Inactivation kinetics of alkaline phosphatase and lactoperoxidase, and denaturation kinetics of β-lactoglobulin in raw milk under isothermal and dynamic temperature conditions

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Summary. A detailed kinetic study of alkaline phosphatase, lactoperoxidase and β-lactoglobulin was carried out in the context of identifying intrinsic time–temperature indicators for controlling the heat processing of milk. The heat inactivation or denaturation of alkaline phosphatase, lactoperoxidase and β-lactoglobulin under isothermal conditions was found to follow first order kinetics. Experimental results were analysed using both a two step linear regression and a one step non-linear regression method. Results obtained using the two statistical techniques were comparable, but the 95% confidence interval for the predicted values was smaller when the one step non-linear regression method was used, indicating its superiority for estimating kinetic parameters. Thermal inactivation of alkaline phosphatase and lactoperoxidase was characterized by z values of 5·3 deg C ($D_{60\,^{\circ}C} = 24{\cdot}6$ min) and 4·3 deg C ($D_{71\,^{\circ}C} = 38{\cdot}6$ min) respectively. For the denaturation of β-lactoglobulin we found z values of 7·9 deg C ($D_{75\,^{\circ}C} = 49{\cdot}9$ min) in the temperature range 70–80 °C and 24·2 deg C ($D_{85\,^{\circ}C} = 3{\cdot}53$ min) in the range 83–95 °C. D_{ref} and z were evaluated under dynamic temperature conditions. To estimate the statistical accuracy of the parameters, 90% joint confidence regions were constructed.

Keywords: Milk, alkaline phosphatase, lactoperoxidase, β-lactoglobulin, kinetics, isothermal, non-isothermal, joint confidence region.

The heat treatment most widely applied to milk products is pasteurization (71·7 °C for 15 s or 62·7 °C for 30 min). Properly applied pasteurization results in the almost complete destruction of any pathogenic bacteria initially present in raw milk. Quantitative measurement of the impact of a thermal process in terms of food safety is of great importance in process design, evaluation, optimization and control. One way to study the process impact is by using intrinsic time–temperature indicators (TTI). These are heat-sensitive components present or formed irreversibly in the product during heat processing, which are time- and temperature-dependent, easily measured and possible to correlate with the changes of a target attribute of a food (a safety or a quality characteristic) undergoing the same treatment. In addition to practical and thermophysical requirements, some kinetic requirements are necessary.

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Firstly, the temperature sensitivity of the TTI and the target attribute should be the same and, secondly, the reaction rate of the TTI must be sufficiently small to guarantee a measurable response after heating (Taoukis & Labuza, 1989; De Cordt *et al.* 1992; Van Loey *et al.* 1996). Consequently, extensive kinetic studies are required to evaluate a TTI. Kinetics describes the way a reaction proceeds (inactivation, denaturation, production) as a function of time. *How* the reaction proceeds (linear, log–linear and so on) is expressed by the mathematical form of the kinetic model. The *rate* of the reaction is represented by kinetic parameters, which are obtained by fitting a model to experimental information. Thus, accurate prediction of the thermal behaviour of a TTI depends on the development of an exact kinetic model. Prediction errors can occur because the model is not of the correct functional form, because factors not considered by the model influence the kinetics, or because the model parameters are not estimated precisely. The first two reasons require increased accuracy and additional experimental work. The final reason demands more efficient re-analysis of data.

In this context, we have undertaken a detailed examination of the kinetic behaviour upon heating of three intrinsic milk components of technological significance in the dairy industry: alkaline phosphatase (ALP, EC 3.1.3.1), lactoperoxidase (Lpo, EC 1.11.1.7) and β-lactoglobulin (β-lg). ALP is widely used to establish whether milk pasteurization is adequate: ALP activity remaining after pasteurization indicates improperly operating pasteurization units or possible contamination by raw milk (Murthy *et al.* 1990; Painter & Bradley, 1997). Lpo is present at relatively high concentrations in raw milk (0·03 g/l) and catalyses the oxidation of thiocyanate by hydrogen peroxide, resulting in an antimicrobiological effect (Hernández *et al.* 1990; Muir, 1996). Lpo has been suggested as a possible indictor for monitoring thermal processes at > 72 °C and could be used to distinguish pasteurized and high-temperature treated milk (Guha & Roy, 1973; Griffiths, 1986; Hernández *et al.* 1990). β-Lactoglobulin is the most abundant whey protein (on average, milk contains ~ 3 g β-lg/l) and is important as it can have a marked influence on the functional properties of milk products. Moreover it appears that β-lg plays an important role in fouling heat exchangers (Jeurnink, 1991; De Jong *et al.* 1992). Heating milk above 65 °C induces denaturation of β-lg and its complex association with casein micelles. The quantitative determination of undenatured β-lg has been proposed for distinguishing between different categories of heat-treated milk. A minimum content of 2600 mg undenatured β-lg/l pasteurized milk is the limit proposed by the International Dairy Federation (Schlimme *et al.* 1996).

