in acid whey (diluted 1/5 in 0·1* M*-phosphate buffer, pH 6·4). at 73 °C and different pressures*

| Pressure, MPa | $k$-value, min$^{-1}$ | $r^2$ |
|---|---|---|
| 0·1 | $(55{\cdot}49 \pm 4{\cdot}00) \times 10^{-2}$ | 0·961 |
| 150 | $(-0{\cdot}29 \pm 0{\cdot}05) \times 10^{-2}$ | 0·860 |
| 400 | $(0{\cdot}18 \pm 0{\cdot}02) \times 10^{-2}$ | 0·938 |
| 700 | $(0{\cdot}59 \pm 0{\cdot}06) \times 10^{-2}$ | 0·939 |
| 750 | $(0{\cdot}85 \pm 0{\cdot}09) \times 10^{-2}$ | 0·934 |

in whey. However, in this case, pressures equal to or higher than 700 MPa again induced inactivation (Fig 4*b*), but the inactivation rate constants remained much smaller than at atmospheric pressure (Table 2). In whey, even a small increase in activity was noted after pressure treatment at 150 MPa, i.e. a negative $k$ value was found (Table 2).

*Activation of LPO in diluted whey*

From Fig. 4 it can be seen that LPO activity increased with increasing temperature (at 0·1 MPa) up to about 50 °C, and then decreased with further increase in temperature, becoming nearly zero at temperatures higher than 70 °C. These findings correspond with earlier results on inactivation of LPO in whey in absence of its substrate, where substantial inactivation occurred at temperatures higher than 70 °C. This indicates that the substrate has no protective effect against enzyme inactivation. The statistical accuracy of activity determination was higher at lower temperatures, i.e. the standard error on the activity value increased from about 9% at ambient temperature to about 24% at 70 °C.

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Table 3. *Lactoperoxidase* (*LPO*) *activity* (*values relative to the activity at atmospheric pressure and 20 °C*) *in acid whey* (*diluted 1/5 in 0·1* M*-phosphate buffer, pH 6·4*) *at different pressure-temperature conditions*

| | Relative LPO activity at selected temperature-pressure conditions | | | | |
|---|---|---|---|---|---|
| Pressure, Mpa | 20 °C | 30 °C | 40 °C | 50 °C | 60 °C |
| 0·1 | 1·00 ± 0·099 | 1·604 ± 0·178 | 1·822 ± 0·193 | 2·261 ± 0·287 | 2·043 ± 0·302 |
| 50 | 0·926 ± 0·153 | 1·366 ± 0·175 | 1·666 ± 0·145 | 1·525 ± 0·329 | 1·173 ± 0·207 |
| 100 | 0·853 ± 0·106 | 1·461 ± 0·167 | 1·758 ± 0·236 | 1·316 ± 0·136 | 1·303 ± 0·253 |
| 200 | 0·529 ± 0·088 | 1·168 ± 0·117 | 1·223 ± 0·258 | 0·913 ± 0·183 | 0·762 ± 0·111 |
| 300 | 0·275 ± 0·065 | 0·817 ± 0·263 | 0·673 ± 0·151 | 0·789 ± 0·136 | - |

Because of the extreme pressure stability of LPO in milk and diluted whey, it seemed worthwhile to investigate whether the enzyme could likewise retain its catalytic activity under pressure. The activity values relative to the activity at 20 °C and atmospheric pressure, together with the standard errors are summarized in Table 3. Regression coefficients of activity determination were situated between 0·870 and 0·990. From Table 3, it becomes clear that LPO remains active at pressures up to 300 MPa although at pressures higher than 100 MPa, the activity was significantly reduced. Analogously to atmospheric pressure, the activity at elevated pressure is dependent on temperature (Fig. 4). The optimal temperature at elevated pressure is shifted to somewhat lower values (30–40 °C) as compared to atmospheric pressure (50 °C).

## DISCUSSION

The most remarkable observation was the pronounced antagonistic effect between pressure and high temperature, i.e. application of pressure up to 700 MPa at 73 °C, a temperature where inactivation at atmospheric pressure occurs rapidly, completely inhibits LPO inactivation. The fact that pressure greatly retards the rate of inactivation during the early stage of thermal treatment indicates that a considerable increase in volume of the molecules takes place in going from the native to the activated state (Johnson & Campbell, 1945). A first explanation in molecular terms is that stability of native proteins is achieved by matching the volume of the residues to the volume of an incompressible cage formed by a covalently linked backbone. At high temperature the change in standard entropy is positive in accordance to the high flexibility of the unfolded protein (Weber & Drickamer, 1983). A second explanation refers to the concept of microscopical ordening stating that pressure increase intensifies the degree of organisation of the protein molecule. At high temperature, disordering of the protein molecule or unfolding of the polypeptide chain occurs due to breaking of bonds by thermal vibrations. Such disorderliness is counteracted by pressure (Suzuki, 1960; Heremans, 1982). At the level of the interactions within the protein, pressure stabilization of proteins/enzymes against thermal denaturation/inactivation arises from the counteracting effects of pressure and temperature on the formation/disruption of intramolecular interactions and/or interactions with the solvent. Hydration of protein functional groups is enhanced by pressure but loosened at high temperature. Hydrophobic interactions are strengthened by temperature up to 70 °C, but greatly weakened by pressure as a consequence of exposure of non-polar amino acid residues and reorganisation of the loosely packed water structure. Where hydrogen bonds are largely destabilised at

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high temperature, minor stabilization by pressure is observed (Gross & Jaenicke, 1994; Damodaran, 1996; Mozhaev *et al*. 1996).