Although these three milk components have already been extensively examined, detailed quantitative kinetic studies are lacking. In the present study, the rates of inactivation–denaturation of ALP, Lpo and β-lg together with their temperature dependence were estimated by isothermal experiments and evaluated under non-isothermal or dynamic temperature conditions. We have considered two different statistical approaches: the individual two step and the global one step. The precision and correlation of the estimated kinetic parameters were examined by constructing individual 95 % confidence intervals and 90 % joint confidence regions.

MATERIALS AND METHODS

Milk

A batch of fresh raw bovine milk was purchased from a local dairy farm, divided into small portions (30–50 ml) and stored frozen at -18 °C.

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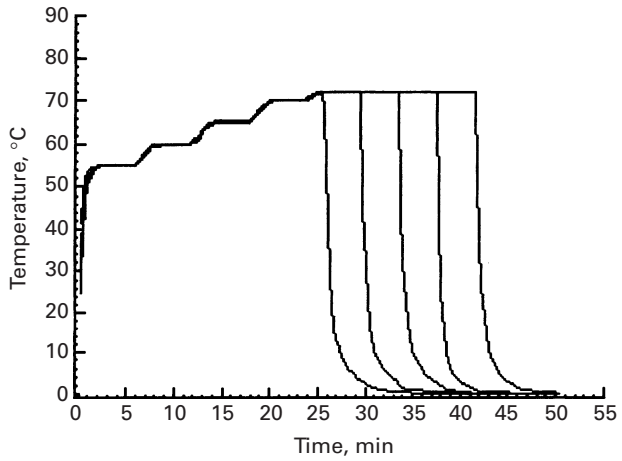

Fig. 1. Example of a time–variable temperature profile used in the non-isothermal experiments in this study (results for five milk samples).

Isothermal treatment

Samples of milk were heated in closed screw-capped vials (12 × 32 mm) immersed for pre-set time intervals in a thermostatted water bath at constant temperature. The samples were then immediately cooled in ice water to stop thermal inactivation or denaturation. After storage on ice for < 2 h, the residual activity or concentration of the indicator was measured. In preliminary experiments, we verified that there was no reactivation of ALP and Lpo and that denaturation of β-lg was irreversible within a period of 2 h.

Thermal inactivation kinetics of ALP were studied at temperatures between 54 and 64 °C and of Lpo between 69 and 73 °C. Denaturation kinetics of β-lg were examined at temperatures between 70 and 95 °C.

Non-isothermal treatment

Samples of milk were subjected to a time–variable temperature profile; an example of such a profile is given in Fig. 1. Similar profiles were used in each assay, with temperatures ranging up to 62 °C for ALP, up to 73 °C for Lpo and up to 80 °C for β-lg. Time–temperature values were recorded at regular time intervals (2, 5 and 20 s) using thermocouples connected to a data-logger (TM 9616; Ellab, DK-2610 Roedovre, Denmark). At different pre-set times the samples were taken out of the water bath and put into ice water. Finally the activity or concentration of the milk component was analysed.

Analytical methods

The activity of ALP was determined spectrophotometrically at 425 nm (Pharmacia LKB-Biotechnology, S-751 82 Uppsala, Sweden) using *p*-nitrophenyl phosphate as substrate and following the procedure of International Dairy Federation (1987).

Lpo activity was determined using a slight modification of the procedure of Hernández *et al.* (1990). Milk proteins were precipitated by adding 1·75 M-acetic acid and 1 M-sodium acetate and Lpo activity was measured spectrophotometrically at 412 nm (Pharmacia) with 2,2′-azinobis(3-ethylbenzothiazoline)-6-sulphonic acid and H_2O_2 as substrate.

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The acid-soluble β-lg content was quantified by HPLC according to International Dairy Federation (1996) with the following operating conditions. A reversed-phase PLRPS column ($10 \times 4{\cdot}6$ mm, 8 μm particle size, 300 Å pore size, Alltech, Deerfield, IL 60015, USA) was placed in a thermostatted oven at 40 °C. Solvent A was trifluoroacetic acid solution in water (1 ml/l) and solvent B trifluoroacetic acid solution in acetonitrile (1 ml/l). Samples of 20 μl were injected and β-lg was eluted with a linear gradient from 340 to 450 ml B/l at a flow rate of 1 ml/min.

data analysis

Kinetic parameter estimation: isothermal experiments

A common method of estimating kinetic parameters is based on isothermal experiments at different constant temperatures in order to facilitate analysis of the kinetic results. The inactivation rate of enzymes and the denaturation rate of proteins can often be described by first order kinetics (Ahern & Klibanov, 1985; Hernández *et al.* 1990),

$$\frac{\mathrm{d}X}{\mathrm{d}t} = -kX, \tag{1}$$

where X is the response value at time t, and k the reaction rate constant.

There are two concepts used to quantify the effect of temperature on the reaction rate. The first is the Arrhenius model (Arrhenius, 1889), used particularly for chemical reactions in which the temperature dependence of the rate constant k is expressed by the activation energy E_a(J/mol) according to eqn (2),

$$k = k_{\mathrm{ref}} \exp\left(\frac{E_a}{R}\left(\frac{1}{T_{\mathrm{ref}}} - \frac{1}{T}\right)\right), \tag{2}$$

in which k_{ref} is the reaction rate constant at reference temperature T_{ref}. The second is the thermal death time model (Bigelow, 1921), which describes first order heat inactivation kinetics in the area of food processing and preservation. In this model the decimal reduction time D is defined as the time required to reduce the initial response value X_0 by 90 % at a constant temperature. The relation between D and k is given by the equation

$$D = \frac{2{\cdot}303}{k}. \tag{3}$$

Substituting k (from eqn 3), and since isothermal heating assumes constant extrinsic/intrinsic factors and thus D to be constant over time, integration of eqn (1) leads to the expression

$$\log\left(\frac{X}{X_0}\right) = -\frac{t}{D}. \tag{4}$$

The temperature dependence of D is characterized by z, which is the temperature increase necessary to decrease D by 90 % to one-tenth of its original value,

$$D = D_{\mathrm{ref}}\, 10^{\left(\frac{T_{\mathrm{ref}} - T}{z}\right)}, \tag{5}$$

where D_{ref} is the decimal reduction time at reference temperature T_{ref}.

From eqns (4) and (5), the kinetic parameters D and z can be calculated by an individual linear regression approach (two step method) from the logarithmic values.

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Since some claim that a global fit is superior (Cohen & Saguy, 1985; Haralampu *et al*. 1985), the kinetic parameters were also estimated in a global fit using non-linear regression analysis of relative residual response values (one step method) according to

$$\log\left(\frac{X}{X_0}\right) = -\left(\frac{t}{D_{\text{ref}}}\right)10^{\left[\frac{T-T_{\text{ref}}}{z}\right]}. \tag{6}$$

To verify the validity of the first order kinetic model and to measure the linearity, coefficients of determination (R^2) were calculated and residual plots checked for the absence of trends or correlations. (If the model is appropriate for the data, the residuals represent only the experimental error and are randomly distributed when plotted.)

Kinetic parameter estimation: non-isothermal experiments

The kinetic parameters estimated isothermally were also evaluated under variable or dynamic temperature conditions. Because in these experiments the temperature varied with time, the assumption of a constant value for D no longer applies. Thus, by integrating and replacing the D value with its temperature dependence (eqn 5), eqn (1) becomes

$$\log\left(\frac{X}{X_0}\right) = -\int_0^t \frac{\mathrm{d}t}{D_{\text{ref}}10^{\left(\frac{T_{\text{ref}}-T}{z}\right)}}. \tag{7}$$

When time–temperature profiles are registered, the kinetic parameters D_{ref} and z can be calculated according to eqn (7) using a numerical integration routine, for example Simpson (Carnahan *et al*. 1969).

Parameter confidence intervals: joint confidence regions

The statistical method most commonly used to measure the precision of an estimate is the use of individual confidence intervals. Confidence intervals are exactly defined and symmetric, and their determination is straightforward. However, because of the possible high correlation between the model parameters, these intervals are only appropriate for specifying ranges of an individual parameter irrespective of the other parameters. The reported error values are based on linearizing assumptions, and will always underestimate the true uncertainty of the parameters (Motulsky & Ransnas, 1987). Alternative techniques, taking into account the possible correlation between simultaneously estimated parameters, are the Monte Carlo technique and joint confidence regions (Bard, 1974; Johnson, 1992). In the present study, $100(1-\varphi)$ joint confidence regions were constructed using the expression (Draper & Smith, 1981)

$$SSQ \leqslant SSQ(\vartheta)\left\{1+\frac{p}{n-p}F(p, n-p, 1-\varphi)\right\} \tag{8}$$

where SSQ represents the error sum of squares at a specific parameter combination, $SSQ(\vartheta)$ the error sum of squares associated with the least squares estimate ϑ at optimal parameter values, p the number of parameters estimated simultaneously, n the number of observations and F the classic F distribution with $(1-\varphi)$ the upper quartile. The validity of this equation is based on two assumptions: independent observations and consequently $(n-p)$ degrees of freedom, and a linear fitting function.