Antagonistic effects of temperature and pressure have been frequently encountered for protein denaturation/enzyme inactivation (Johnson & Campbell, 1945; Suzuki, 1960; Suzuki & Kitamura, 1963; Zipp & Kauzmann, 1973; Balny & Masson, 1993; Heremans, 1993; Weemaes *et al*. 1998; Ludikhuyze *et al*. 2000; Van den Broeck *et al*. 2000*a*, *b*), although in most cases retardation rather than complete inhibition of thermal inactivation was observed. In case of LPO in milk and whey, even a small increase in activity due to pressure treatment was observed. This has not been reported for other milk enzymes, but various reports on this aspect have been published for fruit and vegetable enzymes (Butz *et al*. 1994; Jolibert *et al*. 1994; Anese *et al*. 1995; Cano *et al*. 1997; Van den Broeck *et al*. 2000*b*). Since all these reports concern cell free extracts, cell disruption and decompartimentation cannot be at the base of activity enhancement. Other explanations for enzyme activation by pressure that have been put forward, include (i) limited conformational changes, (ii) release of active enzyme from an enzyme-inhibitor complex, and (iii) liberation of a second active site by limited proteolysis (Butz *et al*. 1994). The observed antagonistic effect and the enhancement of LPO activity at high temperature by low pressure (50–100 MPa) might have potential in exploiting the antimicrobial potency of milk. At temperatures higher than 70 °C, where activity of LPO is strongly reduced, application of relatively low pressure might lead to preservation of LPO activity and hence antimicrobial potency. However, not only the effect of pressure on enzyme activity, but also on the enzymic conversion reaction is important, as the antimicrobial activity is linked to the products formed in this reaction. In this context it was observed that the catalytic reaction of LPO in whey can be enhanced by a controlled pressure and/or temperature treatment. The higher temperature (70 °C) reported by Kussendrager (1993) for maximal activity of LPO in milk between pH 5·0 and 6·5 might be explained by the higher thermostability of LPO in milk compared with whey. Also Barrett *et al*. (1999) observed the LPO system to retain its antimicrobial activity upon milk pasteurisation, provided the time-temperature combination was not too excessive.

At room temperature, LPO displayed very high pressure resistance, both in milk and whey. Similar results were obtained by Rademacher *et al*. (1998*b*), who reported residual LPO activities higher than 50% after treatment for 4 h at 800 MPa and 25–60 °C. They attributed the high pressure stability of LPO to its monomeric structure, which is stabilized by eight disulphide bonds. Sionneau *et al*. (1997) reported that LPO in milk is not significantly affected by continuous pressure treatment at 380 MPa for 5 min. Seyderhelm *et al*. (1996) found pressure treatment of raw bovine milk at 800 MPa and 25–40 °C for 30 min to result in about 20% reduction of LPO activity. In Tris buffer at pH 7, on the other hand, about 70% activity loss was found under similar processing conditions. For many other milk enzymes, much lower pressure stability has been reported. Inactivation of alkaline phosphatase, $\gamma$-glutamyl-transferase and phosphohexose-isomerase in milk at 20 °C could be achieved by treatment for 8 min in the pressure range 400–750 MPa, 400–600 and 300–500 MPa respectively (Rademacher *et al*. 1998*a*, 1999; Ludikhuyze *et al*. 2000).

Opposed to the extreme pressure resistance, a low thermostability was observed for LPO, which was even more pronounced in acid whey than in whole milk. This difference might be attributed to the presence of some protective factors which are lost upon preparation of the whey. Opposite results were found by Hernandez *et al*.

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(1990) for thermostability of LPO in sweet whey. In this case, preparation of whey was accomplished by addition of $CaCl_2$ before renneting, which could be at the base of the observed increased thermostability. Indeed, mono- and divalent cations have been found to protect LPO against thermal inactivation (Sciancalepore *et al*. 1996). In both cases, a very high temperature sensitivity was found as indicated by the high values for the activation energy. In agreement with our results, Hernandez *et al*. (1990) reported an activation energy value for LPO inactivation in buffer of 602 kJ/mol, but higher values for whey (1030 kJ/mol) and raw milk (800 kJ/mol). Also Olszewski & Reuter (1992) found a similar activation energy for inactivation of LPO in raw milk ($E_a = 634$ kJ/mol), although in this case the order of the inactivation reaction was stated to be 1·5. Barrett *et al*. (1999) calculated a somewhat lower activation energy ($\pm 500$ kJ/mol).

Where LPO in milk and diluted acid whey can readily be inactivated at temperatures above 69 °C, it was extremely pressure resistant and withstood treatments at 700 MPa combined with temperatures between 20 and 60 °C. Application of pressure at 73 °C even protected LPO against thermal inactivation. However, although LPO itself was not affected by pressure up to 300 MPa in combination with temperature up to 60 °C, its catalytic activity in presence of the substrate was lower under pressure compared with atmospheric pressure. Nevertheless, the obtained results may indicate possible exploitation of the natural antimicrobial properties of the LPO system, at least when combining pressures up to 300 MPa with temperatures of about 40–50 °C.

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