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All statistical procedures were carried out using the SAS software package, version 6.12 (SAS Institute, Cary, NC 27513, USA).

Results and discussion

Thermal inactivation of ALP and Lpo, and denaturation of β-lg could accurately be described by a first order model, as indicated by the linear relationships obtained by plotting the retention values as a function of treatment time on a semi-logarithmic scale (Fig. 2) and confirmed by the R^2 and residual plots. The kinetic parameters, together with their SE, are summarized in Tables 1, 2 and 3. It can be seen that D decreased with increasing temperature, indicating a more rapid inactivation or denaturation at higher temperatures. The corresponding z values, determined from the slope of the semi-logarithmic plot of D as a function of temperature (Fig. 3), increased from Lpo to ALP to β-lg, indicating the distinction in temperature sensitivity with Lpo being the most sensitive. Notable in Fig. 3 is the discontinuity in the curve representing the temperature sensitivity of β-lg, which is possibly due to the complexity of the irreversible thermal denaturation process of β-lg, which involves a number of successive reaction steps. As first reported by Lyster (1970) and later by Dannenberg & Kessler (1988), thermal denaturation of β-lg showed a marked change in temperature dependence at $\sim$ 83 °C, resulting in a clear distinction of the z values in the two temperature ranges studied.

Comparing the two step linear and the global one step non-linear estimated D and z values, it can be seen that although they were very similar, the parameter estimation procedure had an effect on the estimates, both in accuracy and precision. The SE for the parameters estimated by linear regression were higher than those estimated by non-linear regression (Tables 1, 2 and 3). For performing successive linear least squares fits of the data, the errors of the first regression (log (X/X_0) *v.* t to obtain D) will influence the exactness of the second regression (log (D) *v.* T to obtain z), since while using a global non-linear regression approach the data set is considered as a whole. As a consequence the number of degrees of freedom increases, making the confidence intervals for D_{ref} and z smaller (Cohen & Saguy, 1985; Haralampu *et al.* 1985).

To be useful as an indicator of thermal treatment, the estimated kinetic parameters were evaluated under more realistic, varying temperature conditions. The kinetic parameters re-estimated on the basis of non-isothermal data are reported and compared with those calculated by non-linear regression from the isothermal data in Tables 1, 2 and 3. Using kinetic parameter estimates from isothermal and non-isothermal data, the retention values were calculated by integration of the recorded time–temperature profile and compared with the experimental values in Fig. 4 (*a–c*). A straight line with slope 1 represents the ideal situation, in which the correlation between calculated and experimentally determined values is unity.

From Table 1 it can be seen from the individual 95% confidence intervals that there were no significant differences between the kinetic parameters for isothermal and non-isothermal inactivation of ALP. This is also discernible in Fig. 4 (*a*), in which the experimental activity retention values after non-isothermal treatment are compared with those calculated using eqn (7) by means of isothermal or non-isothermal data. In both cases there was a good correlation between experimental and calculated values (R^2 0·984 and 0·983 respectively).

Although ALP is widely applied as an indicator of efficient pasteurization, only a few detailed quantitative kinetic studies on thermal inactivation of ALP in raw

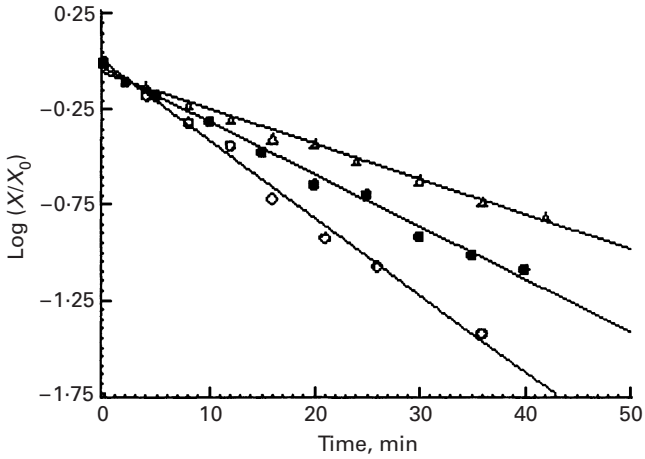

Fig. 2. First order thermal inactivation of ○, alkaline phosphatase at 60 °C and ●, lactoperoxidase at 71 °C, and △, first order thermal denaturation of β-lactoglobulin at 75 °C in raw milk. X, response value at time t, X_0, initial response value. For details, see Data Analysis section.

Table 1. *Kinetic parameters for the first order thermal inactivation of alkaline phosphatase in raw milk under isothermal and non-isothermal conditions*

Temperature, °C	Isothermal		Non-isothermal
	D, min	D_{ref}, min	D_{ref}, min
54	304·04 ± 24·85		
56	129·62 ± 5·65		
58	59·69 ± 2·89		
60	24·62 ± 0·90	24·62 ± 0·54	24·72 ± 0·90
62	9·65 ± 0·82		
64	4·32 ± 0·67		
z, deg C	5·38 ± 0·06	5·30 ± 0·10	4·80 ± 0·24

D, decimal reduction time: the time required to reduce the initial response value by 90 % at a constant temperature; D_{ref}, decimal reduction time at a reference temperature; z, the temperature increase necessary to decrease D by 90 %. For details, see Data Analysis section.

Table 2. *Kinetic parameters for first order thermal inactivation of lactoperoxidase in raw milk under isothermal and non-isothermal conditions*

Temperature, °C	Isothermal		Non-isothermal
	D, min	D_{ref}, min	D_{ref}, min
69	188·25 ± 26·09		
70	57·21 ± 3·17		
71	38·92 ± 2·69	38·63 ± 1·05	49·69 ± 3·28
72	19·88 ± 1·51		
73	12·38 ± 0·62		
z, deg C	3·74 ± 0·31	4·25 ± 0·16	3·32 ± 0·33

D, decimal reduction time: the time required to reduce the initial response value by 90 % at a constant temperature; D_{ref}, decimal reduction time at a reference temperature; z, the temperature increase necessary to decrease D by 90 %. For details, see Data Analysis section.

milk have been reported. Murthy *et al.* (1990) found a value of 9·4 s for D_{ref} at a reference temperature of 71·7 °C and a value of 5·39 deg C for z. They compared their results with three other studies in which the $D_{71·7\,°C}$ ranged between 6·9 s and 9·4 s and the z was ~ 5 deg C. Results reported by Eckner (1992) correspond to a $D_{71·7\,°C}$

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Table 3. *Kinetic parameters for first order thermal denaturation of β-lactoglobulin in raw milk under isothermal and non-isothermal conditions*

Temperature, °C	Isothermal		Non-isothermal
	D, min	$D_{ref,}$ min	$D_{ref,}$ min
70·0	215·01 ± 20·68		
72·5	104·37 ± 7·23		
75·0	63·76 ± 6·54	49·86 ± 1·55	49·31 ± 1·84
77·5	27·67 ± 2·36		
80·0	11·10 ± 0·82		
83·0	6·62 ± 0·55		
85·0	4·56 ± 0·29		
90·0	2·97 ± 0·22	3·53 ± 0·08	
95·0	1·79 ± 0·12		
z_1, deg C	7·94 ± 0·56	7·89 ± 0·22	13·61 ± 1·19
z_2, deg C	21·99 ± 1·85	24·24 ± 1·16	

D, decimal reduction time: the time required to reduce the initial response value by 90 % at a constant temperature; D_{ref}, decimal reduction time at a reference temperature; z_1, z_2, *the temperature increase necessary to decrease* D by 90 % in the temperature ranges 70–80 and 83–95 °C. For details, see Data Analysis section.

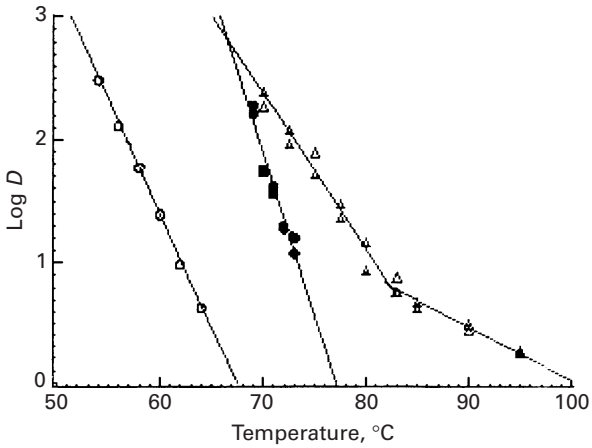

Fig. 3. Thermal sensitivity of ○, alkaline phosphatase ●, lactoperoxidase and △, β-lactoglobulin in raw milk. D, decimal reduction time: the time required to reduce the initial response value by 90 % at a constant temperature. Note the discontinuity in the β-lg curve, possibly reflecting the complexity of the denaturation process.

of 18·2 s and a z of 6·7 deg C. Generally, our results are of the same order of magnitude: a $D_{71·7\,°C}$ of ~ 8·7 s and a z of 5 deg C (assuming that the observed first order inactivation kinetics are still valid outside the temperature region studied).

For Lpo, conclusions similar to those for ALP could be drawn (Table 2, Fig. 4*b*). No significant differences were observed between the z values obtained from isothermal and dynamic experiments and the correlation between the experimentally determined activity retentions and those calculated by means of isothermal and non-isothermal kinetic parameters was sufficiently high (R^2 0·919 and 0·925 respectively). However, from the 95 % confidence intervals the values of D were significantly different. This can be explained by the low z for the inactivation of Lpo, rendering the estimation of D sensitive to even small deviations in temperature.

Previous reports on Lpo largely concern single observations of its activity after thermal treatment or its role in the lactoperoxidase anti-microbiological system (Wolfson & Sumner, 1993; Ravanis & Lewis, 1995). The findings of Olszewski &

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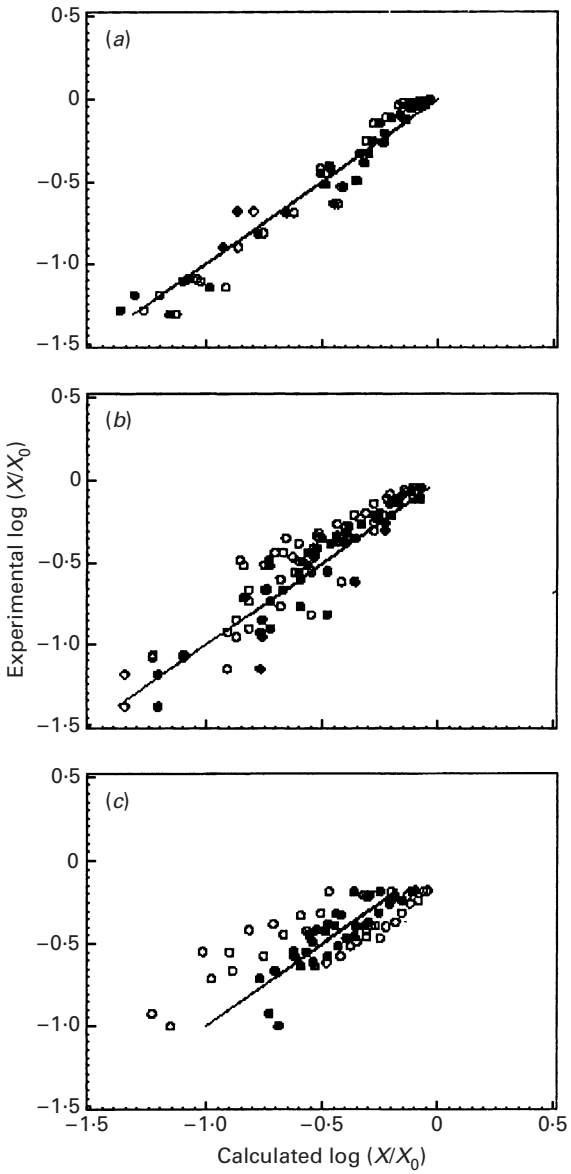

Fig. 4. Correlation between experimentally determined retention values after non-isothermal treatment and those calculated by means of ○, isothermal and ●, non-isothermal kinetic parameter estimates for (*a*) alkaline phosphatase, (*b*) lactoperoxidase and (*c*) β-lactoglobulin. X, response value at time t, X_0, initial response value. For details, see Data Analysis section.

Reuter (1992) are consistent with our results (a z of 3·7–4·3 deg C and an E_a of ~ 640 kJ/mol). They reported a z of 3·7 deg C and an E_a of 643 kJ/mol in the temperature region 73–78 °C for the inactivation of Lpo. Hernández *et al.* (1990) studied the thermal inactivation of Lpo in different media and obtained an E_a of 800 kJ/mol in skim milk and 1030 kJ/mol in whey, pointing out the influence of the composition of the medium on the thermostability of Lpo.

In contrast to the inactivation of ALP and Lpo, isothermally and non-isothermally derived z values for the denaturation of β-lg at temperatures 83 °C

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differed significantly. The values of D, on the other hand, were comparable (Table 3). A possible explanation is that in different temperature regions different thermal denaturation mechanisms predominate, and these are characterized by different values of z. Isothermal experiments were conducted over a limited temperature range of $\sim$ 10 deg C, whereas non-isothermal experiments were conducted over a much broader temperature range, from room temperature up to $\sim$ 80 °C. This may explain the differences found in the kinetic parameters for heat denaturation of β-lg (the upper temperature range was not studied under non-steady state conditions). Moreover, the history of the whey protein system prior to thermal denaturation is clearly different in isothermal and non-isothermal experiments. This might influence the irreversible denaturation mechanism and hence the observed kinetics. Protein denaturation in the temperature range between 60 and 100 °C is considered to be a two-step process; an unfolding step that may be either reversible or irreversible and an aggregation step that mostly follows irreversible unfolding (De Wit & Klarenbeek, 1984).

The correlation between the experimentally determined residual protein concentrations after variable processing and those calculated by means of the non-isothermal kinetic parameters was higher compared with that calculated using the isothermally derived kinetic parameters (R^2 0·864 *v.* 0·789; Fig. 4*c*). Thus it seems that the kinetic parameters based on isothermal data are not simply applicable to the thermal denaturation of β-lg under dynamic conditions.

There are conflicting reports on the kinetics of β-lg denaturation, possibly reflecting the complexity of the process. Several have reported first order kinetics (Gough & Jenness, 1962; De Wit & Klarenbeek, 1981; Harwalkar, 1986; Verheul *et al.* 1998), while others reported second order kinetics (Lyster, 1970; El-Shazly *et al.* 1978; Harwalkar, 1986). Others have found 1·5-order kinetics (Dannenberg & Kessler, 1988; Schlimme *et al.* 1996) or consecutive first order reactions (Harwalkar, 1980) for the thermal denaturation of β-lg. These discrepancies may be explained by variations in procedures and experimental conditions, such as the medium for the protein solution (skim milk, whey or isolated protein in buffer solution), the type of heat treatment, and the method of assay for determining the residual native protein concentration (El-Shazly *et al.* 1978; Anema & McKenna, 1996; Prakabaran & Damodaran, 1997).

To obtain a realistic measure of the statistical confidence for the simultaneously estimated parameters D_{ref} and z, 90% joint confidence regions were constructed based on the parameters calculated by non-linear regression (Fig. 5*a–c*). The extremes of the 90% joint contour plot correspond approximately to the 95% confidence intervals for the individual parameters (the joint probability of two events at 95% probability is $\sim$ 90%, as $0{\cdot}95^2 \approx 0{\cdot}90$). If the parameters are highly correlated, the ellipse is a more accurate representation than the separate confidence intervals, because a parameter pair may be well within the separate 95% confidence intervals, but may be very unlikely to occur since it is far outside the 90% joint confidence ellipse (Haralampu *et al.* 1985; Van Loey *et al.* 1997). Individual confidence intervals are suitable only for describing the limits of a single parameter, without regard to the value of the other parameter.

This is clearly demonstrated in Fig. 5, which shows both the 90% joint regions and the 95% individual confidence intervals. When based on the 95% individual confidence intervals the z values for thermal inactivation of Lpo under steady and non-steady conditions were similar, but when based on the 90% confidence ellipses they were significantly different (Fig. 5*b*). Comparison of the 90% contour plots for

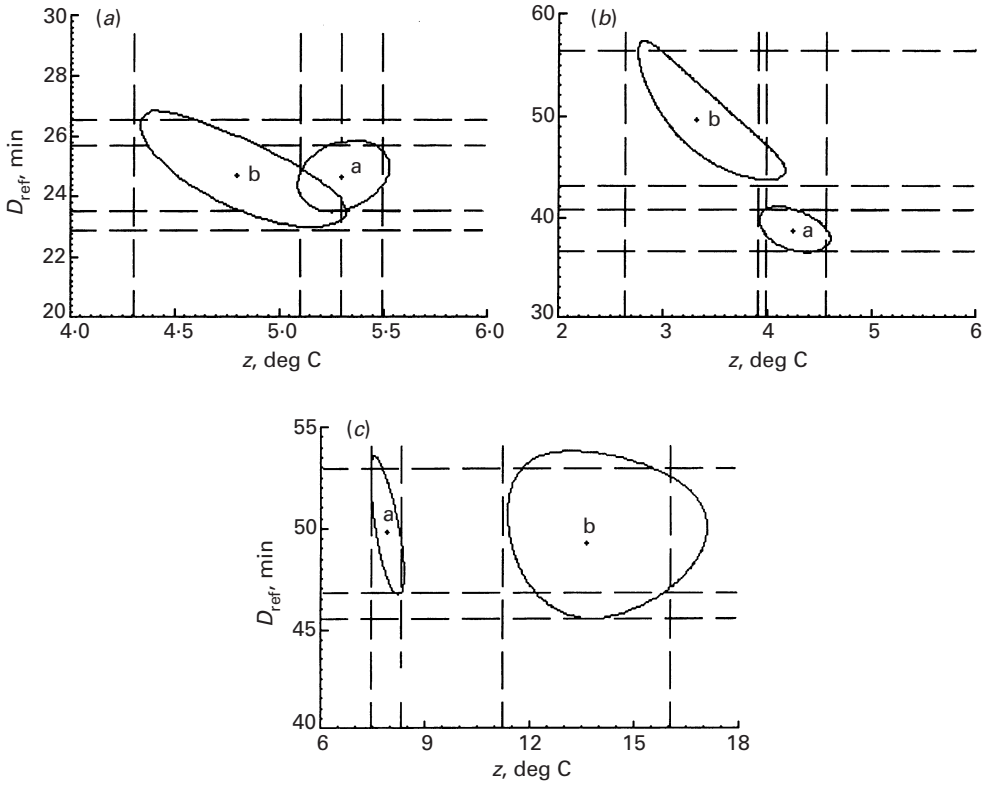

Fig. 5. – – – , Individual 95% confidence intervals and 90% joint confidence regions (indicated by ellipses) for the simultaneously estimated kinetic parameters D_{ref} (the time required to reduce the initial response value by 90% at a reference temperature, T_{ref}) and z (the temperature increase necessary to decrease D by 90%) under a, isothermal and b, non-isothermal conditions at a reference temperature T_{ref} for (*a*) alkaline phosphatase ($T_{ref} = 60$ °C; correlation between D_{ref} and z: a, 0·239; b 0·786), (*b*) lactoperoxidase ($T_{ref} = 71$ °C; correlation between D_{ref} and z: a, 0·417; b, 0·881) and (*c*) β-lactoglobulin ($T_{ref} = 75$°C; correlation between D_{ref} and z: a, 0·696; b, 0·091).

the kinetic parameters for β-lg obtained from non-linear regression with isothermal and non-isothermal data confirmed the significant difference implied by the individual 95% prediction intervals (Fig. 5*c*). The joint regions for thermal inactivation kinetics of ALP overlap, indicating that when based on a 90% significance level parameters derived under steady and non-steady conditions did not differ (Fig. 5*a*).

With regard to the correlations between D_{ref} and z, the rectangular region defined by the individual confidence intervals will approximate to the correct confidence region only if the correlation between the simultaneously estimated D_{ref} and z is close to zero. This is obvious in Fig. 5(*c*), where the correlation between $D_{75\,°C}$ and z is 0·091.

The main objective of this study was to present a detailed kinetic study of ALP, Lpo and β-lg, using two different experimental approaches as well as considering possible approaches to statistical analysis. Concerning their use as an intrinsic TTI, the proteins studied seem to be sufficiently thermostable to provide quantitative measurements of the impact of a thermal process on milk. However, if they are to be used as markers of industrial processes, calibration at an industrial level is necessary. Because kinetics only give pairs of D and z values, and only residual activity or

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concentration can be measured in industrial milk samples, absolute initial concentrations need to be measured and checked for possible variation. In addition, the effect of variation in milk composition on kinetics must be studied, and the kinetic data obtained compared with microbial inactivation kinetics and with industrially applied or legally defined temperature–time conditions. Further experimental work will be undertaken in the future.

To summarize, we found that the inactivation of ALP and Lpo and the denaturation of β-lg could be described by a first order process; that is, the enzyme activity or the undenatured protein concentration decreased in a log linear manner as a function of time. The kinetic parameters D and z were calculated using both a two step linear and a one step non-linear regression approach. Although the results were generally comparable, more accurate predictions of the kinetic parameters were obtained using the global non-linear regression method.

Next, isothermally estimated parameters were evaluated under time–variable temperature conditions. Particularly with the denaturation of β-lg, significant differences were observed between the parameters resulting from the steady state and non-steady state experiments. Possible explanations are proposed from both a mechanistic and an experimental point of view. For ALP and Lpo it seemed that kinetic parameters computed from isothermal experiments could be used to predict thermal inactivation under non-isothermal conditions.

Finally, with regard to the procedure for estimating kinetic parameters carried out in a global fit with inactivation data obtained under isothermal and under non-isothermal conditions, the importance of taking into account the correlation between the simultaneously estimated D_{ref} and z by construction of joint confidence regions was clearly demonstrated.

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